UTILIZATION OF SISAL BOLE JUICE – INULIN AS AN ALTERNATIVE CHEMICAL FEEDSTOCK IN CITRIC ACID PRODUCTION

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By

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy (Chemical and Mining Engineering) of the University of Dar es Salaam

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for examination a thesis entitled **Utilization of Sisal Bole Juice-Inulin as an Alternative Chemical Feed-stock in Citric Acid Production**, in fulfilment of the requirements for the Degree of Doctor of Philosophy of the University of Dar es Salaam.

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I, **Consolatha Joachim Ngonyani**, declare that this thesis is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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"Girls you have done in different ways".

"Dzhikhomu dzhambeyi my first Teachers Dadi-Mwl Joachim, Majengakaya, Charles-Salesi, Michael-Maiku, Augustin-Mzee, Angelo-Mzilasi, Gogo Augustin, Gogo Wilbard, Gogo Gigimanjani; yeniyi indabha, mwalekili nyikuvayila...pang'anda ya pambongo pakulya."

ABSTRACT

Tanzanian Sisal industry is characterised by a severe biomass production for wastage that is (2:98) product to waste ratio. Among the waste, 100% of plant residues in a form of sisal boles, that are made of hydrolysable-fermentable sugars are slashed and burned thus causing environmental emission concerns. The present study on utilization of sisal bole juice-inulin as an alternative chemical feedstock in citric acid production was divided into three parts. The first part involved the isolation and characterisation of the *Aspergillus niger* fungal strains generic to sisal boles for citric acid production using sisal inulin hydrolysate-fructose. A high biodiversity of microorganism with industrial potential were isolated and identified, including the best performer *A. niger* (BYF KT) fungal strain which was used in citric acid fermentation studies.

The second part involved the extraction and hydrolysis of inulin sugars from sisal boles. HPLC analysis revealed that sisal boles had total sugar concentrations values between (26.4±0.24 and 26.9±0.31 g/100ml) with the corresponding fructose values between (24.9±0.02 and 25.6±0.03 g/100ml). Hydrolysis experiments were performed by cooking at temperature range of 30-132°C and pH range of 2-5. The effects of initial conditions (pH and temperature) were evaluated using 2^2 full factorial design (FFD), with varying levels of pH and temperature. The factorial fit and the analysis of variance (ANOVA), indicated that initial conditions for both pH and temperature significantly affected hydrolysates-fructose yield, at confidence interval (CI) of 95%. The optimum hydrolysates yields value of 97.2% (82.4%-fructose) was obtained by cooking at temperature 110±5°C ~ (115°C) and pH 3.

The third set of experiments involved 10l pilot scale fermentation of sisal inulin-hydrolyisates-fructose using *A. niger* (BYF KT) generic to sisal, at initial fructose concentration range of 102-203 g/l, pH range 2-5 and nutrients additives values coded between -1 and 1. The effects of initial hydrolysate-fructose concentration, pH and nutrients additives were evaluated using a 2^3 full factorial design (FFD), with varying levels of initial hydrolysates-fructose concentration, pH and nutrients additives; The factorial fit and (ANOVA) for citric acid yield, indicated that initial hydrolysate-fructose concentration and pH significantly effected citric acid yield, at confidence interval (CI) of 95%, while nutrients additives was not significantly affecting the yields. Results for pilot scale fermentation of 203g/l sisal juice hydrolysate-fructose, in absence of nutrients additive and pH 5 produced highest yields value of 46.66% (103.15±13.08 g/l). On the contrary, minimum citric acid yield value of 9.51% (9.70±2.40 g/l) was obtained at low initial fructose concentration of 102 g/l, pH 2 and with nutrient additions. The current findings supported that sisal inulin has a great potential use as an alternative feedstock in citric acid production, adding value to sisal industry and could be one way of dealing with waste management.

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LIST OF ABREVIATIONS, SYMBOLS AND ACRONYMS

$k_L a$	Volumetric oxygen transfer coefficient (h^{-1} or s^{-1})
(γ)	Yield
(μ_{ap})	Broth apparent viscosity
ΔH	Heat energy
A	Pre-exponential factor
a_1, a_2,	Value for constants in equation [17]& [18] may vary considerably, depending on geometry, variables covered and methodology used
Acetyl-CoA	Acetyl-Coenzyme A or Oxalacetate
Adj MS	Adjusted Sum of Square Mean of Square
Adj SS	Adjusted Sum of Square
AKGDH	α-ketoglutarate dehydrogenase
ANOVA	Analysis of Variance
AOAC	Association of official analytical chemists
ATP	Adenosine triple phosphate
b_1, b_2,	Value for constants in equation [17]& [18] may vary considerably, depending on geometry, variables covered and methodology used
BYF KAT	Best performing *Aspergillus niger* strain
C	Residual inulin concentration (g/l)
C_L	Concentration of Dissolved O_2 in the broth in steady-state (mmol $O_2 l^{-1}$)
C^*	Concentration of saturated O_2 in the broth under given conditions (mmol $O_2 l^{-1}$)
CFU.	Colony Forming Units
c_1, c_2	Value for constants in equation [17]& [18] may vary considerably, depending on geometry, variables covered and methodology used
CA	Citric acid
CAS	Chemical abstracts service registry
CFC	Common Fund for Commodities
CI	Confidence I interval

CIP	Cleaning performed in-place
CMS	Condensed Molasses Soluble
C_o	Initial inulin concentration
CoefVar	Coef of Variation
CPE	Chemical and Process Engineering
Ct Pt	Central Point
CYA	Czapek yeast autolysate agar
DF	Degree of freedom
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen (Mg/l)
DOE	Statistical Design Of Experiments
DP	Degree Of Polymerization
DSMZ 8	Standard A. niger strain from the German Collection of Microorganisms and Cell Culture (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.)
E_a	Activation energy (J/mole/K)
F	Fisher coefficient
FAD	Flavin Adenine Dinucleotide
FFDs	Full factorial designs
FFT	Fructan-Fructan Fructosyl Transferase
F_m	"m" number of fructosyl units
FU	Fructose Units
G	Terminal glucosyl unit
GF_n	Molecular formula of Inulin, Glucosyl unit with "n" number of fructosyl units
GF_{n-1}	Glucosyl unit with "n-1" number of fructosyl units
HPLC	High performance liquid chromatography
HSE	Highlands sisal estate
ICDH	Iso-Citric Dehydrogenase
ICL	Iso Citric Lyase
IPCC	International panel for climate change

ITS	Internal Transcribed Spacers
IUPAC-IUB	International Union of Pure and Applied Chemistry-International Union of Biochemistry
JCBN	Joint Commission on Biochemical Nomenclature
kJ	Kilo Joules
R	Universal molar gas constant = 8.314472(15) $JK^{-1}mol^{-1}[1.8x10^{-6}]$
k_r	Rate constants
KAT	KATANI Ltd
KTH	Royal Technical University of Sweden
KT	Katani/Mkonge
MillQ-H_2O	Millipore filtered sterile water
tons	Metric Tons
N	Population
N*	Omitted population
NA	Nutrient Agar
NADH	Nicotinamide adenine dinucleotide-H
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NDO	Non-Digestible Oligosaccharides
NTP	Normal Temperature and Pressure
OGYE	Oxytetracycline Yeast Extract Agar
OUR	Oxygen uptake rate (s^{-1})
P	Probability
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
P_g	Gassed power consumption (W)
QO_2	Specific oxygen uptake rate (mmol $O_2g^{-1}h^{-1}$)
QO_2^{max}	Maximum specific oxygen uptake rate (mmol $O_2g^{-1}h^{-1}$)
$Q_{O2}X$	Global oxygen uptake rate (mmol $O_2g^{-1}h^{-1}$)
Res. Inulin	Residual inulin
RPM	Revolutions per minutes
R-Sq	Sum of Square

R-Sq(adj)	Adjusted Sum of Square
SB	Sabouraud Dextrose Agar
SBJ A	Sisal Bole Juice (darker liquid)
SBJ B	Sisal Bole Juice (clearer liquid)
SBJ-F	Sisal bole juice-fructose
SBJ-I	Sisal bole juice-Inulin
SE Coef	Standard error of coefficients
SEMean	Standard error of means
Seq SS	Sum of Square
SIP	Sterilisation performed in -place
SST	Sucrose- Sucrose Fructosyltransferase
StDev	Standard Deviation
StdOrder	Standard order
t	Reaction time (minutes)
T	Temperature (K)
T	*T* test
TCA	Tricarboxylic acid
TCT	Tricarboxylate transporter
(tCO2 eq)	baseline methane emissions (Tones of CO_2 equivalent)
TD	Taguchi Designs
TDTC	Technical Development Transfer Centre
TN	Total Nitrogen
total resid	Total residual
TP	Total Nitrogen
TS	Total Sulphur
TSA	Tanzania Sisal Authority
TSB	Tanzania Sisal Board
UDSM	University of Dar Es Salaam
V	Broth volume (l or m^3)
$v_{s.}$	($= 4Q/(\pi D_t^2)$) superficial gas velocity (ms^{-1})
α	Constant of proportionality

α-level	level of significance
ΔG^o	Gibbson activation energy (J/mole/K)
v_{ap}	Apparent kinematic viscosity (m^2s^{-1})
ρ	Density (kg m^{-3})
σ	Surface tension of filtrate (Nm^{-1})
UNIDO	United Nations Industry Development Organisation
CFC	Common Fund for Commodities
FAO	Food and Agriculture Organization of the United Nations
NIST	National Institute of Standards and Technology
SEMATECH	SEmiconductor MAnufacturing TECHnology
UNFCCC	United Nations Framework for Climate Change Conventions
UNISPAR	University-Industry Science Partnership in Africa Research
TGES	Tanzania Government, Economical Survey

CHAPTER ONE

INTRODUCTION

1. BACKGROUND OF SISAL INDUSTRY

The Germans introduced sisal agronomy to Tanganyika (now mainland Tanzania) in 1893, by successfully propagating the exotic species *Agave sisalana*. The first sisal farm was established at Kikogwe (Mwera estate), which is located at Amani, Pangani District Tanga Tanzania. Conducive Tanzanian calcareous coastal soils found within altitude of 0-300 m along Tanga railway line could be the reason. In addition, average annual rainfall ranging between (500-1500 mm) and high average annual temperatures ranging between (25-32°C) suited growth of sisal (Lock, 1969; Kimaro *et al.,* 1994). The *Agave sisalana* was later, followed by the *Agave* hybrid H 11648 which was obtained after crossing an indigenous species "blue sisal" *A. amaniensis* and *A. angustifolia* (Nowell, 1933; Lock, 1969). However, both indigenous *A. amaniensis* and *Agave* H 11648 were vulnerable to sisal bole-rot damage by various microorganisms including notorious black spore fungus *Aspergillus niger* (Lock, 1969).

Manifestation of sisal boles rot seems to suggest that biochemical processes facilitated the survival of *A. niger* colonies, with formation of metabolites such as enzymes and carboxylic acids including citric acid (Lock, 1969; Hosea 1996). For example during the wet seasons, *A. niger* fungi have a tendency to bloom among injured plants. This suggested easy isolation and screening of the *A. niger* fungi, and

eventually utilizing them in fermentation of sisal boles sugars for production of metabolites e.g. citric acid. Also in his report, Lock (1969) alleged that, ruminant animals such as goat, giraffe and zebras were grazing on juvenile sisal plants, and thus the plants are probably rich in carbohydrates, and could be used as animal feed.

The sisal hybrid H 11648 became so successful that it becomes widely used in commercial plantations in East Africa (Lock, 1969; TSB, 1994). In the 60s Tanzania was the largest producer in the world with nearly 40% share of the total global production until the discovery of synthetic fibres which compounded the decline of Tanzanian sisal industry which still relied only on fibres. There were no concerted researches to add value to sisal. This study in some way is addressing the appeal to a serious need for expansion of the sisal market by introducing non-traditional products such as biochemicals from underutilized large part of the plant that remains in the fields (TSB, 1994; TSB, 2007).

It is fair to expect the once non-traditional, high valued products are introduced in the sisal agronomy allowing the market for both the new products such as pulp, biogas-fuel, geo-textile materials, chemical feed materials and fibre production to recovery hence the sisal industry (CFC/UNIDO, 2003; Shamte, 2007; KATANI Ltd. 2007; TSB; 2007).

1.1. Tanzanian Sisal Production History and Projections

During independence, sisal fibre industry proved to be not only major foreign cash earning but also continued to position Tanzania among the leading producers worldwide. Sisal industry reached its peak in 1964/65, when 230,000 tons were produced out of the world production of 602,800 metric Ton as shown in Figure 1.1 (TSB, 1994 CFC/UNIDO, 2003; TSB, 2007). By 1967, already 201 estates in Tanzania were growing sisal on 42,000 hectares thus producing over 216,000 tons of line fibre that corresponded to 10,584,000 tons of decortications wastes.

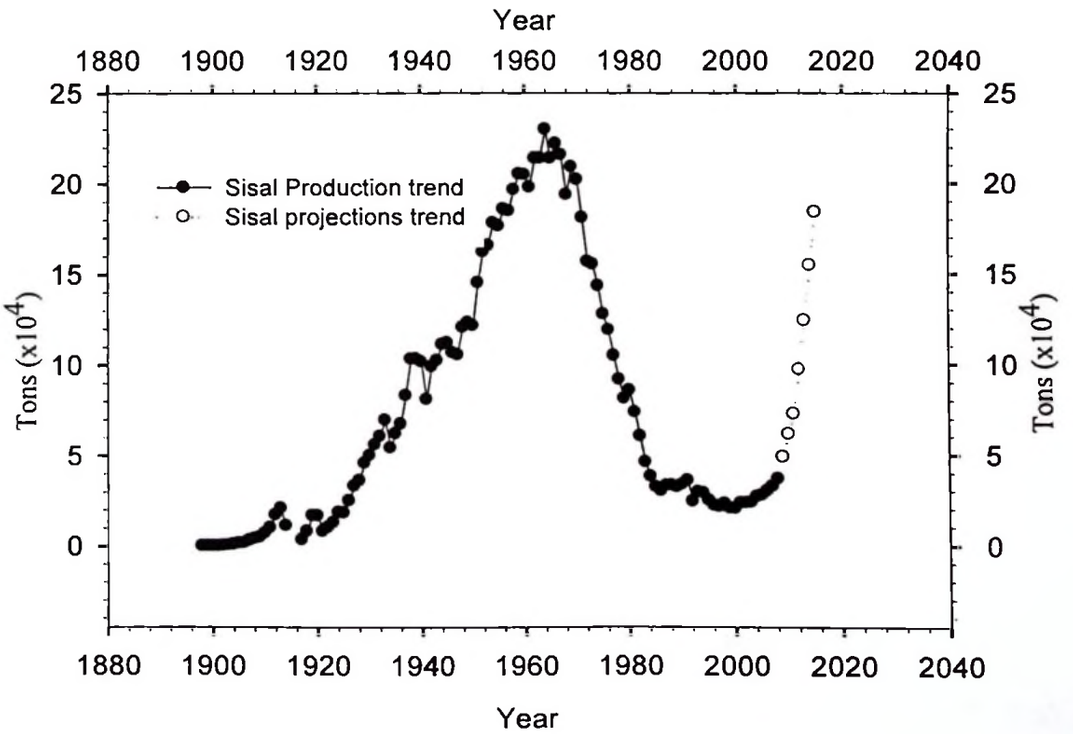


Figure 1.1: Sisal production history and projection in Tanzania to 2015 (TSB, 2007).

By 1996, Tanzania had 52 estates engaged in sisal production on 48,745 hectares, which gave between 21,400-29,997 tons of fibre. This amount corresponded to 1,048,600-1,469,853 tons of wastes (decortications and those left in the field) based on 98% discarded wastes (CFC-UNIDO, 2003; TGES, 2004; TSB, 2007).

The economic importance of sisal declined in the 1970s due to the fall in world market prices caused by the introduction of synthetics fibres. This resulted in the decline of the industry with severe fall in revenue and decline of provision of social services such as education and health. These problems have remained unresolved up to now (TSB, 1994; CFC/UNIDO, 2003; TSB, 2007).

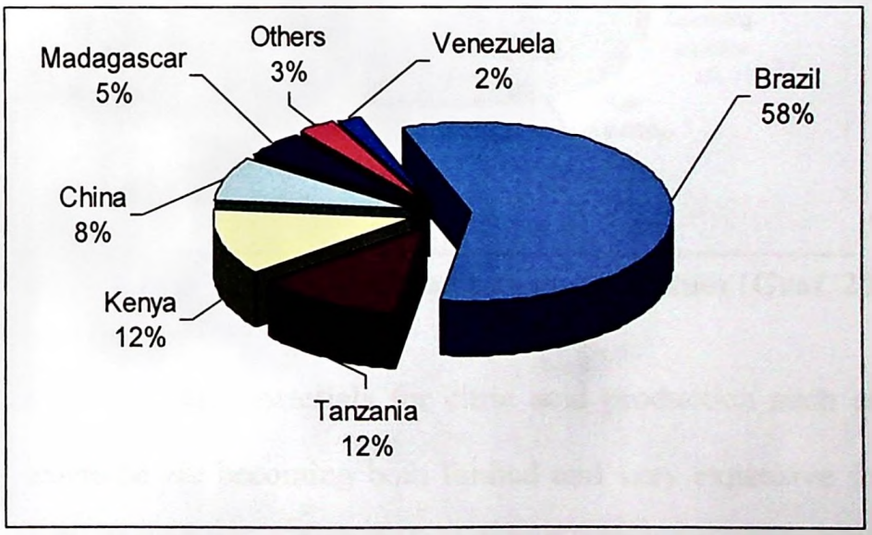


Figure 1.2: World Sisal production in percentiles

Tanzanian sisal industry is currently improving, for example in 2000 Tanzania produced only 23,858 tons while the world sisal production stood at 281,100 tons (CFC/ UNIDO, 2003). By year 2006, Tanzania produced 30,934 tons, which shows a recovery trend with projections that in ten more years will reach the summit, hence will continue to be among the top ten leading producers (Figure 1.1 and 1.2).

With prospects of adding more value to the crop and commercialization of non-traditional products, sisal agronomy will bounce back and so do the Tanzanian economy (TSB, 1994; CFC/UNIDO, 2003; TSB, 2007). There is strong evidence that sisal bole juice could produce Inulin rich liquid, the potential feedstock to industrial processing (Minja, 1991; Hosea, 1996; Massala, 2003; Masanja *et al*, 2003).

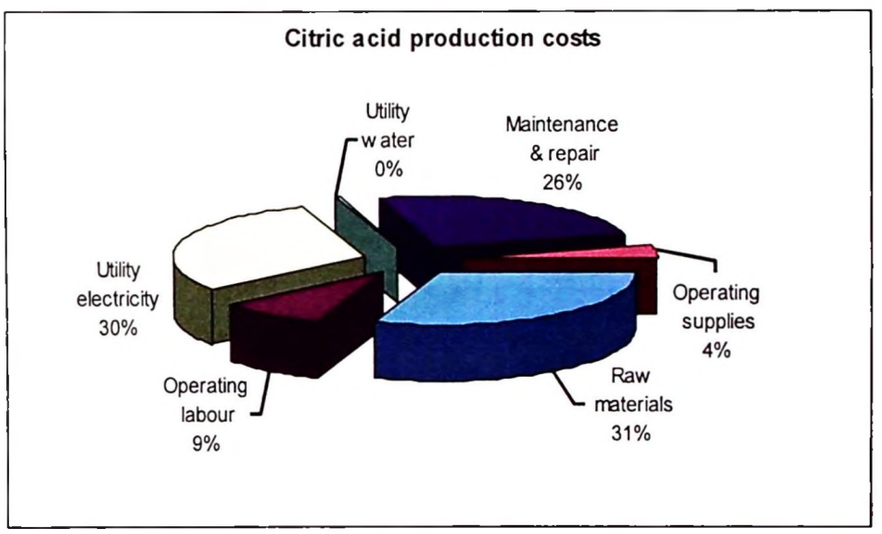


Figure 1.3: Citric acid production costs in percentiles (Graf, 2005)

Apparently, traditional feed materials for citric acid production such as molasses, glucose and limejuice are becoming both limited and very expensive for example; Bagajewicz *et al.,* (2004) determined the profitability of the bio refining investment for the carboxylic acids using corn as the chief raw material (Table 1.1 and Figure 1.3). Results showed that the profitability was positive despite the fact that citric acid price and demand were stable.

Worldwide production costs are still high especially when glucose is the feedstock (Graf, 2005). The reason could be the conversion of corn into glucose escalated the

raw material costs thus making citric acid production least profitable, as compared to other products such as bio ethanol. This significantly emphasised the need for the exploration of other cheaper raw materials that do not need the intermediate processing.

Table 1.1: Demand for citric acid and other products (Bagajewicz *et al.*, 2004)

Product Micro-organism	Yield	World Demand /yr	Growth Outlook /yr	World Price /kg	Production Costs /10^6kg
	%	x10^6kg	%	USD	Million USD
Acetic acid (*C. thermocellum*)	100	2.8	3-4	0.21-0.24	13.26
Citric acid (*A. niger*)	66	2.4	3-5	0.24-0.29	13.61
Fumaric acid (*Rizophus*)	67	2.0	1.5	0.24-0.39	
Succinic acid (*A. succinici procucens*)	87	9.1	6-10	0.91-1.36	15.96
Lactic acid (*L. delbrueckii*)	95	136	3.5-5	0.45-0.68	
Ethanol (*S. cervisiae*)	67	10.6	10.5	5.87-10	64.00
Propionic acid Sodium Propionate	67	99.3	1.8	0.23-0.24	15.79

Findings from this research revealed that while sisal boles inulin processing is simple, resultant hydrolysates sugars are of high quality and costs less. Concurrently the current results indicated that, citric acid production is both more viable and profitable if produced using sisal inulin feed stocks. In a wake of the environmental concerns, Tanzania has been struggling to achieve industrialization in a much greener perspective. Therefore, utilization of sisal bole juice-inulin as an alternative chemical feedstock is providing an opportunity towards industrialization using

affordable green technology.

1.1 The Agro-Economical Value of Sisal

According to report by UNIDO (2006), sisal is essentially an estate crop run by about 82 estates, which are located mainly in Tanga and Morogoro regions thus providing employment, and reduces poverty to more than 6 million people.

Nkuba (2007) reported that sisal crop could thrive in drought conditions, extreme landforms and in typical soils with little nutritional composition. Excellent cover crop sisal, controls soil erosion and survives bush fires. While sisal, is planted any time of the year and harvested throughout the year, it has very few diseases and seldom needs any pesticides.

Sisal can be inter-cropped with foodstuffs thus, this plant is a best candidate once the land use conflicts arises (Figure 1.4). There are very few crops in this world with all these rare qualities (Palutikof, 1986; Nkuba, 2007). These qualities are advantageous to economy of underdeveloped countries like Tanzania, whose agriculture is predominantly rain fed.

Figure 1.4: Juvenile sisal plants intercropped with maize

Being the backbone of the country's economy, Agricultural activity in Tanzania contributes to about 50% of the total Tanzanian economy of which sisal fibre alone contributes to about 10% of the total Tanzanian Agricultural economy (Figure 1.5). By utilizing remaining 98% of the discarded biomass, sisal contribution would definitely increase exponentially.

Larger portion of sisal residue is still underutilized. For example, as small fraction of the flume and tow is spun and woven to produce textile material for gunny bags while about 0.5-1% of sisal crop mainly the poles finds its application in either a local construction industry or as an energy source. The rest of un-accounted for biomass-wastes which are left behind (about 2.88×10^6 tons) of needs to be salvaged (TSB, 1994).

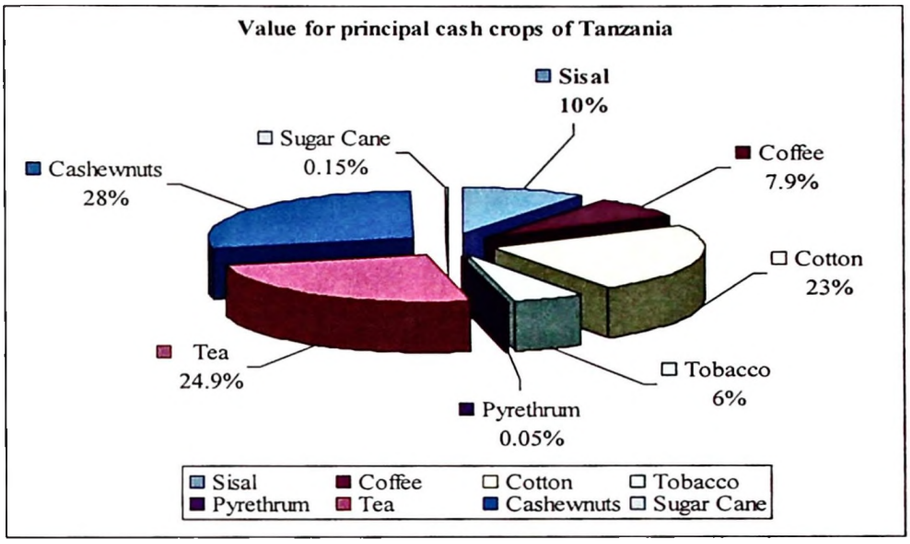


Figure 1.5: Summary of marketed value of principal crops of Tanzania year 2004

The exploitation of $2.88x10^6$ tons of un-accounted for biomass, could produce readily marketable chemicals products e.g. citric acid, enzymes, lactic acid, itactonic acid, acetic acid, ethanol, carbohydrates, proteins, pulp for paper making, biogas, animal feed, organic fertilizer, wax, bio plastics, cement foaming agents, furfural, haecogenin and animal sex hormones and medicinal products (CFC/UNIDO, 2003; TSB, 2007). Products from sisal wastes such as citric acid have good demand both in local and export markets therefore efforts should be made to venture in.

Sisal is still cheaply produced at 300,000 Tanzanian shillings TAS /hectare (ha), whereas the sales are about 1,000,000/ = TAS/ha (Anon, 2000). This attribute has greatly attracted interest of private investors, thus brightened future of sisal industry. Likewise as from the past decade sisal agriculture has been regaining its popularity, small farmers have been encouraged and their response is good (KATANI LTD;

2006; Nkuba, 2007; Kasita, 2008). With this new interest, question of wastes management by either converting sisal boles into marketable chemicals and exploration of other uses of sisal plant becomes inevitable. This will make the industry more vibrant and profitable.

Like other agricultural produce in Tanzania, sisal production deteriorations with wide fluctuations in world market prices, could also been worsened by underutilization of the crop and emergence of synthetics polypropylene substitutes (TSB, 2004). The acquisition of 55 percent of the sisal market shares since the 70s to 80s by the later also exacerbates the situation. This necessitate the need for utilization of whole sisal crop as currently average price of East African grade UG sisal fibre is about US$550 per ton-the same as it was 15 years ago despite of increased farm inputs cost.

1.2 Environmental Impacts of Sisal Wastes

Sisal fibres accounts for only 2% of the total sisal plant and have been the central focus for the industry in Tanzania for over a century now. The remaining 98% of the total biomass is thrown away in form of solid and liquid waste, including a high biological oxygen demand effluent that is discharged into streams (Kasita, 2008). This industry brings about a serious case of biomass waste in a form of both liquid and solid, in particular fibrous sisal boles; the stems that are left after leaves are cut.

In some sisal estates, a small fraction of the biomass wastes such as the decortications solids spread in the sisal nursery as compost matter. In others, they are

used as part of raw materials for biogas production (Kissaka, 2007). However, most all that remaining are left haphazardly, which prompts the urgency for a sensible disposal strategy.

Despite the fact that, decortications process produces an average of 1.5 tons of extracted sisal fibre per hectare per year, high biodegradable decortications liquid wastes are discharged haphazardly into receiving water bodies, at the rate of 3-5 tons/day (Kissaka, 2007; Kasita, 2008). Between 4.19 and $13.96x10^4$ tons/year of decomposing decortications wastes at the Highland emit significant amount of (CH_4) into the atmosphere.

Tanzanian sisal-estates have similar corona-decorticator models, thus the have similar characteristics; in his work Salum, (2008), estimated that per each corona-feed, 130 tons of sisal leaves are processed thus making a total of 260 tons per day (2 shifts * 130 tons = 260 tons). Likewise, the Highland Sisal Estates has 260 tons of leaves processed daily

Since out of the 260 tons of leaves processed per day 98% remain as waste (the rest 2% is sisal fibre). This means that the factory generates 254.8 tons of sisal waste per day (260 tons * 98% = 254.8 tons). The annual total waste production is thereby obtained by multiplying daily waste production by 365 days, which gives 93,002 tons/year of decorticator wastes (APPENDIX XII). Based on First order decay (FOD) model of IPCC (UNFCCC, 2007), the baseline methane emissions value of 4128 (tCO2-eq) was calculated (APPENDIX XII, equation [33]).

Meanwhile the current method of disposal of the boles which involves slashing and burning in the fields, is both labour intensive and adds extra costs at the stake of waste emissions, whilst their decomposition causes significant environmental and amenity consequences (Nkuba, 2007; Kasita, 2008).

At the Highland sisal estate a total amount of about 1000 hectares constituting of mature sisal are harvested annually. About 200 hectares out of 1000 are composed of sisal plants at their end of life cycle and are subjected to slashing as part of land preparation for a new growth cycle.

The recommended planting density at the Highland is 4000 seedlings /ha and normally, 200 ha are planted annually. One can easily estimate that for the 200 hectares targeted for replanting produces 800,000 waste plants in a form of sisal poles and fibrous sisal boles, which are left behind to rot [production to waste] (Mashauri *et al.,* 2004). The slashed solids are normally burned, and as the boles contain a fructose polymer inulin, according to equation [1], the burning will produce gaseous pollutants and the resulting heat will be wasted as well.

fructose complete combustion

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 12H_2O + \Delta E \qquad [1]$$

Considering that the sisal boles normally weigh 20-100 kg, with an average of 60 kg, annually about 4000 plants x 200 Ha x 60 kg (4.80×10^7 kg) of these boles plus other fibrous waste (pole, roots), are burned or left to decompose slowly (Kassita, 2008).

This makes the total mass value of both liquid effluents and solid wastes in form of boles is about (93,002+48,000 = 141,002 tones per year).

Table 1.2: Fructose combustion and citric acid production mass balances estimates

	TSB-2007/8 estimates (/ha year)	HSE -2007/8 estimates for 200 ha (/year)
Wt of sisal boles (x10^5 kg)	2.4	480
Fructose (x10^4kg)	5.76-7.20	115-144
Citric acid(x10^4 kg)	6.14-7.68	123-154
76% Yield citric acid (x10^4 kg)	4.67-5.84	93.4-117
CO_2 (x10^4 kg)	9.22-11.5	184-230
H_2O (x10^4 kg)	6.91-8.64	103.8-173
ΔH (x10^{10} kJ)	3.37-4.21	674-843
CH_4 (x10^4 tCO2-eq)	*1.5152	0.4128

TSB = Tanzania sisal authority * = (Salum, 2008) HSE = Highlands sisal estate

citric acid production

$$C_6H_{12}O_6 + 15H_2O + 32ADP + 32P_i \longrightarrow C_6H_8O_7 + 2H_2O + 32ATP \quad [2]$$

As individual matured boles are composed of 24-30%w/v Inulin sugars and weighs 60 kg, during the annual replanting of 200 hectares burning of solid waste produces about 1.84-2.30 kg of CO_2 and 1.04-1.73x10^6 kg of H_2O. In addition to that, 6.74-8.43x10^{12} kJ of heat would be lost (equation [1] and Table 1.2).

Harvested matured sisal boles could produce roughly between 1.15-1.44x10^7 kg of sugars/year. Since one molecule of fructose produces one molecule of citric acid (Equation [2]; Table 1.2), these sugars have a potential to for produce about 1.23-1.54x10^6 kg of citric acid /year (i.e. 6.14-7.68x10^4kg of citric acid /ha).

1.3 Statement of the Problem

Today Tanzanian sisal industry has reached an annual production of 30,000 tons of sisal fibres leaving about $2.88x10^6$ tons of wasted biomass (UNIDO, 2006; TSB, 2007). This amount of waste is bound to increase, as there is an indication that Tanzanian sisal industry is regaining its prosperity, therefore the question of sisal wastes management is inevitable (UNIDO, 2006; TSB, 2007; Nkuba, 2007).

The prevailing century old technology in sisal fibre production recovers only 2% of harvested crop, leaving behind 98% in form of liquid wastes plus decortications effluents trailing into the nearby receiving rivers. These liquid are biodegradable; therefore, they can deplete dissolved oxygen and hence severely degrade the aquatic life. The worse part in this industry is that, 100% of the plant solids residues in form of sisal poles and boles are either left to decompose in the field or slashed and burned consequently causing environmental concerns. Alternative uses of sisal waste will add value to sisal plant for example Massala (2003), successfully developed laboratory scale citric acid production process from sisal bole sugars. Since conditions in small-scale productions differed from large-scale production, the pilot scale study of citric acid production by fermentation of sisal bole juice-inulin using indigenous *A. niger* fungi is an important step towards industrial production.

This research therefore was aimed to address the above challenges, while mitigating solid wastes from the sisal industry by using them in selective fermentation to produce chemicals. Research findings may promote utilization of the whole sisal

plant, which could lead to the expansion of production and marketing of non-traditional sisal products such as citric acid.

1.4 Significance of the Study

Tanzanian sisal industry represents one of the most serious cases of biomass wastes in a form of solid residues and effluents at a tune of (2:98 product waste ratios). To prevent biomass production to waste, sugars present in the solids residues could be used to produce different products such as citric acid, thus adding value to the sisal. Tanzanian sisal industry is regaining its prosperity with its growth trend increasing exponentially (seen in Figure 1.1). However, there is evidence that most of effluent receiving water bodies around sisal estates have died from decorticator pollutants and cannot support aquatic life due to extremely low levels of dissolved oxygen, which measures at an average value of 5 mg/l (Mashauri *et al.,* 2004; Mziray, 2010). This situation is not environmentally acceptable. Therefore, there is an urgent need for institution of a waste management strategy to solve this problem. The management of effluent problem by recycling after treatment using either constructed wetland and/or biogas production sounds very appropriate for controlling noxious gases e.g. methane and hydrogen sulphide which are of environmental concerns (UNIDO, 2006; TSB, 2007; Kissaka, 2007; Mziray, 2009).

As part of farmland preparations at the Highland sisal estate, about 200 hectares per year are harvested, leaving 100% of the plant solids residues in form of sisal poles and boles either left to decompose in the field or slashed and burned consequently

emitting more green gases such as CO_2, H_2O, NO_x, ash and particulate matter. Burning of sisal boles or leaving them to decompose results into sugars and energy loss, along with generation of tones of emissions of unwanted gasses such as methane. Hence, the need for using the inulin sugars present in sisal boles; as a raw material for producing relatively cheap sources of reducing sugars e.g. fructose become important.

Molasses and glucose the traditional feed materials for fermentation industries, are currently becoming both limited and expensive, because their recovery involves costly multistage processing and are energy intensive (Graf, 2005; Bagajewicz *et al*, 2004). One example is that despite to the fact that citric acid is highly demanded worldwide; the conversion of corn into glucose escalates raw material costs and makes citric acid production least profitable. This emphasized the exploration of cheaper feed materials such as sisal inulin sugars.

Studies of citric acid production via fermentation process using glucose and molasses are well-documented (Kristiansen, 1981; Hossain *et al.,* 1984; Kubicek *et al.,* 1994; Röhr, 1998). Industrial production of citric acid from the Tanzanian *Agave* hybrid H 11648 sugars not been tried. The laboratory small-scale results by Massala (2003) justified the need for large-scale production (Seletzky, 2007).

1.5 General and Specific Objectives

The overall objective was to investigate the potential of using sisal bole juice-inulin as an alternative chemical feedstock in citric acid production at the pilot scale. Specific objectives were therefore, aimed at the following:

(a) To determine the fermentation potential of *A. niger* fungi from sisal bole rot infected plants;

(b) To determine the kinetics and mechanisms of sisal bole juice inulin hydrolysis for optimizing total hydrolysates yield; and

(c) To develop a process for optimizing citric acid production at pilot scale level.

1.6 Hypotheses

This study was based on the following hypotheses:

i. The *A. niger* fungi from sisal bole rot can produce citric acid with a comparable or superior performance to the commercial strain;

ii. The increase of initial hydrolysis temperature of sisal bole inulin can improve total hydrolysates yield by 90% and consequently citric acid yield; and

iii. The decrease of initial sisal inulin concentration, pH and nutrients additives decreases citric acid yield by 20%.

CHAPTER TWO

LITERATURE REVIEW

2. PREVIOUS AND CURRENT RESEARCH TRENDS

In chapter two; literature review provides an insight on what was done in previous research work on citric acid fermentation processing and sisal industry. Several documented work for example; Saeman, (1945); Smart and Whistler, (1953); Edelman and Jefford, (1968); De Leenheer, (1996); Kalliat, (2008) provided information regarding Inulin sugars and their conversions into fructose. In all literature cited information on feedstock from the *Agave* hybrid H 11648 was not covered in depth. The outstanding researches by others for example; Kubicek and Röhr, (1978); Kubicek and Röhr, (1980); Hossain *et al.,* (1984); Kubicek and Röhr, (1985); Kubicek, (1988); Kubicek *et al.,* (1988); Kubicek *et al.,* (1994); Röhr, (1998); Kristiansen *et al.,* (1999); Bizukojc and Ledakowicz, (2003); Karaffa and Kubicek, (2003), covered comprehensive works on citric acid processing by *A. niger* fungi using traditional feedstock, such as glucose sucrose and molasses.

Information on the sugar metabolism by *A. niger* for citric acid production and regulation, of the α-ketoglutarate dehydrogenase in optimization of citric acid production was provided by many e.g. Meixner *et al.,* 1985; Meixner-Manori *et al.,* (1985); Röhr and Kubicek, (1996); Taiz and Zeiger, (1998); Ruijter *et al.,* (1999); Alvares-Vasquez *et al.,* (2000); Jianlong, (2000); Alves da Silva, (2009).

3.4.8 Vegetative inoculum transfer procedure and fermentation technique

Culturing was done by aseptically harvesting and transferring of 5% (v/v) of the vegetative mycelia pellets of approximately 1-2 mm diameters into the main pilot scale production fermenter which contained fresh sterile broths of desired initial pH, nutrients additive and sugar concentrations as described in section 3.4.9. The incubation temperature was kept at 30 ± 1°C throughout the fermentation period of 168 hours after inoculation. Air sparging and agitation were done by using a magnetic driven spinning impeller. Fermentation conditions were maintained throughout for 168 hours after inoculation (Datta and Bergemann, 1996; Ruijter *et al.*, 2002).

3.4.9 Preparations of fermentation media and nutrients additives

Fructose rich-Inulin hydrolysates were obtained by autoclave cooking of sisal bole juices at 115±5°C, and pH adjusted to value of 3.5 for 180 minutes. The calculated total hydrolysate sugars had average value of 25.07±0.21% (v/w).

Nutrients additives concoction was prepared using salt solution as per modified method by Datta and Bergemann, (1996). Therefore 100% concoction of nutrients additives per one litre feedstock was composed of the following: 3.1 g/l $NH_4 NO_3$, 0.15 g/l $KH_2 PO_4$, 0.15 g/l NaCl, 1.1 g/l $MgSO_4$, 6.6 mg/l $ZnSO_4$, 7 H_2O, 0.1 mg/l $FeCl_3$ (Table 3.7).

Table 3.7: Fermentation broth compounding

	SBJ(%w/v)	Fru (g/l)	pH	YE (g/l)	*Nutrients (%)	SBJ (ml)	Vol (ml)
High	80	200	5.0	2	100	8000	10000
Central	60	150	3.5	2	50	6000	10000
Low	40	100	2.0	2	0	4000	10000

* = levels for concoction of nutrients additives (1 g/l) = 100% (0 g/l) = 0%

3.5 Production of Citric Acid from Sisal Bole Juice

The conditions for fermentation were adopted from several works e.g. (Datta and Bergemann, 1996; Alvarez-Vasquez *et al.*, 2000; Ikram-Ul *et al.*, 2001; Ali, *et al.*, 2001) and were fixed at optimum levels and maintained throughout, for instance oxygen tension was maintained at value of (1.5-2±0.2 l/l/min) and fermentation temperature (30±1°C).

Since on average, sisal boles are composed of 70-80% (w/v) juice, pure sisal boles juice 100% (w/w) consists of hydrolysable sugars corresponding to fructose 25.07±0.21%, (about 250.70g/l). The initial sisal bole juice concentrations were 40, 60 and 80% (sisal bole juice: water ratio), these corresponded to total hydrolysates sugars equivalent to 11.66±1.15, 17.49±1.72 and 23.32±2.30 g/100ml (fructose 10.18±0.38, 15.27±0.57 and 20.37±0.75 g/100ml sisal bole juice inulin respectively); and each were fortified with 2 g/l yeast extract.

Adding 0%, 50% or 100% levels of nutrients media concoction studied the effect of

world trade and through farm subsidy systems (CFC/UNIDO, 2003).

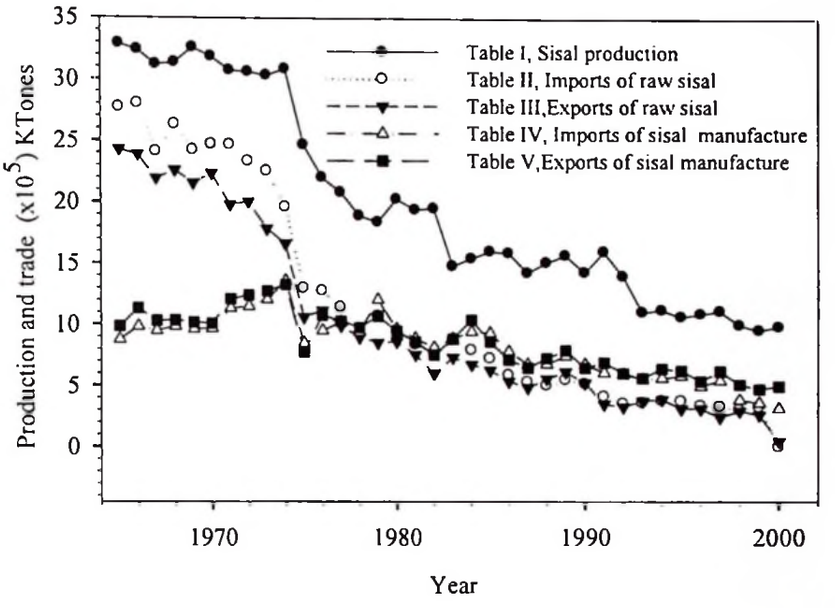


Figure 2.1: FAO statistical data on the World production and trade of sisal and henequen trend 1965-2000 (CFC/UNIDO, 2003).

China has started to infiltrate the world market share for example; as Brazil produced 145,000 tons, China produced 55,000 tons of fibre. In 2006, China exported approximately 12,000 tons of fibre to the Middle East, which was not enough as the local market demand was estimated at about 35,000 tons (CFC/UNIDO, 2003). Tanzania will face competition from other sisal producers in Africa as well. For example, South Africa, which used to import sisal fibres, is now an established producer. Kenya and Madagascar have continued to maintain their production levels of 2005.

Improvement of political stability in these countries could lead into increased production. Mozambique and Angola are re-starting sisal production after the end of

civil wars (CFC/UNIDO, 2003; TSB, 2007). This suggests that, the World demand for sisal product is on the rise again and therefore Tanzanian sisal industry should take proactive steps to access it, while making necessary efforts for marketing of other non-traditional products such as citric acid.

2.2 Morphology of the Sisal Plant

Sisal plant looks like an overgrown pineapple with a pineapple-like bole from which leaves extend from (Figure 2.2).

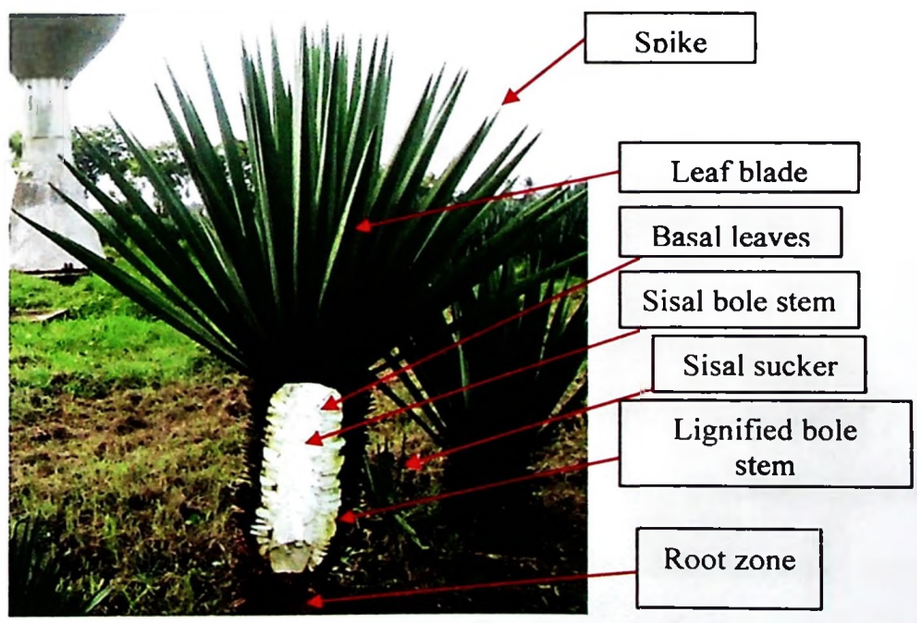


Figure 2.2: Sisal bole morphology, *Agave* hybrid H 11648 at ARI-Mlingano

2.2.1 Sisal roots

Sisal roots seldom penetrate deeper than 60 cm below the sub soil horizon (30-60 cm). The root system of sisal is adventitious arising from age-lignified leaf scar and takes a considerable time to rot after completion of life cycle. This makes sisal crop

an excellent cover crop in soils with few perennials and shrubs, and an excellent host to microorganism's biodiversity throughout its life span (Acland, 1972; Hartemink and Wrenk, 1995; Hartemink, 1997).

2.2.2 Sisal suckers

Sisal suckers grow from the rhizome produced from the base of the bole, near the parent stem while some can grow as far as to about 18 m away (Figure 2.2). Sucker starts to appear a year after planting and they are usually prolific during the first half of the cycle. Rhizomes have stored sugars to sustain the emerging suckers. About 20 suckers can grow during the life cycle of a plant (Lock 1969; Acland, 1972). As seen apart from the plant support, suckers helps in plant propagation and are cover crop in soils with few perennials.

2.2.3 Sisal bole

Sisal bole is a hard woody structure that looks like a pineapple. In a healthy plant, it reaches and maintains a width of about 20 cm two years after planting. At maturity reaches a height of about 12 m and weighs about 20-100 kg. They normally appear to be thicker than 20 cm owing to the presence of basal ends of the cut leaves (Lock 1969; Acland, 1972; Massala, 2003). The growing point is at the top of the bole. Healthy plants have wide boles, plants with narrow boles produces short leaves at long intervals of time.

Sisal bole fibres are not in the form of fibre bundles except in the leaf butt ends attached to the boles. Base of the bole is woody and fibrous, but the ultimate pulp fibres are shorter and the actual fibre content is low. An upper portion becomes increasingly pithy and contains almost no fibrous material toward the growing tip (Lock 1969; Acland, 1972; Hurter, 1997), which minimizes energy requirements in making sisal bole paste and juices.

As the bases of leaves containing leaf fibre, remain attached to the bole when leaves are cut, highly lignified cortical layer covers the sisal bole to places which leaves were attached. The cortical layer of bole forms an excellent barrier to pests and microorganisms feeding on sisal bole juices. Therefore, microorganisms seldom spoil stems unless there is some injury (Lock 1969; Acland, 1972).

2.2.4 Sisal leaves

Leaves from a healthier sisal plant are about 12 cm wide, tipped with a sharp highly lignified spike (Lock 1969; Acland, 1972). However, leaf tissue of this plant yields hard flexible fibres, which are suitable for making rope and twine, cord, matting, padding and upholstery (Hurter, 1997). A well-developed epidermis contains carbohydrates and cutin waxy surface covers the outside of the sisal leaf. Long fibre bundles (70-130 cm) existing under the epidermis in three or four parallel rows are extracted for line fibre, another row of thinner fibres runs across the median of the leaf with length ranging from (1.5-4 mm). The centre of the leaf consists of pithy material containing acidic plant juices (3.5-6 pHs), with the dominant chemical

species identified being amino acids, malic, citric, ascorbic, sugars, sapogenins and some amorphous cellulose pectin material (Lock 1969; Acland, 1972; Kimaro *et al.*, 1994; Hartemink and Wrenk 1995; Masanja *et al.*, 1995; Hurter, 1997)

During the 7-12 year of life in the field, sisal plant can produce between 200 to 250 leaves at a rate of 2-3 per month. As they mature, they tend to be horizontally oriented and wither if they stay uncut for much longer time. Each mature leaf contains about 1100 creamy white fibre, which runs the whole length of a blade from basal to apex. Although very numerous fibres from about 3% of the leaf and 2% of the total, plant the remainder consists of a cuticle vascular tissue and fleshy mesophyll (Lock 1969; Acland, 1972). On decortications these makes most of the biodegradable liquid discharged into the nearby streams. Also discarded is a highly cutinised and extremely sharp spine at the leaf apex.

2.2.5 Sisal pole and bulbils

Sisal pole appears at the end of the sisal life cycle, which could also weigh 15-25 kg on a wet basis. At this point, no much leaves are produced and pole normally grow very rapidly and may reach 6 m length. At this stage, an inflorescence occurs but seeds are seldom formed (Figure 2.3).

Figure 2.3: Matured sisal plant and bulbils for propagations (Hurter, 1997)

Instead of seed on the inflorescence, the miniature plants called the bulbils are formed (Lock 1969; Acland, 1972; Hurter, 1997). One pole can produce as many as 3000 bulbils (Figure 2.3). These when matured they reach about 3-5 cm and falls to the ground with their rudimentary roots. As they are easier to collect, they form excellent planting materials (Acland, 1972; Hurter, 1997).

2.3 Sisal Bole Juice – Inulin

Sisal inulin from *A. tequila* Azul Weber variety is the most studied of all *Agave sp.* Several workers for example Praznik *et al.,* (2002) analyzed plants harvested before flowering after 6 years from planting. Analyses resulted into the following; (a) in the heart of *Agave* and basal region of leaf no monosaccharide were found but very low

concentrations of sucrose (0.5-1.3%) was detected. (b) 5.2% mono (glu/fru) and 9.3% sucrose was found in the middle region of the leaf. (c) The concentration of fructo-oligomers with degree of polymerization (DP) 3-10 in heart and basal region of the leaf was 35-43%, while 60% was in the middle region. Thin layer chromatograms identified trisaccharides neokestose, 1-kestose and 6-kestose (Praznik *et al.*, 2002).

Table 2.1: Characteristics of sisal juice (*Hosea, 1996; **Masanja *et al.*, 1995)

*Trace elements	Amount
Magnesium	23.21±3.03 (mg/kg)
Iron	0.37±0.06 (mg/kg)
Zinc	0.09±0.01 (mg/kg)
Copper	0.02±0.01 (mg/kg)
Manganese	8.94±1.05 (mg/kg)
**Component	Sisal Juice Bole
Lactose	21.10 (mill mol/litre)
Glucose	13.27 (mill mol/litre)
Galactose	78.29 (mill mol/litre)
Siccinate	5.91 (mill mol/litre)
Lactate	8.68 (mill mol/litre)
Glycerol	3.95 (mill mol/litre)
Acetate	57.04 (mill mol/litre)
Ethanol	13.24 (mill mol/litre)
Pro-Syre (poly-alkencarboxyl-syre)	57.88 (mill mol/litre)
Inulin	14.3-24.1 (%)
Water	62-70 (%)

The higher fructo-polymers content 56-64% with DP 11-60 were found between heart and basal region of the leaf and only 30% was found in the middle region of the leaf, with calculated percentage weight (DPw) value of 14 and polymerization (DPn) value of 8 for heart and basal region of the leaf. DPw and DPn for middle of the leaf was 8 and 4 (Praznik *et al.*, 2002). This evidence suggested utilization of fructo-

oligosaccharides from other species for example *Agave* hybrid 11648 (Masanja *et al.*, 1995; Hosea, 1996; Massala, 2003; Ngonyani *et al.*, 2006).

2.4 Inulin-Fructooligosaccharides Background

Inulin is a naturally occurring carbohydrate in form of non-digestible oligosaccharides (NDO) produced naturally in some bacteria and in over 36,000 plants worldwide including 1,200 native grasses belonging to 10 families (Edelman and Jefford, 1968; Hill and Kolb, 2001). These plants are widely spread within the *Liliaceae* (3,500 species), such as the *Agaves* and vegetables e.g. onion, garlic, and asparagus. The frequently encountered inulin plants belongs to the *Compositae* (25,000 species) for example the *Jerusalem artichoke* and *chicory*. Others are wheat, rye and banana (De Leenheer, 1996).

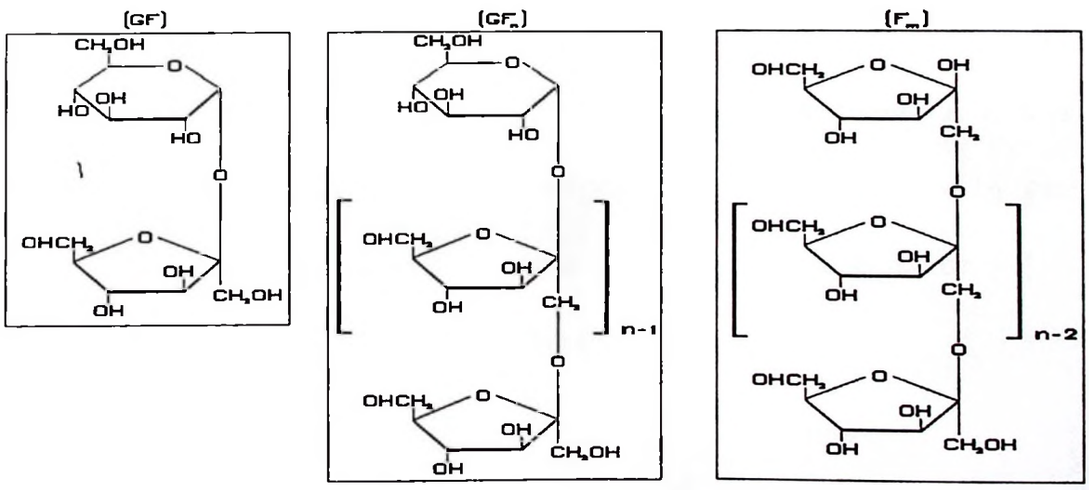


Figure 2.4: Structures of sucrose (left) Inulin (centre) oligofructose (right)

The joint International Union of Pure and Applied Chemistry, International Union of Biochemistry (IUPAC-IUB) and Joint Commission on Biochemical Nomenclature

(JCBN) agreed the gross molecular formula of inulin (Figure 2.4), to be GF_n, while G being a terminal glucosyl unit. While letter, F stands for the fructosyl units and "n" representing the number of fructosyl units hence both GF_n and F_m compounds are inulins (De Leenheer, 1996).

$$GF + GF \xrightarrow{SST} GFF + G \qquad [3]$$

As one plant of special status, the *Agave azul Tequila* Weber (*Liliaceae*) when analysed indicated that it contained 24% inulin. Its highly branched inulin molecule contains β (2→1) and β (2→6) linkages (De Leenheer, 1996). This suggested exploration of more *Agave* species such as the Tanzanian *Agave* hybrid H 11648.

In vivo production of inulin by living organism such as fungi occurs via sucrose: sucrose fructrsyltransferase (SST) route Equation [3], where trisaccharides (Isokestose kestose and neokestose) and a glucose molecule from two sucrose molecules which are released through tri-saccharide production (Edelman and Jefford, 1968; Hill and Kolb, 2001). The polymeric chain is lengthened by vacuolar enzyme fructan-fructan fructosyl transferase (FFT) to sucrose equation [4], which suggests possibility of enzyme bioprocessing of inulin.

$$G(F)_n + G(F)_m \xrightarrow{FFT} G(F)_{n-1} + G(F)_{m+1} \qquad [4]$$

The degree of polymerization (DP) of inulin as well as the presence of branches influences the bioprocess functionality. Internal hydrolysis of inulin by endoinulinase

lowers degree of polymerisation F_m and GF_n molecules allowing plants to osmoregulate surviving in winter and drought stricken regions (Edelman and Jefford, 1968). Lowering of DP under wet conditions triggers sisal putrefactions.

2.4.1 Inulin hydrolysis

Inulin hydrolysis is a very important aspect in citric acid production because the mega molecule is thereby reduced into relatively smaller molecules that are easily assimilated into microbial cells (Table 2.1). The general stoichiometry reaction for Inulin hydrolysis is shown in equation (catalyst could be either enzyme or acid).

$$2n(C_6H_{10}O_5)+nH_2O \xrightarrow{\text{catalyst}} n(C_{12}H_{22}O_{11}) \qquad [5]$$

Hydrolysis of Inulin at normal temperature and pressure (NTP) proceeds at slower pace due to steric hindrance and/or inductive effects to the glycosidic bonds between the fructose-glucose monosaccharide molecules. The increase in n-number of attached groups may contribute to conformation in the structure of the monosaccharide resulting in lower hydrolysate yield of glucolysic bonds as degree of polymerization increases (Edelman and Jefford, 1968; De Leenheer, 1996; Hill and Kolb, 2001).

The hydrolysis equilibrium rate for a given polymer sugar depends up on pH and temperature of the reactant mixture. For example, it takes 360 minutes (6 hours) for Inulin to attain 50% hydrolysis, when treated with 1N H_2SO_4 at 20°C. Thus low pH

and elevated temperatures results in high hydrolysates yield (Smart and Whistler, 1953; Edelman and Jefford, 1968). Rise in temperature, increases activation energy that facilitates molecular bond breaking, hence producing free sugar monomer molecules. This, allude to the need for studying hydrolysis-yield optimization procedures.

2.4.2 Factors affecting Inulin hydrolysis

Since Inulin is a collection of fructose molecules joined by a fructosyl moiety, when hydrolysed it behaves more like fructose as opposed to glucose (Lindhorst, 2007). Fructose differs from glucose by the position of a carbonyl functional group; the differences in the functional groups more clarified in the linear forms (Figure 2.5).

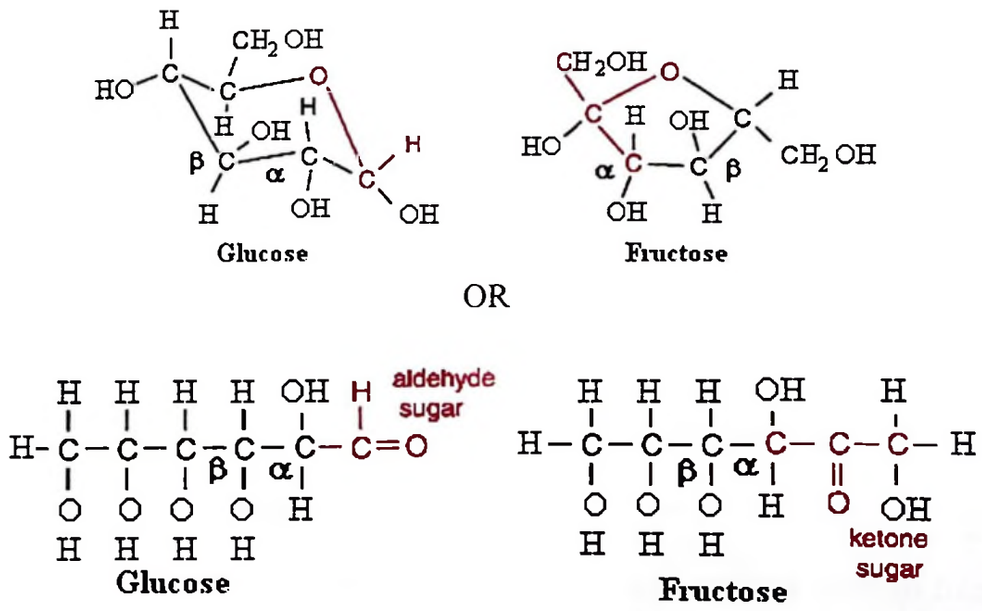
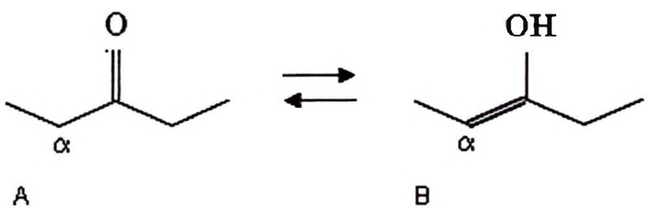


Figure 2.5: Presentation of glucose and fructose molecules.

The carbonyl group in both fructose and glucose are very electron withdrawing, thus affecting the adjacent carbons by induction. Using the carbonyl group as a reference, adjacent carbons are named after Greek letters in order of closeness to the carbonyl group. Alpha (α) carbons are directly, attached to the carbonyl group; beta (β) carbons are connected to alpha carbons, gamma (γ) to beta (β), and so on (Morrison and Boyd, 1992).

Due to the inductive effect of the partial positive charge on the carbonyl carbon of a ketone or aldehyde, as well as the stabilizing resonance of the double bond between the hydroxyl group and conjugated carbons to the carbonyl group (ketone molecule A), the alpha (α) hydrogen's are acidic, meaning they are prone to removal.



[6]

In presence of an acid (H^+) or a base (OH^-), the aldehyde or ketone group migrate to form double bond between the carbonyl and the alpha (α) carbon (enol molecule B). In presence of an acid, protonation of the oxygen group occur, thus water molecule is attracted to an alpha (α) hydrogen. Where as in the presence of a base, deprotonation of the alpha hydrogen occur, and the carbonyl oxygen abstracts hydrogen from water (Kotz and Purcell, 1991; Morrison and Boyd, 1992).

This is an important feature of ketone and aldehydes, and is known as the keto-enolic

tautomery or keto-enol tautomerism, which also occurs during the hydrolysis of Inulin molecule in aqueous acidic conditions equation [6] (Kotz and Purcell, 1991; Morrison and Boyd, 1992).

Inulin like other carbonyl compounds and their enols interchange rapidly by proton transfers catalyzed by acids or bases, mediated by the enolate or the proton source, even if they are in trace amounts. The explanations would be that, if the difference in pK_b between the base and the enolate is so large (then at low pH i.e. at high concentrations of H^+) the equilibration leading to the thermodynamic product a ketone (fructose molecules) is likely. Kotz and Purcell, (1991) and also Morrison and Boyd, (1992) explained that, in such reactions proton exchange occur between the kinetic enolate e.g. (Inulin) and as-yet-unreacted ketones e.g. (fructosyl units).

The converse is also true i.e., if the difference in pK_b between the base (ketone) and the enolate is so small (then at high pH low concentration of H^+), the reaction is essentially reversible, so the equilibration leading to the thermodynamic product a ketone (fructosyl units) is unlikely (Morrison and Boyd, 1992).

Such reactions are described thermodynamically as the distinction phenomenon (also known as selectivity). The distinction phenomenon thermodynamically is when kinetically controlled chemical reaction pathways (e.g. Inulin hydrolysis) occurs and the kinetically controlled product **A** (GF_{n-1}) forms faster than thermodynamically controlled product **B** (F). The reason could be that the activation energy for product **A** is lower than that for product **B** (Kotz and Purcell, 1991), shown by equation [6].

At low temperature, the temperature-catalysed hydrolysis of Inulin molecule is controlled by the thermodynamic reaction, thus producing the thermodynamic product-ketone (fructosyl units) or aldehyde (glucosyl unit). During that temperature range, protonation of an enolate ion Inulin (GF_n) produces kinetic product-enol (GF_{n-1}) at a fast rate, while producing the thermodynamic product-ketone fructosyl units (F) or aldehyde (glucosyl unit) at a very slow rate. In this case, equilibrium maintains the formation of product-enol (GF_{n-1}) of different degrees of polymerisation.

At higher temperatures, temperature-catalysed hydrolysis of Inulin molecule is controlled by kinetic reaction, producing the kinetic product-enol (GF_{n-1}). During that temperature range protonation of an enolate ion (Inulin), produces the thermodynamic product-ketone fructosyl units (F) or aldehyde (glucosyl unit) at a fast rate, while producing kinetic product-enol (GF_{n-1}) at a very slow rate. In this case, equilibrium maintains the formation of the thermodynamic product-ketone (fructosyl units) or aldehyde (glucosyl units).

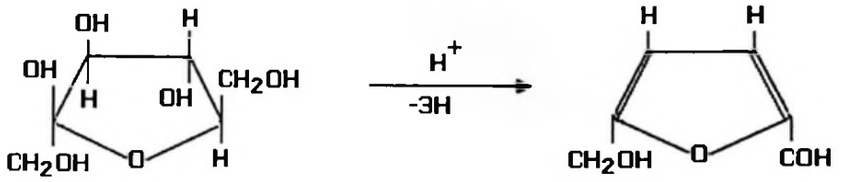
As the major constituent of Inulin hydrolysates is therefore fructose, a more ketone-enolic characteristics dominate during the hydrolysis, thus thermodynamically the distinction phenomenon is assumed to prevail. Therefore, the rate of Inulin hydrolysis is affected by reaction temperature, pH and time:

Reaction pH: Hydrolysis at low pH is considered suitable for the hydrolysis as the protonation of the carbonyl group occurs in acidic environment. This in turn weakens

the glycolysic bonds by Vanda Waal forces, which pulls water molecules towards an alpha (α) hydrogen. Sufficient values of pH reported were between 1 and 2 (Lee, 1995; Abasaeed and Lee, 1995; Abasaeed and Lee, 1996). However, extremely low pH and excess (H^+) in other hand results in formation of stable hydrides such as hydroxymethylfurfural, which lowers the total sugars yield.

Reaction Temperature: Hydrolysis at high temperature increases the activation energy, which in turn favours Inulin hydrolysis. However, other side reactions like Maillard, caramelization and pyrolysis results in undesired products. Böhm *et al*, (2005) worked on heat induced Inulin degradation, in their work they found that thermal treatment of Inulin leads to a degradation of the long fructose chains and formation of new products, most likely di-D-fructose dianhydrides. However, degradation products of Inulin were normally cleavable by acid to fructose monomers, but their glycosidic bonds were no longer accessible for β-fructosidase, thus the combined heat and enzyme treatments could equally produce lower fructose yield (Böhm *et al*, 2005; Lindhorst, 2007).

Reaction time: Hydrolysis at prolonged reaction time favours product formation. However, other side reactions occurs to the resultant monomer sugars, thereby forming undesired products in a way that more time does not necessarily increase yield. In his findings Böhm *et al*, (2005) demonstrated that di-D-fructose dianhydrides together with some minor compounds are likely to occur after thermal treatment of inulin especially in the presence of citric acid. Their concentrations increased with heating time and temperature (Lindhorst, 2007).



Fructose

Hydroxymethyl Furfural
(HMF)

In many cases, excessive Maillard products are not appreciated in culture media. Some of them for example Hydromethyl Furfurals are growth inhibitors thus obstructs metabolism of culture consequently metabolite yields are lowered (Nirupama *et al.,* 1981; Kim and Lee, 2004).

2.4.3 Different methods for Inulin hydrolysis

Fructose is gaining popularity as feedstock in fermentation production processes hence there is a need for finding alternative sources for example from sisal boles. Recovery processes of fructose from Inulin are either by thermal, enzymatic or chemical hydrolysis. Both methods have merits and demerits (Lindhorst, 2007).

Enzyme hydrolysis requires commercial hydrolytic enzymes such as Fructanase and inulinase. These are expensive when considering a big production volume. Enzymes optimum operating conditions for example salt tolerance pH range, temperature and concentration of medium hinders hydrolysis efficiency. Low level of enzyme activity prolongs processing time and encourages microorganisms' contamination. Study by Zhang *et al.,* (2004), discussed that during enzyme hydrolysis, glucose inhibits inulinase-catalyzed hydrolysis of Inulin, thus for the process, optimization glucose

needs to be removed from hydrolysates matrix, either by simultaneously application of both exoinulinase and endoinulinase gives better results.

The advantage of enzymatic hydrolysis is the production of high purity fructose syrup that has a desirable taste, smell and colour. It produces lower levels of undesired by-products those frequently encountered during the acid-catalyzed hydrolysis e.g. fructose di-anhydride and other products from base-catalyzed aldol condensation. However, they tend to increase hydrolysis cost, because of an increased processing time, while the recovery process by using organic solvents, may not be preferred under certain circumstances (Zhang *et al.,* 2004).

Thermal hydrolysis by autoclaving or oven cooking is a well known method in fructose syrup recovery from *Agave* plants, especially where colour, taste and aroma of caramelized fructose syrup may not be critical for example as feedstock in industrial processing. In this case, the capital investment necessary to run a thermal process is low and the processing time is acceptable, resulting fructose syrup has a relatively low purity as it is typically yellow or brownish, and often includes contaminants, such as hydroxymethylfurfural, which may be toxic (Böhm *et al.,* 2005).

Acid hydrolysis process for producing fructose syrup from plant and cellulose materials, involves the use of a mineral acid such as sulphuric or hydrofluoric acid. While the capital investment necessary to run an acid hydrolysis process is low and the processing time is favourable, the resulting fructose syrup is generally of a

medium purity and having a yellowish or brownish tint. Such syrup may also contain contaminants such as hydroxymethylfurfural (Lindhorst, 2007).

2.4.4 Combined acid and thermal hydrolysis

Combined acid and temperature hydrolysis compromises use of larger quantities of mineral acid in one hand and the use of excessive heat applications. A number of researchers such as Saeman (1945), studied effect of dilute acid hydrolysis at elevated temperatures and strong acids at lower temperatures.

Saeman (1945) showed that the formation of glucose from cellulose is of the first order reactions equation [7]:

$$Cellulose \xrightarrow{k_1} Glucose \xrightarrow{k_2} Degradation\ product \qquad [7]$$

Where k_1 and k_2 are first order rate constants.

Therefore maximum glucose concentration is functions of the rate constants only see equation [7] and [9]. The calculated maximum potential yield increased from 10% at $k_r = 0.2$, 50%, at $k_r = 2.5$ and 80%, at $k_r = 14$ (Saeman, 1945).

Since ratio of rate constants increases with temperature therefore yield increases. Goldstein (1981), found that selectivity in acid hydrolysis increases with acid concentration (higher concentrations of H^+). Acid hydrolysis begins with the

formation of conjugate acid by H^+, which leads to cleavage of glycosidic bonds to form two fructose molecules on water addition, which is highly appreciated. Similar cleavage when formed by thermal hydrolysis tends to shift polymerization into oligomer (poly fructose molecules); therefore, combined pH and temperature could assist in maintaining the forward equilibrium reaction favouring fructose formation (Kotz and Purcell, 1991; Morrison and Boyd, 1992; Hill and Kolb, 2001).

2.4.5 Hydrolysis reaction kinetics and rate constant

Reaction rate constant is evaluated as in equations [8] to [11], assuming first order reaction:

$$\frac{dC}{dT} = -kC \qquad [8]$$

Integrating Equation [8] gives

$$\frac{\ln C_0}{C} = -k_t \qquad [9]$$

Where:

k = reaction rate constant
C_o = initial Inulin concentration
C = residual Inulin concentration (g/l)
t = reaction time (minutes)
T = temperature (K)

Activation Energy

The rates of most hydrolysis reaction are very sensitive to temperature, an increase in temperature by 10°C causes an approximate doubling of rate constant (Kalliat, 2008).

Arrhenius proposed an empirical equation [10] based on many observations (Kotz and Purcell, 1991; Moran and Shapiro, 2000).

$$k = A\exp(\frac{-Ea}{RT}) \quad [10]$$

Where:

A = pre-exponential factor
E_a = activation energy (J/mole/K)
T = Temperature (K)
(R) = Universal molar gas constant ($JK^{-1}mol^{-1}$)

Recall: $\Delta G^{o} = -RT \ln K$ & $\ln k = \frac{\Delta G^{\circ}}{-RT}$; Re-writing Equation [10] gives Equation [11]

$$k = \exp(\frac{-G}{RT}) \quad [11]$$

Where:

G = Gibbs activation energy

K bears a strong resemblance to equilibrium constant *k*, a function of Gibbs free energy. Experimentally equation [10] can be rewritten using equation [12]

$$\ln(k) = \ln(A) - \frac{E_a}{R}\left(\frac{1}{T}\right) \quad [12]$$

When plotted as ln (k) Vs (1/T), the value of "y-intercept" corresponds to ln (A), which defines, pre-exponential factor (A) a constant of proportionality to be calculated from intercept and the universal molar gas constant (R) = 8.314472(15) $JK^{-1}mol^{-1}[1.8x10^{-6}]$ (Mohr, *et al* 2008). The activation energy is the minimum amount of energy required to initiate a reaction ($KJmol^{-1}$), the gradient of the line

will be equal to ($-E_a/R$) from which the activation energy (E_a) can be calculated (Kotz and Purcell, 1991; Kyle, 1992; Moran and Shapiro, 2000).

2.5 The fungi *Aspergillus niger*

A. niger is a filamentous ascomycete fungus that is found everywhere in the environment and has been implicated in opportunistic infections of humans. This organism is normally found in soil organic layer (humic layer) with a wide array of hydrolytic and oxidative enzymes involved in breaking down of plant lignocelluloses' into simpler molecules such as carboxylic acids (Kubicek *et al.*, 1994). *A. niger* is a common member of the microbial communities found in soils; therefore, it is known to play a significant role in the global carbon cycle through production of a variety of enzymes that are important in biotechnology industry and in studying eukaryotic protein secretions for biomass degradation. This fungus belongs to a group that includes the notorious *A. flavus* that often causes aflatoxins (Kubicek *et al.*, 1994).

Sequenced genomes of different strains of *A. niger* for example of a wild type *A. niger* ATCC 1015, has resulted in the first patented citric acid process that was accepted for sequencing through the US Department of Energy (DOE) Microbial Genome Program (MGP) (Kubicek *et al.*, 1994). Citric acid is a carboxylic acid produced at over one million tons annually making *A. niger* a model in fungal fermentation process.

2.5.1 *Aspergillus niger* fungi in citric acid production

Until about 1920 all commercial citric acid were from lemon and lime. Because of its numerous applications, the volume of citric acid production by fermentation has increased exponentially (Jianlong and Ping, 1998). Various yeasts moulds and fungi have been used in industrial citric acid production (Pazouki *et al.*, 2000). Among the moulds and fungi species that are used in citric acid production such as yeast, *A. niger* has been preferred because of many aspects including its widely distribution in nature and persistent spores (Table 2.2).

Table 2.2: Comparison of citric acid yield (γ) from common feedstocks

Raw material in citric acid submerged fermentation	Strain	γ(citric acid) kg/m^3	Yield%
Beet molasses[1]	*A. niger* ATTC 9142	109	
Sisal bole juice[2]	*A. niger* GCM 7	83.65	67-83
Sisal bole juice[3]	*A. niger* DSMZ 8	54	68.7
Black strap molasses[5]	*A. niger* GCM 7	86	-
Brewery wastes[5]	*A. niger* ATTC 9142	19	78.5
Cane molasses[5]	*A. niger* GCMC-7	11.36	100
Carob pod extract[4]	*A. niger*	86	
Coconut oil[4]	*C lipolytica* N-5704	-	99.6
Corn starch[4]	*A. niger* IM-155	-	62
Date syrup[4]	*A. niger* ATTC 9142	-	50
Glycerol[4]	*C lipolytica* N-5704	-	58.8
Hydrolysate starch[4]	*Y lipolytica* A-101	-	75
	A. niger UE-1	74	49
Palm oil[5]	*C lipolytica* N-5704	-	15.5
Rapeseed oil[5]	*Y lipolytica* A-101	-	57
	A. niger	-	11.5
Soybean oil[5]	*Y lipolytica* A-101	-	63
Wood hemicelluloses[5]	*A. niger* IMI-41874	27	45
Yam bean starch[5]	*A. niger* YW-112	-	74

Note: Referred in Table 2.2 [1, 4, 5] based on sugar consumed (Soccol *et al.*, 2006), [2]reported by (Massala, 2003), [3]sisal Inulin feed stocks (Ngonyani, 2010).

In their findings, Schuster, *et al.*, (2002), Soccol *et al.*, (2006), indicated that *A. niger* has a profound ability to ferment a variety of cheap raw materials, easy in handling and comparatively high citric acid yields per unit substrate consumed at low pH without the secretion of toxic by-products besides citric acid. However, some strains of *A. niger* accumulate other organic acids as well, e.g. oxalic, malic, tartaric and pyruvic acids under specific fermentation conditions (Schuster, *et al.*, 2002).

In contrary, industrial strains that produce commercial citric acid are not freely available and only a few can be obtained from international culture collections. Screening of the native well-acclimatized fungi from sisal bole rot is the best strategy in the establishment of the future production strains (Table 2.2).

2.6 Properties and Production of Citric Acid

Citric acid is a chemical with a chemical abstracts service (CAS) registry number [77-92-9] and IUPAC name (2-hydroxy-1, 2, 3-propanetricarboxylic acid). This tri-carboxylic organic acid has a chemical formula $C_6H_8O_7$ and is soluble in water with a pleasant taste; therefore, it is the most versatile and widely used in the food industries (Bailey and Ollis, 1986; Othmer, 2001). Although it occurs in rather high concentrations in citrus fruits, citric acid is ubiquitous in nature; it is formed as an intermediate in the citric acid cycle. Being a natural common constituent metabolite of plants and animals citric acid occurs widely in for example in fruits of the citrus species such as lemons (4.0-8.0%) and grapefruit (1.2-2.1%).

Citric acid occurs in all animal tissue and fluid e.g. human whole blood is about 15 ppm where as in the human blood serum is approximately 1 mg/kg of body weight, while the daily excretion totals (0.2-1.0g). During the oxidative metabolism process commonly referred as "Krebs cycle", citric acid is produced by different microorganisms for example bacteria yeasts and moulds including *A. niger*, *Citromyces pfefferianus* and *C. glaber* (Othmer, 2001).

2.6.1 Physical properties of citric acid

Anhydrous citric acid has the molecular weight of 192.13 and crystallizes from hot concentrated aqueous solution. While producing anhydrous translucent holohedral monoclinic crystals with melting point value of 153°C and density of 1.66 g/cc. They are optical inactive and manifests no piezoelectric effect. Citric acid is a relative strong acid; the 1st, 2nd and 3rd dissociation constants at 18°C are, $K_1 = 8.2 \times 10^{-4}$, $K_2 = 1.77 \times 10^{-5}$ and $K_3 = 3.9 \times 10^{-7}$, respectively. The pK values at 25°C are $pK_1 = 3.128$, $pK_2 = 4.128$ and $pK_3 = 6.396$ (Othmer, 2001).

2.6.2 Chemical properties of citric acid

When heated to 175°C citric acid is partial converted to aconitic acid by elimination of water and to acetonedicarboxylic acid by loss of carbon dioxide and water. Acetonedicarboxylic acid in turn decomposes to form acetone an important solvent of many industrial applications (Othmer, 2001). As a tri-basic acid, citric acid manifests the polybasic properties by forming a variety of salts and organic compounds such as esters amides and acryl halides. In aqueous solution, citric acid

can be mildly corrosive towards carbon steel and therefore precaution should be taken in assembling processing equipments. In aqueous media citric acid's hydroxyl and carboxylic acid groups acts as multi-dentate cheleate ligands which supports many industrial processes for example in polymer chemistry.

2.6.3 Production of citric acid and uses

Citric acid is produced either in an anhydrous form or as a monohydrate. The transition temperature between the two forms is 366°C. As the anhydrous form is obtained by crystallization from hot aqueous solutions, the monohydrate is obtained by crystallization at temperature below 366°C. Both forms have a high market demand. The worldwide sales distribution is estimated to be; (75%) food industry, (10%) pharmaceutical industry and (15%) chemical industries (Othmer, 2001).

The widespread presence of citric acid in the animal and plant kingdoms is an assurance of its non-toxic nature and it has long been used as acidulate in the manufacture in food, in toiletries, pharmaceutical and detergents industry, as a sequestering, neutralization agent for bases and as a pH control buffer. In the pharmaceutical field, citric acid stabilizes ascorbic acid and bicarbonates in antacid drugs. In preparations employing basic substances, citric acid is used as anionic active agent. Trisodium citrate is widely used as a blood preservative where it prevents clotting by complexing calcium. Ferric ammonium citrate is widely used in the treatment of anaemia. Citric acid esters, in particular the triethyl, tributyl and acetyltributyl esters are used in manufacturing of non-toxic food grade plastic films.

Likewise, monostearyl citrate is used instead of citric acid as an antioxidant in oils and fats as it is more easily incorporated than the free acid. Ammonium ferric citrate is used in photography for making blueprint "Turnbull's blue" (Othmer, 2001).

The use of citric acid as a good acidulate depends in part on its strength as an acid. A polybasic citric acid is able to complex heavy metals such as iron and copper. This property has led to its increasing use as a stabilizer of oils and fats where it greatly reduces oxidation catalysed by these metals; the ability to complex metals combined with its low degree of attack on special steels allows the use of solutions of citric acid in the cleaning of power station boilers and similar installations. In the areas where there are restrictions on phosphates in detergents, trisodium citrate is replacing phosphates in heavy-duty cleaning liquids.

In bioremediation of polluted soils and in removal of sulphur dioxide from flue gases of power stations and metal smelters, a buffer solution containing principally H_2Cit is used as scrubbing agent. A complex ion $H_2CitHSO_{2/3}$ formed in a second stage reacts with H_2S to produce elemental sulphur that is recycled (Wasay *et al.*, 1998).

2.6.4 Citric acid cycle and its physiological role

Citric acid occurs in the terminal oxidative metabolic system commonly referred as "Krebs tricarboxylic acid cycle" a metabolism process presented by Figure 2.6. This process involves the conversion of carbohydrates, fats or proteins into CO_2 and H_2O, with release of copious amount of energy (Jianlong, 2000; Taiz and Zeiger, 1998).

Tricarboxylic acid (TCA) cycle plays an important physiological role in generating high-energy fuel molecule (ATP) or producing intermediate metabolites such as organic acids for various purposes in the cell functioning through catabolic or metabolic process (Figure 2.6).

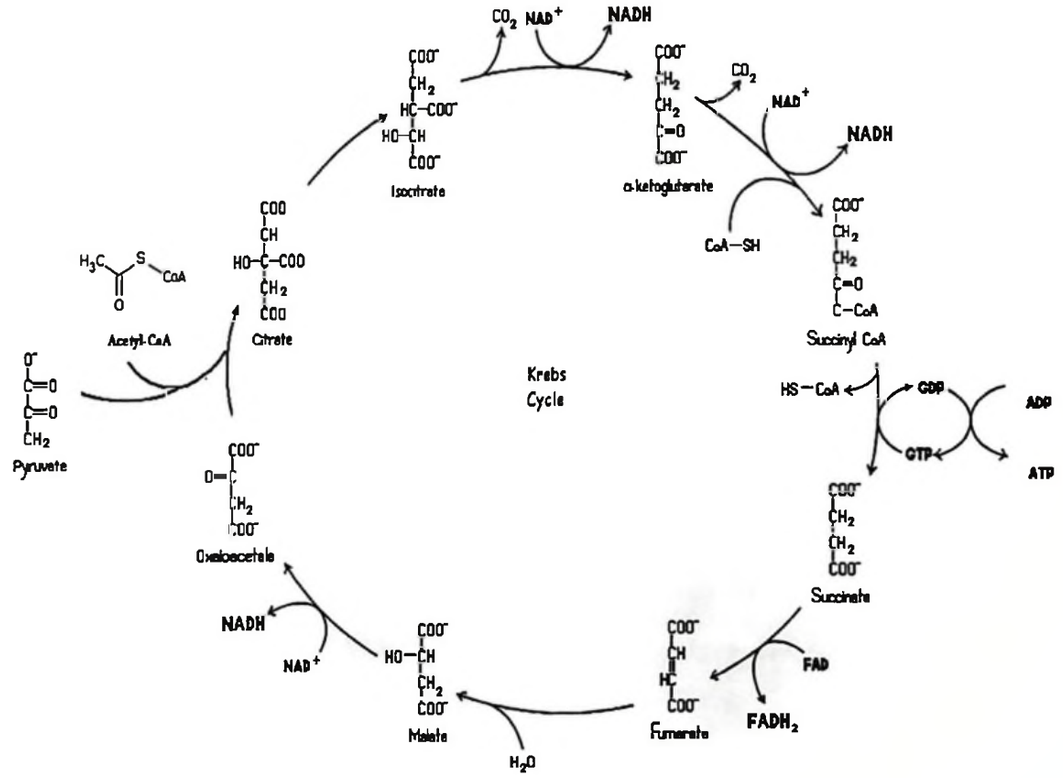


Figure 2.6: Krebs carboxylic acid cycle

When cell requires excess energy for cell propagation or maintenance, TCA cycle and electron transport/oxidative phosphorylation, harnesses all three processes for more energy generation (Figure 2.6). It is during cellular respiration process, where a complete oxidation of glucose to CO_2 and H_2O produces high-energy fuel molecules (32 ATPs) per one molecule of glucose shown by equation [13].

Complete respiration energy is produced

$$C_6H_{12}O_6 + 6O_2 + 6H_2O + 32ADP + 32P_i \longrightarrow 6CO_2 + 12H_2O + 32ATP + \Delta E \quad [13]$$

Citric acid cycle occurs during the catabolic pathway of hexoses to pyruvate and Acetyl-Coenzyme A (Acetyl-CoA) by glycolysis (Alvares-Vasquez *et al.*, 2000). Glucose is an important starting carbohydrate source in glycolysis hence it plays an important role in citric acid production. According to Wayman and Mattey, (2000) over 90% of glucose can be converted into citric acid mainly under optimal fermentation conditions described by stoichiometric equation [14]. Full respiration process stops when the cell produces citric acid instead of CO_2 during glucolysis.

Incomplete respiration citric acid is produced

$$C_6H_{12}O_6 + 15H_2O + 32ADP + 32P_i \longrightarrow C_6H_8O_7 + 2H_2O + 32ATP \quad [14]$$

The reason for production of citric acid by strains of *A. niger* is not clear, however several theories related to biological competition against enemies and the increase of ionic mobility of insoluble forms of trace elements or nutrients such as copper calcium and zinc prevails. Phosphate can easily solubilised by fungal citric acid, and their mobility is thus increased.

2.6.5 Chemistry behind citric acid metabolic pathways

During metabolism of glucose, pyruvate CH_3-$(CO)_2$-O- which is produced by glycolysis and contains a lot of reducing power is formed (recall oxidation states for each of its carbon atom as compared to carbon's oxidation state in CO_2). This reducing power is utilized by cell through the citric acid cycle as seen in Figure 2.6.

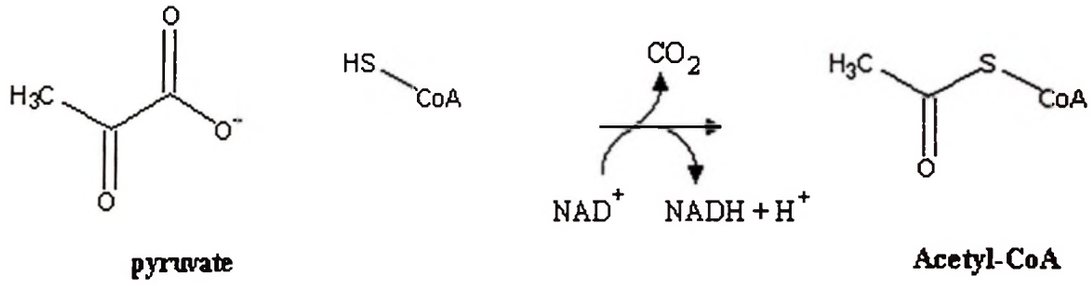


Figure 2.7: Decarboxylation of pyruvate to acetyl-CoA

During the first stage of citric acid cycle, decarboxylation of pyruvate to acetyl-CoA "an activated form of acetate (CH_3COO-)" takes place. The forward reaction is catalyzed by pyruvate dehydrogenase, a complex enzyme with several cofactors named lipoamide, flavin adenine dinucleotide (FAD) and coenzyme-A (Figure 2.7). The hydrolysis of thioester bond (S-C = O) is highly exergonic and it demands high energy of formation (Meixner-Manori *et al.,* 1985; Alves da Silva, 2009).

Energy required for that reaction comes from pyruvate decarboxylation (in which three carbon atoms from pyruvate are involved, as compared to the acetyl portion of acetyl-CoA that posses only two). In the decarboxylation process, decarboxylate group is released in a form of CO_2 (Ruijter *et al.,* 1997). Therefore, energy from decarboxylations process is often used by cell to push equilibrium towards product

formation according to the Le-chatter's principle. This is an important link reaction as it forms an important link between the metabolic pathways of glycolysis and the citric acid cycle via a key enzyme acetyl-CoA.

2.6.6 Citric acid formation from pyruvate

The acetyl-CoA from the first reaction of the citric acid cycle, attacks the oxaloacetate ion to form citrate by an aldol addition. Thus, the thioester hydrolysis occurring helps to direct displace equilibrium towards citrate ion formation Figure 2.7. Without this reaction citrate would have been decarboxylated to yield a branched carbon compound, which is much harder to metabolize.

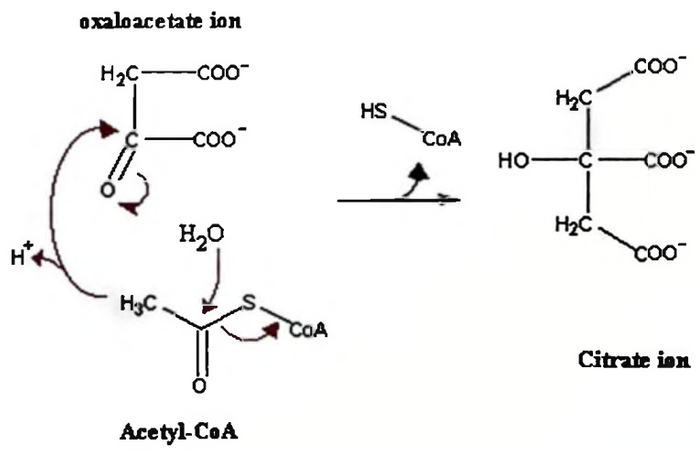


Figure 2.8: An aldol addition of acetyl-CoA to oxaloacetate to form citrate ion

During citric acid accumulations, the cycle is to a greater or lesser degree blocked, while another, so-called anaplerotic reaction that supplies the oxaloacetate takes over. This anplerotic reaction is the carboxylation of pyruvate-by-pyruvate carboxylase. Carboxylic cycle continues after citrate is being decarboxylated to α-ketoglutarate in presence of NAD^+ while producing CO_2 (Figure 2.9).

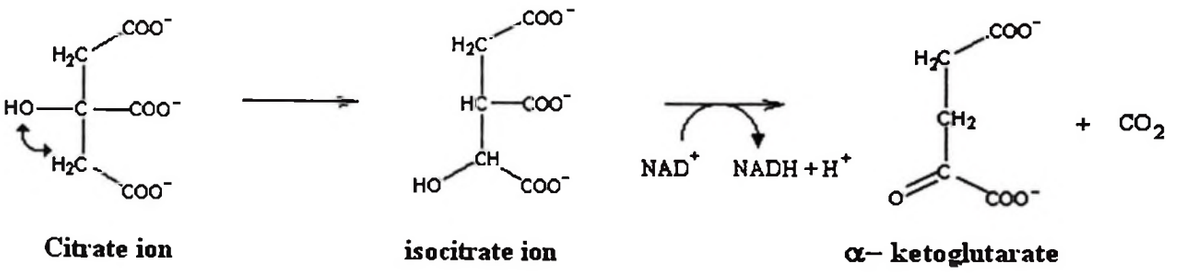


Figure 2.9: CO_2 Removal and α-ketoglutarate formation (Alves da Silva, 2009)

This reaction suggests that in order to achieve higher citric acid accumulations in industrial fermentation processing; α-ketoglutarate formation has to be minimized or stopped at this stage, because higher citric acid yield is achieved by the condensation of carbon dioxide and pyruvate to form oxaloacetic acid by pyruvate carboxylase. This is supported by studies by Martin and Wilson (1951), on the uptake of $^{14}CO_2$ by *A. niger* during the formation of citric acid, which suggested that somehow CO_2 fixation occurs at this stage. These findings suggest that recycling of CO_2 in fermentation broths would be of desired outcome, because carboxylase formation reverses equilibrium away from the formation of other carboxylic acids such as succinic acid etc.

2.6.7 Bio-chemistry of citric acid metabolic pathways

Work by Meixner-Manori *et al.*, (1985) indicated that pyruvate carboxylase could be isolated from *A. niger* thus justifying the existence of the condensation reaction. The pyruvate carboxylase enzyme required no acetyl coenzyme-A in its action, and pyruvate carboxylase inhibition by aspartate was minimal. Since thus, α-

ketoglutarate dehydrogenase was practically absent during citric acid accumulation therefore, aspartate activity was kept low hence citrate ion inhibition was minimized.

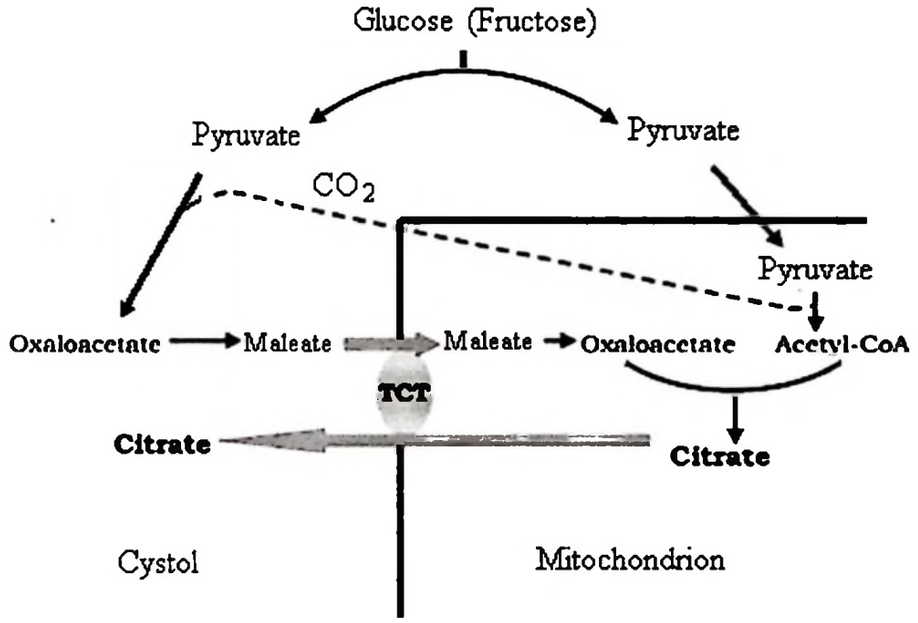


Figure 2.10: Citric acid formation by oxaloacetic acid through Tricarboxylate transporter (TCT), note a dotted line suggesting CO_2 fixation

Studies by several (Kubicek-Pranz *et al.*, 1990; Wolschek and Kubicek 1997; Wolschek and Kubicek, 1999; Karaffa and Kubicek, 2003; Zehentgruber, *et al.*, 2006) indicated that citric acid formation is related only to the oxaloacetic acid available at a time. Since citric synthase was poorly regulated, both citrate synthase and pyruvate carboxylase were not effectively regulated (Figure 2.10). The findings inferred that, the overall rate of citric acid formation is related to conversion rate of carbohydrate into pyruvate. Once the level of citric acid in the cells is elevated, acid has to be excreted to keep the reaction forward (Woronick and Johnson, 1960; Fier and Suzuki, 1969; Kubicek, *et al.*, 1988; Kubicek-Pranz *et al.*, 1990).

These findings are based on a very extensive research on sucrose, glucose and molasses those which are either rich in glucose of are in the ratio of 1:1 glucose: fructose (Kubicek-Pranz *et al.,* 1990). However, very scanty footage has been done when fructose is being utilized as the only carbon source in citric acid fermentation processing. In the present research, therefore the overall rate of citric acid formation was presumed to relate to rate of conversion of hydrolysate fructose into pyruvate just like in other traditional feedstock's (Figure 2.10).

2.6.8 Constraints for inhibition of citric acid catabolism

Tri-carboxylic acid cycle interruption at any stage by enzyme inhibition would cause a non-cyclic operation, which then leads to accumulation of its intermediates. Inhibition of α-ketoglutarate dehydrogenase, in this case will trigger citric acid overflow according to the equilibrium of the enzymes involved at this metabolic stage. Apart from the overflowing theory, Kubicek and Röhr (1985), verified that once citrate has accumulated to a certain concentration, it tends to inhibit its own catabolism thus facilitates accumulations of more acid.

This implies that, at this stage citrate will remain the only metabolite, which rapidly accumulates owing to a continuing supply of its precursors, e.g. acetyl coenzyme-A and oxaloacetate, regardless of the absence of iron or the presence of copper (Kubicek and Röhr, 1978; Kubicek and Röhr, 1980; Kubicek and Röhr, 1985; Kubicek, 1988). Therefore, according to the Le charters' principle, accumulation of aconitase is not necessary a hindrance because the equilibrium of the reaction of the

enzyme is strongly in the direction of citrate formation. These findings were supported by Meixner-Manori *et al.,* (1985); Legiša and Kidric, (1989); who suggested that the occurrence of enzymes iso-citric dehydrogenase (ICDH) and iso citric lyase (ICL) facilitated breaking down of iso-citric acid.

However, in these studies, the case of ICDH was more complex; as two enzymes, namely the cytoplasmic Nicotinamide adenine dinucleotide-H (NADH) and the mitochondrial Nicotinamide adenine dinucleotide phosphate ($NADP^+$) were involved. As the chemo Nicotinamide adenine dinucleotide phosphate-H (NADPH) enzyme showed up, the physiological concentrations of citrate seemed to inhibit the mitochondrial NADPH enzyme. This concequently resulted in further decarboxylation of pyruvate to acetyl-CoA, an activated form of acetate (CH_3COO-) which combines with oxaloacetate to form citrate ion.

In their work, Legiša and Mattey (1986) proposed that, the most probable mechanism, for initiation of citric acid formation could be by the accumulation of up to 1% glycerol by enlarged cells of *A. niger*. This accumulation causes the initial inhibition of NADP specific-iso citrate dehydrogenase resulting in citrate a build up via aconitase. Once the concentration of citrate reaches a critical value it brings about the feed-forward inhibition of NADPH specific iso citrate dehydrogenase despite the assimilation of glycerol from the medium (Mischak *et al.,* 1984; Mattey, 2006).

This notion seemed to be valid especially when the influence of glucose concentration on citric acid production is correlated to morphology of *A. niger*

(Papagianni *et al.,* 1999a). Many findings therefore explained that the overall success of citric acid production therefore depends on TCA manipulations. However, such generalisation when using sugars other than glucose, such as hydrolysates sugars from sisal Inulin, fungi glycerol accumulation and in the presence a keto-enolic fructose (the main hydrolysates constituent) during citric acid accumulations necessitated more studies.

2.6.9 Production of other carboxylic acids

The carboxylic cycle continues after the isomerisation of citrate to iso citrate. Iso citrate is being decarboxylated to α-ketoglutarate in existence of NAD^+, thus producing NADH, H^+ and CO_2 (Alves da Silva, 2009). The enzyme α-ketoglutarate is characterised by its α-keto acid nature (i.e. it contains a carbonyl group adjacent to a carboxylic acid) which undergoes decarboxylation (i.e. reacts like pyruvate thus yielding enough energy) during the formation of a thioester bond with coenzyme-A (α-ketoglutarate dehydrogenase).

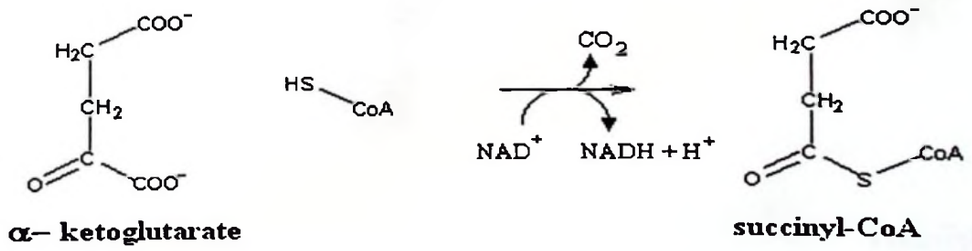


Figure 2.11: Formation of a thioester succinyl-CoA through α-ketoglutarate

The reaction shown by Figure 2.11 is quite similar to pyruvate dehydrogenase in terms of composition, cofactors and mechanism. Like other energetic thioester

bonds, presence of that in succinyl-CoA facilitates the hydrolysis to produce ATP 'the only step in the citric acid cycle where direct production of ATP or equivalent occurs' (Alves da Silva, 2009).

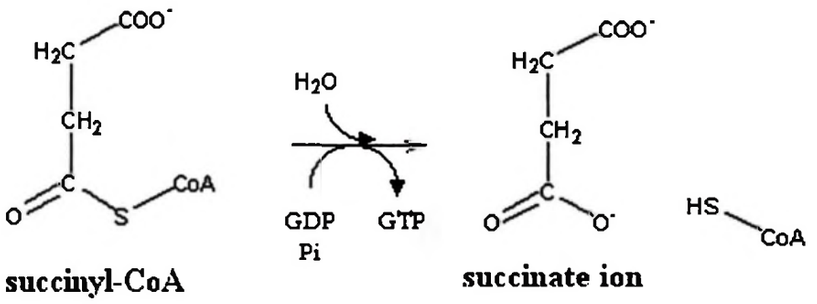


Figure 2.12: Formation of a succinate ion by succinyl-CoA

Like in oxaloacetate, succinate a four-carbon product from the last reactions, which occurred in citric acid cycle regenerates oxaloacetate from succinate Figure 2.12). Succinate is firstly, oxidized to fumarate by the succinate dehydrogenase complex 'also known as complex II, which is present in the amtrix side of the inner mitochondrial membrane' (Alves da Silva, 2009).

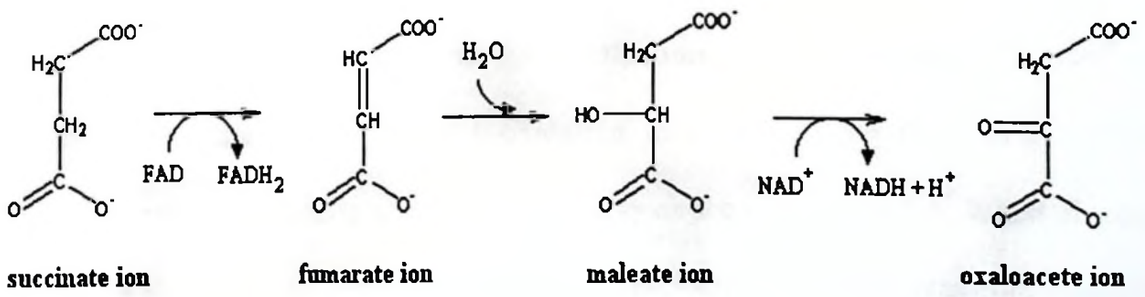


Figure 2.13: Formation of other carboxylic acids ions

Redox potential of the oxidation of a C - C single bond to a C = C double bond (alkanes to alkenes) is too high to enable the involved electrons to be accepted by NAD+ (E_0 = -320 mV). At this stage cell will therefore use flavin adenine dinucleotide (FAD) (E_0 = 0 mV) as electron acceptor instead of Nicotinamide Adenine Dinucleotide NAD (Alves da Silva, 2009). Fumarate hydration yields malate, which is oxidized to oxaloacetate, which thus closes citric acid cycle (Figure 2.6, 2.13 and equation [15]).

$$\begin{pmatrix}(\text{Acetyl-CoA} + \text{oxaloacetate})\\ +3\,\text{NAD} + \text{GDP} + \text{P} + \text{FAD})\end{pmatrix} \rightarrow \begin{pmatrix}\text{oxaloacetate} + 2\,CO_2 + \text{FADH}_2\\ +3\,\text{NADH} + 3\,\text{H} + \text{GTP}\end{pmatrix} \qquad [15]$$

A similar sequence of reactions happens in fatty acids oxidation, which *A. niger* fungi also assimilate (Alves da Silva, 2009).

2.6.10 Citric acid production by yeasts

Whether glucose or n-alkanes are used as substrate, fermentation pattern by yeasts shows a growth phase which is followed by citric acid accumulation phase. The transition is brought about by nitrogen limitation. When *n*-alkanes are used as substrate they are converted by β-oxidation to acetyl coenzyme which is then combined with oxaloacetic acid in the usual way as seen in Figure 2.6. When glucose is used as substrate, pyruvate carboxylase provides the anaplerotic reaction, whereas when n-alkanes are used as carbon sources anaplerosis is provided by the glyoxylate cycle (Kristiansen, *et al.* 1999).

This tendency is shown by the presence of high levels of iso citric lyase (ICL). Mean while levels of acotinase (ACH) are higher than when glucose is utilized. A problem with yeast processing is the yield of high ratios of iso citric acid and citric acid. In some cases, these ratios are much higher than the equilibrium values mediated by ACH; therefore, a selective transport of iso citric acid from the mitochondrion to the cytoplasm is suggested to occur (Legiša and Mattey, 1986; Legiša and Kidric, 1989).

2.7 Fermentation Technology

Fermentation could be referred to as any facultative microbial action controlled by man to make useful products (Othmer, 2001). Modern techniques involve microbial strain selection, large-scale sterilization, aeration and product separation; these allow the cultivation of a specific microorganism under more optimal conditions. Today a variety of primary and secondary metabolites (e.g. carboxylic acids, antibiotics, steroids, vitamins and enzymes) are produced industrially this way, depending on a particular fermentation medium design (Shuler and Kargi, 2001).

Fermentation products can be divided into three broad classes; the first are those, which involves products, produced during the microorganism growth phase i.e. production of ethanol, commonly referred to as primary metabolites. The second are those produced after growth has stopped (or during restricted growth) known as secondary metabolites e.g. production of citric acid and the third class focuses on production of cells or biomass. For that matter, media design is therefore a great tool

for achieving a successful microorganism's growth hence the fermentation process (Anderson, *et al.,* 1982; Shuler and Kargi, 2001).

2.8 Industrial Citric Acid Fermentation by *Aspergillus niger*

The economic and widely used way of citric acid production is by fermentation. More than 90% of the citric acid produced worldwide is obtained by fermentation (Grewal and Kalra, 1995; Othmer, 2001). Advantages for the fermentation includes the following: operations are simple and stable, the plant is generally less complicated and needs less sophisticated control systems, technical skills required are lower, energy consumption is lower and frequent power failures do not critically affect the functioning of the plant. Citric acid production by fermentation is divided in three phases, which include preparation of substrate and inoculation, fermentation, and recovery of the product.

Submerged fermentation technique is a widely used method for citric acid production. It is estimated that about 80% of world citric acid production is by submerged fermentation (Röhr *et al.,* 1996). When in large scale, fermentation process requires more sophisticated installations and rigorous controls.

In industrial citric fermentation, the large-scale spore production is made by using appropriate means and conditions such as direct inoculation in the production fermenter. Sometimes it is necessary to remove the remainder minerals of the raw material and add other nutrients such as phosphorous, magnesium and nitrogen for

development of the mycelium and a good production of the citric acid. On the other hand, it presents several advantages such as higher productivity and yields lower labour costs, contamination risk and energy consumption (Kristiansen, *et al.,* 1999).

Submerged fermentation may be divided into two main types, with various combinations and modifications. These are batch fermentations and continuous fermentations. The batch mode is more frequently used and normally, citric fermentation is concluded in 5 to 12 days, depending on the process conditions and media design.

As glucose is the common starting carbohydrate in glycolysis, under optimal fermentation conditions glucose is converted into citric acid up to over 90% conversion. Other carbon sources are thought to produce comparatively lower yields (Grewal and Kalra, 1995; Othmer, 2001).

The industrial citric acid production is carried in two different ways: by submerged and surface fermentation (solid and semi solid-state fermentation). These methods require excessive of carbon source as raw material and inoculums preparations that maximizes yield (Kubicek *et al.,* 1980; Legiša and Kidric 1989; Kusai *et al.,* 2002).

The surface fermentation process is subdivided according to the state of medium used. Currently only about 20% of the total citric acid worldwide production is produced this way because it is both laborious and costly. The submerged

fermentation process can either be stirred or airlift. The use of deep fermenter begun in 1930's (Othmer, 2001).

2.8.1 Design of submerged fermentation reactors

There are different types of bioreactors for example bioreactor with internal mechanical agitation (impellers, turbines). Others are bubble columns, which rely on gas sparging for agitation and loop bioreactor, in which mixing and liquid circulation are achieved by the motion of an injected gas, by a mechanical pump, or by a combination of the two (Anderson, *et al.*, 1982; Shuler and Kargi, 2001). Internal mechanical agitation that is used in this study has the following main characteristics:

(a) Highly flexible Mechanical agitation that disperses gas bubbles throughout the reactor while increasing the residence time of bubbles.

(b) Shears large bubbles into smaller bubbles, which provide high oxygen volumetric transfer coefficient values ($k_L a$).

(c) Air is supplied by a sparger where mixing is accomplished using disk- or turbine type impellers.

An axial flow hydrofoil impeller are designed to pump liquid either up or down (i.e. in an axial direction). These impellers have shown to provide superior performance compared to Rushton impellers, in terms of lower energy outputs required for

equivalent oxygen transfer also produce less shear stress while augmenting mixing. On dealing with the polarization of the fragile filamentous *A. niger* fungi, are highly recommended (Shuler and Kargi, 2001).

The bubble columns type disperses gas bubbles through the fermenter, but has a disadvantage of cells accumulating at the surfaces of bubbles, and when the bubbles burst, they destroy the cells. Although airlift systems are other most common types of loop bioreactors they involve complex designs, but they are cheaper to operate at volumes <500 L. The largest fermentations (>200,000 L) are usually carried out in airlift bioreactor, as higher oxygen transfer rates and better cooling can be achieved in these systems versus mechanically agitated bioreactor (Shuler and Kargi, 2001).

2.8.2 Geometry, layout and construction of reactor

Bioreactor can be constructed of glass or stainless steel. Typically, glass vessels are only used for volumes < 50 L; structural properties of glass make it unsafe to use glass vessels at > 500 L volumes; 316 stainless steel is used for the full range of fermenter volumes, 304 ss (slightly less corrosion resistant) is used for vessel covers and jackets typical mechanically agitated. For a typical 100,000 L fermenter the following considerations are applied:

Height to diameter ratio of 2-3, sterile air inlet and sparger, baffle plates and impellers, cooling coils, foam breakers, working (liquid) volume ~ 75% of the total vessel volume; the steam lines permit in-place sterilization (SIP) of valves, pipes and

seals so as the input air can be sterilized by heat or filtration (Shuler and Kargi 2001). The effect of foam production by fungi is controlled by using silicon oil. As foam escaping from the fermenter, can wet (and block) air filters, decreasing influent air flow, and/or causing pressure to build up in the reactor and allow for volume increase due to aeration and agitation. Working volume $\approx$ 0.75 total vessel volume prevent foam overflowing otherwise in other type's special foam breakers are provided (Anderson, *et al.,* 1982; Shuler and Kargi, 2001).

Cleaning is often performed in-place (CIP). Highly alkaline detergents are usually used (often at high temperatures), CIP is performed at the end of a run, and then again before a new run is initiated, after which sterilization in-place (SIP) procedures are performed, and then the new run can begin (Shuler and Kargi, 2001).

2.8.3 Fermentation medium and process design

The design is often considering nutrients consumption by the microorganism and incorporation into the biomass while producing metabolites (Othmer, 2001). As a first approximation of the minimum nutrient metabolism of a fermenting organism, in a fermentation media sugar a carbon nutrient $(CH_2O)_n$ is the most abundant energy-releasing source with typical concentration of 0.2-20% (w/v). Nitrogen is the next abundant and its concentration depends on the type of microorganism and the product (Table 2.4). Other important factors are the downstream processing and waste treatment or restrictions because they reflect the product recovery costs (Othmer, 2001).

2.8.4 Substrate pre-treatment

Pre-treatment is very important for the optimization of the fermentation products and minimization of running costs pre-treatment procedures involve sterilization and hydrolysis (Othmer, 2001; Shuler and Kargi, 2001).

(a) Sterilization

Sterilization is the reduction of the untargeted native microorganisms that compete for the available nutrients in a media. The common methods include heating, irradiation and chemical sterilization, while taking precaution on the indiscriminate elimination characteristics to both useful and harmful microorganisms (Othmer, 2001; Shuler and Kargi, 2001).

(b) Hydrolysis

Hydrolysis is a process whereby substrate is broken to products through enzymatic or chemical activity. Some common enzymes involved are the amylases, cellulases and inulinases for glucose, simple sugars and Inulin respectively (Abasaeed and Lee, 1995; Abasaeed and Lee, 1996). Common chemicals used for the hydrolysis are strong oxidizing acids, such as hydrochloric and sulphuric acids followed by heating in dilute alkalis. Currently metal catalysts have been reported (Abasaeed and Lee, 1995; Abasaeed and Lee, 1996).

2.8.5 Air supply temperature and pH control

Growth of fungi is generally aerobic and for good production of citric acid, from cultures proper aeration is necessary. Studies show that respiration becomes impaired in low oxygen concentrations (Shuler and Kargi, 2001; Soccol *et al.,* 2006).

Increased aeration rate enhances high yields and reduces process time; an interruption of aeration during batch fermentation is quite harmful. Dissolved oxygen concentration influences citric acid formation directly. It is therefore important to maintain the oxygen concentration above 25% saturation (Kubicek, *et al.,* 1980; Olsvik and Kristiansen, 1994; Badino *et al.,* 2001; Pandey *et al.,* 2001; Campesi *et al.,* 2009). Critical dissolved oxygen tension is 9-12% of air saturations recommended for growth phase and 12-13% of air saturation for the production phase. This is also dependent on the viscosity of the fermentation broth (Kubicek and Röhr, 1986; Vandenberghe *et al.,* 1999). This could be another reason why small compact pellets are preferred mycelial forms of *A. niger* during the production as they provide low surface area (Soccol *et al.,* 2006).

When the organism develops filaments, the dissolved oxygen tension rapidly falls to less than 50% of its previous value even if the dry mass has not increased by more than 5%. Aeration should be performed through the medium during the whole process with the same intensity. Therefore incorporation of the oxygen together with air in submerged process results in increment of citric acid production, but it is economically unviable (Shuler and Kargi, 2001; Soccol *et al.,* 2006); However, it is

possible to circulate oxygen in the fermenter since the carbonic gas is removed from the process. High aeration rates lead to high amounts of foam demanding addition of antifoaming agents or institution of mechanical defoamers to tackle this problem (Shuler and Kargi, 2001; Soccol *et al.,* 2006).

Studies of citric acid production by *A. niger* in solid-state fermentation (SSF) revealed that an environment with high concentrations of CO_2 had a positive effect on citric acid synthesis (Vandenberghe *et al.,* 1999). High partial pressure of CO_2 probably favour condensation of carbon dioxide and pyruvate to form oxaloacetic acid while retarding spore liberation of the filamentous fungi, hence favouring citric acid synthesis as was experienced in cassava bagasse substrates (Soccol *et al.,* 2006).

2.8.6 Oxygen transfer in broths containing mycelia cells

Oxygen transfer in broths containing mycelia cells for example during batch cultivations exhibit a shear thinning which frequently results in a pseudo plastic non-Newtonian rheological behaviour (Olsvik and Kristiansen, 1994; Badino *et al.,* 2001; Kilonzo and Margaritas, 2004; Campesi *et al.,* 2009). This behaviour exerts a profound effect on the bioreactor performance and consequently the issue of volumetric oxygen transfer (k_La) when filamentous fungi are involved needs considerations in yield maximization (Kubicek *et al.,* 1980). When discussing the regulation of citric acid production, effect of dissolved oxygen tension on *A. niger* respiration is important. Kusai *et al.,* (2002) reported that dissolved oxygen concentration effects intracellular pH and consequently the growth rate of *A. niger.*

Studies by Gavrilescu *et al.*, (1993) indicated that volumetric oxygen mass transfer coefficients in non-Newtonian rheological substrates ($k_L a_b$) changes during biosynthesis processes. This change is mainly due to rheological parameter modifications, i.e. increasing the consistency index (K) and decreasing the flow behaviour index (n). Gavrilescu *et al.*, (1993) reported values of $k_L a_b$ to be 3.0-6.5 times lower than recorded in water because the $k_L a$ values were without consideration of biological liquid and on the nature of fermentation broths.

Under normal circumstances, like in the current experimental set up, the oxygen transfer can be presented by the relationship represented by equation [16].

$$k_L a = Q_{O_2} X / (C^* - C_L) \qquad [16]$$

Where C is the dissolved oxygen concentration in the broth at the steady state given by the galvanic electrode and C^* is the dissolved oxygen saturation concentration in the broth estimated by the method proposed by Schumpe *et al.*, (1982); Quicker *et al.*, (1982);

Correlations for the volumetric oxygen transfer coefficient ($k_L a$) widely used in fermentation systems which do not make use of any dimensional criterion, i.e. $k_L a$ is related to the gassed power consumption per unit volume of broth (P_g/V) and the superficial gas velocity (v_s), just as originally proposed by several; e.g. Montes, *et*

al.,(1999), Cooper *et al.*, (2002). These correlations have been automatically incorporated the dynamic k_La estimations shown in equation [17].

$$k_La = (P_g/V)^{a_1}/(V_s)^{b_1} \quad [17]$$

Values of the constants a_1 and b_1 may vary considerably, depending on the system geometry, the range of variables covered and the experimental methodology used.

$$k_La = (P_g/V)^{a2}/(V_s)^{b2}(\mu_{ap})^{c2} \quad [18]$$

Extended forms of first type of relationship that incorporates terms like impeller speed (N) and the fluid apparent viscosity (v_{ap}), developed by Ryu and Humphrey (1972), who studied the influence of the broth apparent viscosity (μ_{ap}) on k_La in penicillin fermentation and proposed the correlation shown in equation [18]

a_1, a_1	=	constants of equation [17]& [18] [–]
b_1, b_2	=	constants of equation [17]& [18] [–]
c_1, c_2	=	constants of equation [17]& [18] [–]
C_L	=	dissolved O_2 concentration in the broth in steady-state (mmol $O_2 l^{-1}$)
C^*	=	dissolved oxygen saturation concentration in the broth (mmol $O_2 l^{-1}$)
k_La	=	volumetric oxygen transfer coefficient (h^{-1} or s^{-1})
P_g	=	gassed power consumption (W)
QO_2	=	specific oxygen uptake rate (mmol O_2 g^{-1} h^{-1})
QO_2^{max}	=	maximum specific oxygen uptake rate (mmol $O_2 g^{-1} h^{-1}$)
$Q_{O2}X$	=	global oxygen uptake rate (mmol O_2 g^{-1} h^{-1})
$v_{s.}$	=	($= 4Q/(\pi D_t^2)$) superficial gas velocity (ms^{-1})
(μ_{ap})	=	broth apparent viscosity
V	=	broth volume (l or m^3)
α	=	constant of proportionality
v_{ap}	=	apparent kinematic viscosity ($m^2 s^{-1}$)
ρ	=	density (kg m^{-3})
σ	=	surface tension of filtrate (Nm^{-1})
OUR	=	oxygen uptake rate (s^{-1})
DO	=	dissolved oxygen (mg/l)

2.8.7 Measurement of oxygen transfer using dynamic method

A dynamic method as described by Shuler and Kargi, (2004), also uses a fermenter containing active cells just as in the steady state method. It is comparatively simpler than the steady state method, because it requires only a DO probe and a chart recorder, rather than off-gas analyzers (as required in the steady state method).

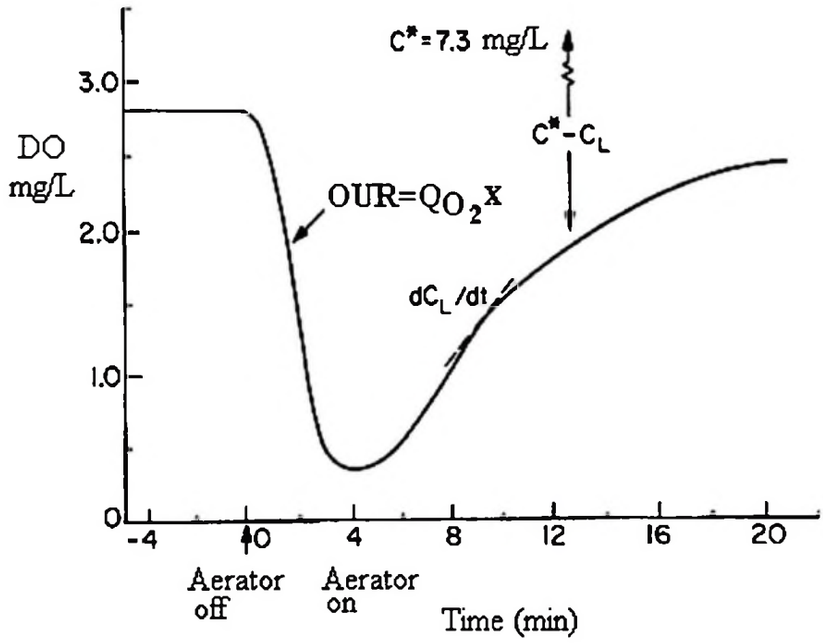


Figure 2.14: Dissolved oxygen Vs Time

Procedure for the dynamic method requires that O_2 supply be turned off for <5 min and then turned back on. Various parameters can then be calculated from a plot of DO vs. time; since $dC_L/dt = k_La\ (C^*- C_L)$ therefore $\ln (C^*- C_L) = - (k_La)$ at $t = 0$ and $C_L = 0$ (Figure 2.14 and Equation [19]).

$$OUR = Q_{O_2}X = k_L\alpha(C^* - C_L) \qquad [19]$$

Using equation [19] for oxygen uptake rate (*OUR*); where as Qo_2 is the specific uptake rate of oxygen (molO_2/g-h), the value of Qo_2 or (*OUR*) is the demand side of the equation; typical values of *OUR* in large-scale culture are 40 to 200 mmol O_2/l-h. However, most fermentation systems are within the 40-60 mmol O_2/l-h range (Table 2.3). Primary advantage of the dynamic method is $k_L a$ can be measured under actual fermentation conditions cultures (Shuler and Kargi, 2004).

Table 2.3: Respiration rates in cultures (Shuler and Kargi, 2004)

Organism	Qo_2 (mmolO_2/g dw-h)
Bacteria	
E. Coli	10-12
Azobactor sp	30-90
streptomyces sp	2-4
Yeast	
Saccharomyces cerevisiae	8
Moulds	
Penicillium sp	3-4
A. niger	ca.3
Plant cells	
Acer pseudoplatanus (sycamore)	0.2
Saccharum (sugar cane)	1-3
Animal cells	
HeLa	$0.4\frac{\text{mmol O}_2/\text{l}-\text{h}}{10^6 \text{ cells / ml}}$
Diploid embryo WI-38	$0.15\frac{\text{mmol O}_2/\text{l}-\text{h}}{10^6 \text{ cells / ml}}$

2.8.8 Heat transfer and temperature control of a bioreactor

Main limitations on bioreactor design are the abilities to provide an adequate supply of oxygen and to remove metabolic heat efficiently (Shuler and Kargi, 2004). Internal coils are used for smaller volumes and cooling jackets for larger volumes. Internal coils they provide a larger surface area for heat transfer, also they boast heat transfer. However, the coils fouling by filamentous fungi minimize growth and thorough mixing (Ryu and Humphrey, 1972; Cooper *et al.*, 2002; Kusai *et al.*, 2002).

2.8.9 Preparations and culture maintenance of inoculums

Inoculums preparation is done by aseptically harvesting spores or by purchasing industrial grade citric acid strains in slant agar tubes. These are propagated aseptically on agar plates and spores formed are tested for productivity (Torres *et al.*, 1997; Torres *et al.*, 1998; Ruijter *et al.*, 2002).

Culture maintenance is done by harvesting spores of pure *A. niger* colonies, after 10 times dilution with sterile distilled water and shaking for 0.5 minutes. Inoculation of spores is made on potato dextrose agar (PDA) plates and incubation done at 30°C for seven days. Spores of *A. niger* strains can subsequently be used in fermentation. For best yield a very careful control of the initial conidia concentrations by inoculating 10^6-10^7 conidia/ml is usually done at 30°C incubation temperature. For the culture maintenance, seven days old spores are inoculated aseptically into PDA plates and re-cultured after every two months (Torres *et al.*, 1998; Ruijter *et al.*, 2002).

2.8.10 Morphology of filamentous fungi in submerged cultivations

Fungi respond differently to culture environments. On soil or on agar substrates for example, they develop penetrating mycelia structures. In submerged processing important parameters such as the spore inoculums level, phosphate and manganese concentrations affects morphology (Kubicek and Röhr, (1977). Starting from a spore, mycelium develops as a mass depending on culture conditions; it turns to a surface or remains as a mass lump. A simple morphologic form could be in dispersed forms of young mycelia produced by dense spore mass lump. Another formation is the pellet morphology, which is characterised by the radially projected growth from the nucleous aggregate. The morphological characteristics can also vary between freely dispersed mycelia and distinct pellets of aggregated biomass (Meixner-Manori *et al.*, 1985; Žnidaršić and Pavko, 2001).

The advantages and disadvantages for mycel or pellet cultivation have to be balanced out carefully. Some researchers for example Papagianni *et al.*, (1999a); Grimm *et al,* (2004), discussed the morphogenesis of filamentous microorganisms as important entity in better understanding of the molecular and cell biology of filamentus microorganisms as well as in biochemical engineering and particle technique, specifically during the characterization of connections between the growth conditions, cell morphology, spore-hyphae-interactions and product formation.

The control, characterisation of the morphology and transport gradients, seemed to have a remarkable relationship to the metabolism of filamentous fungi. Therefore based on their findings, one can conclude that recognition of pellet morphogenesis of *A. niger* within the complex production pathways is very important as may improve the production yield (Papagianni *et al.*, 1999a; Grimm *et al,* 2004).

In their study Grimm *et al,* (2004) demonstrated that pellet morphological development is linear dependent on the growth rate, specifically for coagulating microorganisms like *A. niger*. Mycelia compactness of spherical pellets was found to vary in different size and density depending on the rheological properties of the fermentation broth. The mechanism of pellet formation for non-coagulating microorganisms types starts by a pellet growing out of single spores, where as for coagulating microorganisms could originate either from the same stages or from conidia coagulation aggregates during the early stages of cultivation. Much more defined pellets were found to be formed by mycelia growing out of these aggregates (Papagianni *et al.*, 1999a; Grimm *et al,* 2004).

Hydrodynamic conditions in bioreactors show great influence on particle velocities and therefore on collision frequency; as collision frequency is raised by an increase in the impeller speed of a stirred-reactor, a higher rotation speed also results into stronger shear forces, that counteracts the aggregation of conidia, thus disturbing formation of pellets and productivity (Grimm *et al,* 2004; Papagianni and Mattey, 2006).

Concurrently findings by Papagianni *et al.,* (1999b); Žnidaršić and Pavko, (2001), and Papagianni and Mattey (2006), provided an insight on the correlation between the influence of glucose concentration on citric acid production, which lead to a concept that, the morphology development being a function of the spore inoculums level of *A. niger* and hence a bioprocess parameter in submerged fermentation.

When studying the effect of the conidia concentration on pellet formation Papagianni *et al.,* (1999a); Žnidaršić and Pavko, (2001) found that, low concentrations in the inoculum seemed to cause fewer number of pellets with large diameters, while higher concentrations led to many but smaller sized pellets.

Raising of concentrations of conidia was linked to the disturbance of the hyphal growth and the main reason was thought to be that, less surface is rendered unavailable for aggregation, as more frequent collisions counteract the increased aggregation. Both studies regarded the control of mycelial morphology as a prerequisite to ensure increased productivities in industrial applications (Papagianni *et al.,* 1999a; Žnidaršić and Pavko, 2001; Papagianni and Mattey, 2006).

Aggregated particles were found to decrease with increasing inoculums level, which is a typical characteristic that affects the aggregates compactness (Papagianni, 2006). Compact forms developed at low inoculum levels 10^4 to 10^5 spores/ml and yield was (110 to 103) g/l respectively. For example, cultures with inoculums at 10^7 spores/ml reduced the compactness thus, mycelium developed were mainly in clump forms and yield was 120 g/l. Beyond that, growth was of free filamentous form with single

mycelia trees and yield was 100 g/l at the end of fermentation (Papagianni, 2006). These results emphasised the need for the correct inoculum levels.

2.8.11 Control of pH

Most enzymatic activities in microorganisms occur at moderate pH values of 2 to 8. Culture pH may change in response to microbial metabolic activities, especially when fermentation process results into secretion of organic acids (Shuler and Kargi, 2001; Grewal and Kalra, 1995; Ruijter *et al.,* 2002). Changes in pH kinetics depend on the type of microorganism. When moulds *Aspergillus* sp, *Penicillium* sp and *Rhizopus* sp, are involved pH values can drop very quickly to less than pH 3. Other groups of fungi for example *Trichoderma*, *Sporotrichum*, *Pleurotus* sp, pH could be more stable between 4 and 5. The nature of the substrate and production technique also influences pH kinetics. In this way, optimization of initial pH is crucial (Torres, 1998; Pandey *et al.,* 2000; Pandey *et al.,* 2001).

In some studies, results showed that at pH 4 or above, the formation of oxalic acid is accelerated due to high buffeting capacity of the medium. The pH 2.2 is reported to be optimum for the growth of the mould as well as for the production of citric acid in sucrose rich medium (Shuler and Kargi, 2001; Ruijter *et al.,* 2002). Higher initial pH values of 5.4 and 6.0-6.5 are suggested to be optimum for citric acid production in molasses medium (Grewal and Kalra, 1995; Shuler and Kargi, 2001; Ruijter *et al.,* 2002).

2.8.12 Nature of carbon source in citric acid production

As the physiology and cell metabolism in fungi like in other living organisms requires a well-balanced nutrient to supplement the carbon source. Researchers Röhr and Kubicek, (1981); Röhr and Kubicek, (1996); Ruijter *et al.*, (1999); Ruijter *et al.*, (2000); Soccol *et al.*, (2006), have reported various factors affecting the production of citric acid specifically the media nutritional composition (Table 2.4).

Works by (Mschak *et al.*, 1984; Drysdale and McKay 1995; Tran *et al.*, 1998), on cane sugars and molasses indicated that deficiency of manganese and other metals, with existence of Fe stimulates microbial cells growth at the expense of citric acid production and subsequently meditate the formation. However, such results do not reflect the fact that, the same applies to other carbon sources.

This suggests a thoroughly investigation on the use of alternative sugar sources e.g. fructose rich syrups and sisal Inulin hydrolysates. Traditionally the optimum citric acid production that was achieved commercially using mutant strains of *A. niger* and *A. foeticides* in different carbohydrate media such as Inulin, indicated that yields from Inulin are usually 20-30% lower than from sucrose the traditional substrate. This suggests that the nature of the carbon source affect citric acid yield, and therefore more research work is needed for the maximization of yield from other sugars than glucose for example sisal Inulin hydrolysates (Drysdale and McKay1995; Hossain *et al.*, 1984).

Table 2.4: Chemical factors affecting citric acid production (Soccol *et al.*, 2006)

Factor	Positive effect source	Level	Negative effect
Carbon	Sucrose	14 to 22%	Starch
	Glucose		Xylose
	Fructose		Arabinose
	Galactose		Sorbitol
			Pyruvic acid
Phosphorus	Potassium dehydrogen phosphate	Low	
		0.5 to 5.0 g/l	
Nitrogen	Ammonium nitrate	under 25%	High concentrations
	Ammonium sulphate	0.1-0.4 g (Nitrogen)/l	(biomass production)
	Peptone		
	Malt extract		
	Urea		
Trace elements	Zinc	low levels	Manganese
	Copper	(0.02 -0.025%)	(1 ppm)
	Magnesium sulphate		
Lower alcohols	Methanol	1 to 4%	
	Ethanol	(V/M)	
	n-propanol		
	Iso-propanol		
	Methyl acetate		
Oils and fats		0.05 to 0.3%	
Other compounds	Calcium fluoride		
	Sodium fluoride		Potassium ferrocyanide
	Potassium		Quaternary ammonium compounds
	3-hydroxy-2-naphtoic acid		Amine oxides
	4-methyl-umbelliferone		
	Benzoic acid		
	2-naphtoic acid		
	Iron cyanide		
	EDTA		
	Vermiculite		
	H_2O_2		

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	Peptone		
	Malt extract		
	Urea		
Trace elements	Zinc	low levels	Manganese
	Copper	(0.02 -0.025%)	(1 ppm)
	Magnesium sulphate		
Lower alcohols	Methanol	1 to 4%	
	Ethanol	(V/M)	
	n-propanol		
	Iso-propanol		
	Methyl acetate		
Oils and fats		0.05 to 0.3%	
Other compounds	Calcium fluoride		
	Sodium fluoride		Potassium ferrocyanide
	Potassium		Quaternary ammonium compounds
	3-hydroxy-2-naphtoic acid		Amine oxides
	4-methyl-umbelliferone		
	Benzoic acid		
	2-naphtoic acid		
	Iron cyanide		
	EDTA		
	Vermiculite		
	H_2O_2		

2.8.13 Considerations of nutrients additives

Physiology and cell metabolism in fungi like in other living organisms requires well-balanced nutrients to supplement the carbon source. Growth of *A. niger* requires in addition to a source of carbon, supplies of nitrogen, phosphate, potassium, magnesium and sulphur. Simultaneously small quantities of micronutrients such as zinc, iron, copper, molybdenum and manganese are also necessary. Excess availability of nutrients accelerates mould growth to a sporulation phase. Limitation of nutrients availability to mould maximizes of citric acid accumulations, therefore neither full-growth nor sporulation is required for the commercial scales productions.

On nitrogen limitation studies, it was shown that when ammonium sulphate was used as nitrogen source, citric acid accumulation increased without the formation of oxalic acid (Arts *et al.*, 1987; Grewal and Kalra, 1995; Ruijter *et al.*, 1999: El-Holi and Al-Delaimy, 2003). This emphasised that nitrogen limitation was an essential requirement for citrate accumulation. However, those findings showed that either nitrogen or phosphate limitation depended upon strain and the nature of carbon (Shu and Johnson, 1948; Shu and Johnson, 1984; Röhr and Kubicek, 1996). Retardation of citric acid yield by *A. niger* by lack of potassium phosphate salts suggested that phosphate ion might have been functioning in some way other than a simple nutrient or buffer for example phosphates were reported to act at the level of enzyme activity (Habison, *et al.*, 1983; Chen and Christensen, 1985; Kubicek-Pranz *et al.*, 1990).

The optimum additions of iron and manganese were necessary for best citric acid yields. Experiments using a purified glucose medium and the well-known *A. niger* Wisconsin 72-4 showed that additions of 0.3 zinc and 1.3-ppm iron were optimum. However, the optimum conditions varied from strain to strain (Clark *et al.*, 1966; Shu and Johnson, 1984; Schreferl *et al.*, 1986; Röhr and Kubicek, 1996).

Studies of the necessity for ion-exchange purification of glucose solutions or the treatment of molasses with ferrocyanide ions, verified that additions of as little as 1 ppb Mn to ferrocyanide-treated molasses reduced citric acid yield by 10%. The control of levels of the Zn and Mn concentrations in fermentation medium, were thus established (Grewal and Kalra, 1995; Shuler and Kargi, 2001).

2.9 Recoveries of Citric Acid and Effluent Disposal

Downstream processing for bio refining includes separation and purification (Figure 2.15). Often downstream processing is the most expensive phase of producing a substance of biological origin especially if stringent quality or regulation on quality is regarded. Key reasons for this are the complexity and dilute nature of the fermentation broths (Kristiansen and Charley, 1981).

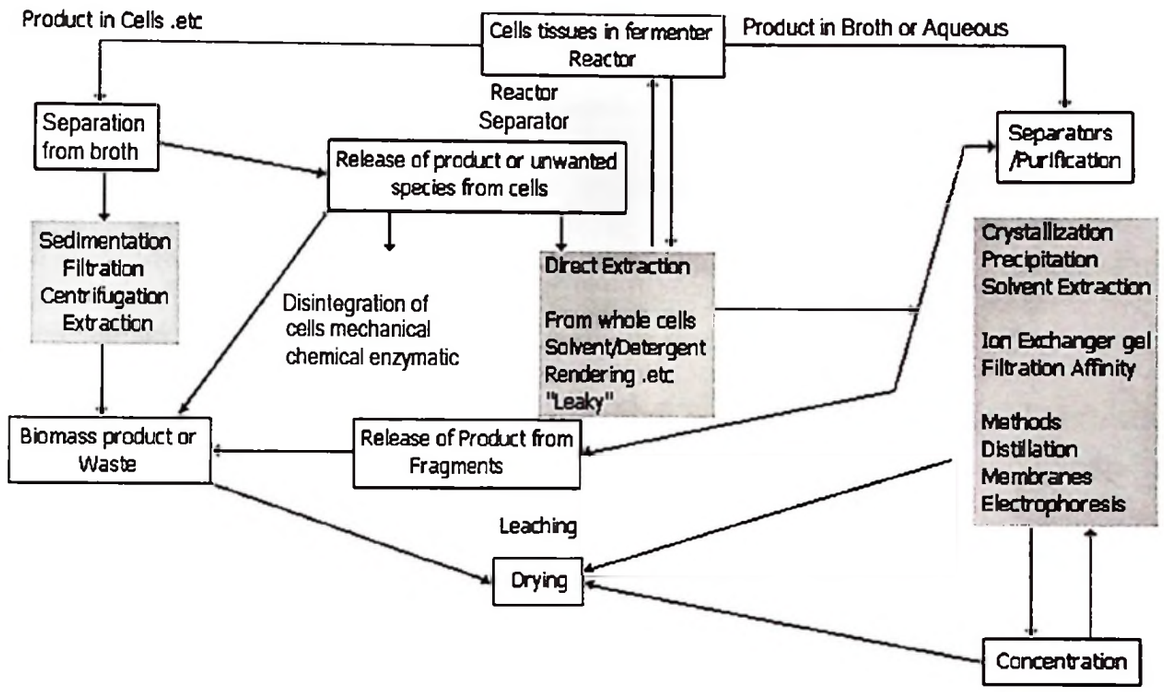

Figure 2.15: A schematic recovery of fermented products Dechow (1989)

2.9.1 Classical recovery process

The classical citric acid recovery process is particularly suitable for use with very impure liquors derived from molasses. This process involves heating the fermented liquor and lime is added to precipitate insoluble calcium citrate (Figure 2.15). The washed precipitate is treated in aqueous suspension with H_2SO_4 yielding an aqueous solution of citric acid and a by-product $CaSO_4$ (gypsum). This set of operations has the effect of removing most of the impurities whether derived from the substrate or those generated in the fermentation (Collins and Mention, 1962).

Conditions of the crystallization steps are varied to produce either the anhydrous acid or the monohydrate as both have a high market demand. Mother liquor containing acid is treated with active carbon, before cation and anion exchangers' treatments. Citric acid monohydrate is formed after vacuum evaporation at 40°C. Concentration of citric acid liquor and finally vacuum crystallization is done at 20-25°C. Traditional method does use and produce stochiometric high amounts of mineral acid and salt co-product gypsum, which escalates labour (Kristiansen, *et al.,* 1999).

2.9.2 Direct crystallization

Recovery of citric acid from the fermented liquor directly by crystallization is hard to achieve because it contains many unwanted materials, which come from the raw material or from the autolysis of the microbial cells. In fermentations with n-alkenes and in particular where sodium hydroxide is used, monosodium or trisodium citrate crystallization is made from the clarified fermented liquor. In this case, citric acid is recovered from the sodium citrate by electro dialysis (Tongwen and Weihua, 2002; Tongwen and Weihua, 2003; Kubicek *et al.,* 1994; González, 1997).

2.9.3 Amine recovery process

Newer citric acid recovery technology takes advantage of the fact that the fermentation takes place at pH below the lowest pK_a of the acid (still in its protonated form), thus a weakly basic tertiary amine can be used to form complexes. The complexes can be separated using the reactive surface exchange column or ion

affinity protocols to fit the scenario (Pazouki and Panda, 1998).

Currently amine extraction is a prospering method of citric acid separation from aqueous solution for example organo-phosphorus and aliphatic amine extractants. The use of nontoxic substances is highly desired, primary, secondary, tertiary and quaternary amines are used as reactive components. Experiments have shown that tertiary amines should be considered for future works (Kristiansen, *et al.,* 1999; Pazouki and Panda, 2000; Pazouki *et al.,* 2000).

2.9.4 Solvent extraction

Solvent extraction is another method used for the recovery of citric acid. It uses 2-butanol and tributyl phosphate for the extraction of citric acid (Collins and Mention, 1962). Citric acid extracted by solvent extraction using a mixture of n-octyl alcohol and tri-dodecylamine method is suitable for food and drugs (FDA-USA, 1985). Better recovery is realized by extracting citric acid into the solvent at low temperature, subsequently stripping the solvent with hot water. The advantage of the solvent extraction method is that it avoids the use of lime and H_2SO_4 and the problem of gypsum disposing (Pazouki and Panda, 1998).

2.9.5 Ion pair extraction

Another variant of the solvent extraction process is the ion-pair extraction system. Extractant consists of secondary or tertiary amines, having in total at least 20 carbon atoms dissolved in immiscible solvent water. Extraction of citric acid is done at a

lower temperature (20°C) and the stripping at a higher temperature of (80°C), using a mixture of tri-dodecylamine octyl alcohol and iso-alkanes. A further development of this process is the use of N-substituted alkyl amides as extractants (Pazouki and Panda, 1998; Pazouki and Panda, 2000).

2.9.6 Anti solvent extraction

A two-step anti-solvent crystallization process a novel and unique purification process by Shishikura and Takahashi (1992) is in use. It is characterized by organic solvent extraction and precipitation with a poor solvent compressed carbon dioxide (CO_2). First the fermentation broth of citric acid is filtered to remove microorganisms, its water concentration adjusted to about 10-20 wt% by multiple effect evaporation.

Shishikura and Takahashi (1992), developed method that involves citric acid extractions with acetone: then residual impurities are precipitated from acetone solution of citric acid, using the anti-solvent effect of compressed CO_2. Impurities are separated from the supernatant by a settling separation. Finally, crystal grains of citric acid of food additive grade (99.5 wt%) are recovered from supernatant liquid, decolorized with activated charcoal, column re-crystallized then dried (Shishikura, *et al.*, 1994). The alternative to conventional crystallization; after the removal of impurities from the acetone solution; citric acid is crystallized by the anti-solvent crystallization using supercritical liquid CO_2 at 50 kg/cm^2, which yields citric acid of food grade (Shishikura and Takahashi, 1992).

2.9.7 Disposal of effluent

The production of gypsum in the calcium citrate during citric acid recovery by precipitation shows that, the disposal of this solid waste is a problem (Soccol *et al.*, 2006). However, more serious problem is the disposal of filtrate from the calcium citrate precipitation especially when molasses is used as feedstock material. These wastes are non-toxic, but very high oxygen demanding, thus unaccepted in rivers without treatment. The cultivation of yeasts on such the effluent could produce a material suitable for animal feed; therefore, they are commended for such purposes (Soccol *et al.,* 2006). Another possibility is to evaporate the effluent to produce a concentrated molasses-like material usually called condensed molasses soluble (CMS), the material which can be used in feedstuff formulations. Another method of treatment is anaerobic biogas production (Salum, 2008).

2.10 Microbiological experimental techniques

Microbiological experimental set up involves working with microbes that are too small to see with naked eyes, in normal circumstances special techniques, methods and apparatus are used under strict sterile conditions. In particular, special-sterile agar gel and liquid substrates are often employed to grow microorganisms for the purpose of enumeration and characterization. The most accurate method for enumeration is by counting the number of viable cells (living cells capable of replication, divide and form a population or colony). This is done by plating aliquots of cell in diluted form.

There are various ways of plating the aliquots; the spread plate method, the pour plate method, and calibrated loop method are some examples. After a period of time (usually 24-48 hrs), the number of colonies (called colony forming units or CFU) on each plate can be counted (Tomasiewicz *et al.*, 1980; FDA. 2001). Only statistically valid plates, those that have between 25 and 250 colonies per plate, are counted (it used to be 30-300). This method is based on the assumption that each colony arose from a single cell. Hence, by counting the number of colonies and factoring in the various dilutions that were performed, you can estimate (extrapolate) the number of viable cells in the starting material using equations [20].

$$\frac{\text{CFU}}{\text{ml}} = \frac{(\text{no of colonies})(\text{dilution factor})}{\text{plated volume in (ml)}} \qquad [20]$$

$$\text{where as (dilution factor)} = \frac{1}{\text{dilution}}$$

A pour plate for viable cell counting method on agar plate after serial dilutions is the common procedure in fungal enumeration (Pirt, 1975; Collins *et al.*, 1998).

(a) Pour plate method of isolation

Pour plating is a method of separating one species of bacteria from one another by diluting serialy one loopful of organism into cooled melted agar in test tubes. The bacteria are mixed with melted agar until evenly distributed and separated throughout the liquid. The melted agar is then poured into an empty plate and allowed to solidify. After incubation, discrete bacterial colonies can then be growing both on the

agar and in the agar. In a way, that one of the plates poured will provide an ideal sample for isolation (Pirt, 1975; Collins *et al.*, 1998).

(b) Streak plate method of isolation

The most common way of separating bacterial cells on the agar surface to obtain isolated colonies is the streak plate method. This technique involves streaking a loopful of bacteria and diluting the sample by mechanical means. As the loop is streaked across the agar surface after incubation, the area at the beginning of the streak pattern will show confluent growth, while the area near the end of the pattern should show discrete colonies. Each colony will then serve as the source for a pure colony of enetically identical cells. This method is meant for isolating, counting, and to identify bacteria present in a colonal sample (Pirt, 1975; Collins *et al.*, 1998).

(c) Serial dilution for viable cell counting method

Serial dilution for viable cell counting is the most common methods, for enumeration of microbe. The concentration of the original culture solution and the desired concentration will determine how many dilutions are required. Important to note is the total volume of culture solution needed. If only small quantities of culture solutions are needed then greater numbers of dilutions are necessary.

Also the approximate concentration should be known at the start of the experiment before the appropriate number and amount of dilutions can be made. In order to arrive at the desired concentration, serial dilutions are made, instead of making one

big dilution. This method is not only cost effective but it also allows for small aliquots to be diluted instead of unnecessarily large quantities of materials and hence chances for cross contaminations (Pirt, 1975; Collins *et al.*, 1998; Lee, 2008).

This technique involves the drawing of a known small amount of an original volume of culture solution to another container which is then brought up to the same volume as the original volume using the required culture solution. For example, if you have 1 ml of your original culture solution, and you draw 10 µl and place it in a tube containing 990 µl of culture solution you have made a 1:100 dilution. If the original culture solution contained 8×10^{12} organisms or cells/ml, a new concentration will be 8×10^{8}(cells/ml) because we have simply divided our concentration by 100. Similarly if 1ml from a 1:1000 diluted culture solution produced 28 CFU on agar plate then the original culture solution had 28×10^{3} CFU/ml.

Serial dilutions are usually made in increments of 1000, 100 or 10 i.e. at dilutions between 10^{-1}-10^{-7} of the sample and suspension is spread over the surface of growth medium prior to its solidification. The plates are incubated so that colonies are formed and are macroscopically visible to the naked eye (Pirt, 1975; Collins *et al.*, 1998; Lee, 2008).

Despite to the fact that the limitation to the plate count method is selectivity and un viable cells are not counted, the major limitations in this method is the relatively narrow countable range (generally considered to be 25-250 CFU bacteria on a standard Petri dish). The currently prevailing confusion between the Limit of

Detection (1 CFU) and Limit of Quantification (25 CFU) for the plate count method creates a larger degree of variability in microbiology data than is necessary (Pirt, 1975; Collins *et al.,* 1998; Lee, 2008).

2.10.1 Morphological systematics and molecular identification methods of microorganisms

Microorganism identification can be performed either phenotypically using fermentation reactions of sugars or growth on carbon and nitrogen sources or other compounds. These characteristics can vary, depending on physiological state of the cell. Molecular biology techniques are independent of the state of the cell as they analyse the genome of the cell (Pirt, 1975; Collins *et al.,* 1998; Lee, 2008).

Morphology of colonies can have a definite color, shape, edge and elevation. These features are observed with the naked eye by looking at the colony itself. However, cellular morphologies differentiation is done by the differences shown by individual cells as seen under the microscope. Cellular morphology of a cell can be *coccid, bacilli*, spiral etc (Pirt, 1975; Collins *et al.,* 1998; Lee, 2008).

In its modern sense, molecular biology methods attempt to explain the phenomena of microorganism starting from the macromolecular properties that generate them. Two categories of macromolecules in particular are the focus of the molecular biologist:

1) Nucleic acid among which the most famous is deoxyribonucleic acid (DNA) the constituent of genes, and
2) Proteins which are the active agents of living organisms.

The scope of molecular biology identification therefore is to characterize the structure, function and relationships between these two types of macromolecules, in establishment of a date for the so-called "molecular revolution", or at least to establish a chronology of its most fundamental developments (White *et al.*, 1990; Talbot, 2001).

During the past decade, molecular approaches have yielded new insights into the taxonomic relationships of many microbial lineages. Molecular sequence data have become increasingly important in investigating eukaryotic microbial diversity at the community level. However, the popularity of molecular community analyses has also resulted in databases becoming populated largely by sequences from environmental samples for which morphological information is provided. DNA sequence data from the same samples from which reliable morphological identifications have been made, are used in eukaryotic cell extraction and identification techniques (White *et al.*, 1990; Talbot, 2001).

DNA extractions, sequencing and recombination technologies, incorporates the PCR; a scientific technique used to amplify a single or few copies of a piece of DNA across several orders of magnitude, thus generating thousands to millions of copies of a particular DNA sequence. Primers which are basically (short DNA fragments) contain sequences which are complementary to the target region along with a DNA polymerase (after which the PCR method is named) are key components to enable selective and repeated amplification (White *et al.*, 1990; Talbot, 2001).

As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR can therefore be extensively modified to perform a wide array of genetic manipulations as well as genome identities (Mullis and Faloona, 1987; White *et al.*, 1990; Mullis and Erlich, 1988; Higuchi *at al.,* 1992; Talbot, 2001).

Molecular analyses, involving DNA extractions are performed on each of the replicate tubes containing the cell isolates after referring standard protocol relevant to microorganism in question for example procedure for extracting DNA suitable for PCR amplification from fungi mycelium grown on 'Reverse Malt Agar' (RMA) medium containing 30% BASF pluronic polyol F-127, substituted for agar in the Malt Extract Agar medium of Pitt, (1979), is documented by several (Gardner and Jones, 1984; Seifert, 2002) can be used. The sample could either preserved at -20°C until needed or immediately be utilized in PCR reactions.

Many of the molecular biology laboratory techniques that involve DNA sequencing and the polymerase chain reactions (PCR) require DNA fragments amplification by using primers. The primers used are usually short, chemically synthesized oligonucleotides, with a length of about twenty bases. These are hybridized to a target DNA, which is then copied by the polymerase (Mullis and Faloona, 1987; White *et al.*, 1990; Mullis and Erlich 1988; Higuchi *at al.,* 1992; Talbot, 2001). PCR amplification of fungi is usually done to the ITS1-ITS4 region of the ribosomal, using the primers and conditions described in standard protocols by several (White, *et al.,* 1990; Gardes and Bruns, 1993; Piercey-Normore and Egger, 2001; Gardes and

Bruns, 2008). The ITS region, or portions thereof, has been used extensively to distinguish between closely related fungal isolates. Because the ITS region is nonbonding, its size and sequence are less conserved and more useful for examination of closely related populations. Therefore, ITS region is typically used in molecular systematics of the fungal species level, and even identifications of *A. niger* within *Aspergilla* species (Mullis and Faloona, 1987; Mullis and Erlich, 1988; Higuchi *et al,* 1992; Gardes and Bruns, 1993; Peterson, 2000; Mullis, 2007; Gardes and Bruns, 2008).

2.11 Hypothesis testing

In general, a hypothesis test is a process in which you assume an initial claim to be true and then test this claim using sample data. Hypothesis tests include two hypotheses: the null hypothesis (denoted by H_o) and the alternative hypothesis (denoted by H_1). The null hypothesis is the initial claim and is often specified using previous research or common knowledge. The alternative hypothesis is what you may believe to be true or hope to prove true (Lehmann and Romano, 2005; McCloskey, 2008). The alternative hypothesis sometimes is referred to as the research hypothesis. The decision-making process for a hypothesis test can base on the probability value (p-value) for the given test. In this work, the Tukey Method was used for hypothesis testing. Decisions making were based on the levels of p-values as determined from a predetermined level of significance (α) (Tukey, 1993; Jones and Tukey, 2000; Lehmann and Romano, 2005; McCloskey, 2008).

- Should the p-value be less than or equal to a predetermined level of significance (α-level), then the null hypothesis is rejected and the alternative hypothesis is supported.

- Should the p-value be greater than the α-level, then the null hypothesis is not rejected and the alternative hypothesis is not supported.

Four possible outcomes are expected depending on whether the null hypothesis is true or false and whether you reject or fail to reject the null hypothesis. These outcomes were summarized as the follows (Tukey, 1993; Jones and Tukey, 2000):

Null Hypothesis		
Decision	True	False
Fail to reject H_o	correct decision	Type II error
	$p = 1-\alpha$	$p = \beta$
Reject H_o	Type I error	correct decision
	$p = \alpha$	$p = 1-\beta$

When the null hypothesis is true and you reject it, you make a type I error. The probability of making α type I error is called alpha (α) and is sometimes referred to as the level of significance.

When the null hypothesis is false and you fail to reject it, you make a type II error. The probability of making a type II error is called beta (β). The probability of rejecting the null hypothesis when it is false is equal to 1-β. This value is also referred to as the power of the test (Tukey, 1993; Jones and Tukey, 2000).

CHAPTER THREE

EXPERIMENTAL METHODOLOGY

3. METHODOLOGICAL FRAMEWORK

Methodological and experimental procedures provided in this chapter aimed at establishing the comparative and screening statistical designs for studying the potential of utilising sisal bole juice-Inulin as an alternative chemical feedstock, in citric acid fermentation production using *A. niger* fungi indigenous to sisal boles.

Three sets of experimental procedures were performed; the first set involved the isolation and characterization of microorganisms from sisal bole rot. Among the isolates, *A. niger* fungal strains were tested for citric acid production potency against an industrial *A. niger* strain (DSMZ 8).

The second set of experiments involved the extraction and hydrolysis of sisal Inulin by cooking at different pH and temperatures, while identifying suitable extraction and initial conditions for the optimum yield.

The third set covered pilot scale citric acid fermentation studies using *A. niger* (BYF KT) at a 10l volume. The parameters studied included the initial fermentation conditions "hydrolysates-fructose concentration, pH and nutrients additives".

3.1 Experimental Designs

3.3.2. Design of experiments (DOE) for isolation and characterisation of microorganisms

In the isolation, characterisation and determination of indigenous fungi species from sisal bole rot, one factor at a time method was used. Three replicates were made during the determinations of the best performing *A. niger* isolates in terms of their potential in industrial citric acid production. The best performer *A. niger* (BYF KT) was thus tested against DMSZ 8.

3.1.2. Design of experiments (DOE) for hydrolysis and fermentation

A statistical design of experiments (DOE) was used to plan in advance efficient procedures, which would involve conducting the statistically minimum number of experiments. This well-known approach is able to generate enough data that could be analysed to produce the most statistically valid and objective conclusions, at a minimum cost per trial. The design of experiments also allowed for the study of interactions amongst the chosen factors affected the response levels.

During the hydrolysis experiments, initial temperature and initial pH were the two factors investigated. In fermentation experiments, initial sugar concentration, initial pH and nutrients additives were the three factors investigated. Other factor that could influence the fermentation process were not varied (held constant) in order to reduce the number of experiments. These additional factors were fermentation temperature, aeration (dissolved oxygen tension) and initial inoculums concentration. Their levels

were however fixed at levels adopted from optimized protocols for traditional feedstocks i.e. sucrose and glucose (Hossain *et al.*, 1984; Xu *et al.*, 1989; Röhr *et al.*, 1996; Torres *et al.*, 1998; Tsao *et al.*, 1999; Bizukojc and Ledakowicz, 2004; Karaffa and Kubicek, 2003; Papagianni and Mattey, 2006).

In literature, various statistical experimental design methods were covered. Taguchi designs (TD) and Full Factorial Designs (FFD) were recommended (Hicks, 1999; NIST/SEMATECH, 2006). Taguchi designs is characterised by signal-to-noise ratio calculations, which determines the effect of the specified factors, without giving as much statistical information as FFD.

The FFD provides enough information for computation of main effects, interactions and even estimate the error levels. For this reason, FFD approach was implemented. In hydrolysis experiments, two factors: initial temperature and initial pH were investigated at two levels (higher and low), which produced a 2^2 experiments (4 runs). In fermentation experiments three factors: initial sugar concentration, initial pH and nutrients additives were studied at two levels 2^2. All factors at all levels was simply adopted and the number of runs was minimized to 2^3 (8 runs). All experiments had two replicates, thus the total number of experiments (including replicates) were 24 (8 for hydrolysis and 16 for fermentations). Since replicates for central points were also included, the total number of experimental runs was 28. While providing adequate number of data statistically, this number of experiments was also within the research budget and complemented both empirical knowledge and experimental data, within the set of objectives.

The designed FFD experiments were randomized to reduce biasness in the experimental results. Analyses were done using ANOVA (Analysis of variance), to estimate the main effect of all the parameters under study: (initial conditions such that pH, concentration and nutrients). Determination of main effects is described by several (Fisher, 1926; Scheffe, 1959; Yates and Mather, 1963; Neter *et al.*, 1993; Berthouex and Brown, 1994; NIST/SEMATECH, 2006). Two main software's Minitab V 15, and Sigma plot V 10 were used to solve complex algorithms. Main effect from individual factor e.g. "pH" was computed by subtracting the average response of all experimental runs from the average response of all experimental runs for which pH was set at low level (2.0) and (5.0) as its high level. The main effect plots, interaction plots, a normal probability plot of the estimated effects, Factorial Fit and ANOVA were used in interpretation of the results (Tukey, 1993).

In setting the levels of the factors at two levels of high and low, it was assumed that the factors were continuous and linear. However, the fermentation processing experiments involved the summation of physiological conditions and performance of individual fungal cell. This summation provided the overall fermentation pattern and so do the summation of interaction effects. Results thus obtained presented the overall performance of individual cells-pellets performances. Statistical analysis provided closer solutions by using probability values (Miller, 1997; Jones and Tukey, 2000; Wilcox, 2001; Lehmann and Romano, 2005; McCloskey, 2008).

3.2 Materials and Methods

Materials for this research were from the Highland sisal estate, which is located at Ubena Zomozi village about 120 km along the Dar es Salaam to Morogoro road. Highland sisal estate is about 15 km from Chalinze town hardly 3 km off the road and has 5,900 of ha suitable land for sisal cultivation (Figure 3.1). This estate has been replanting about 200 Ha annually. From that, about 800,000 solid wastes in form of sisal boles are generated. Pilot scale unit was assembled at the Chemical and Processing Engineering (CPE) Laboratory, University of Dar Es Salaam.

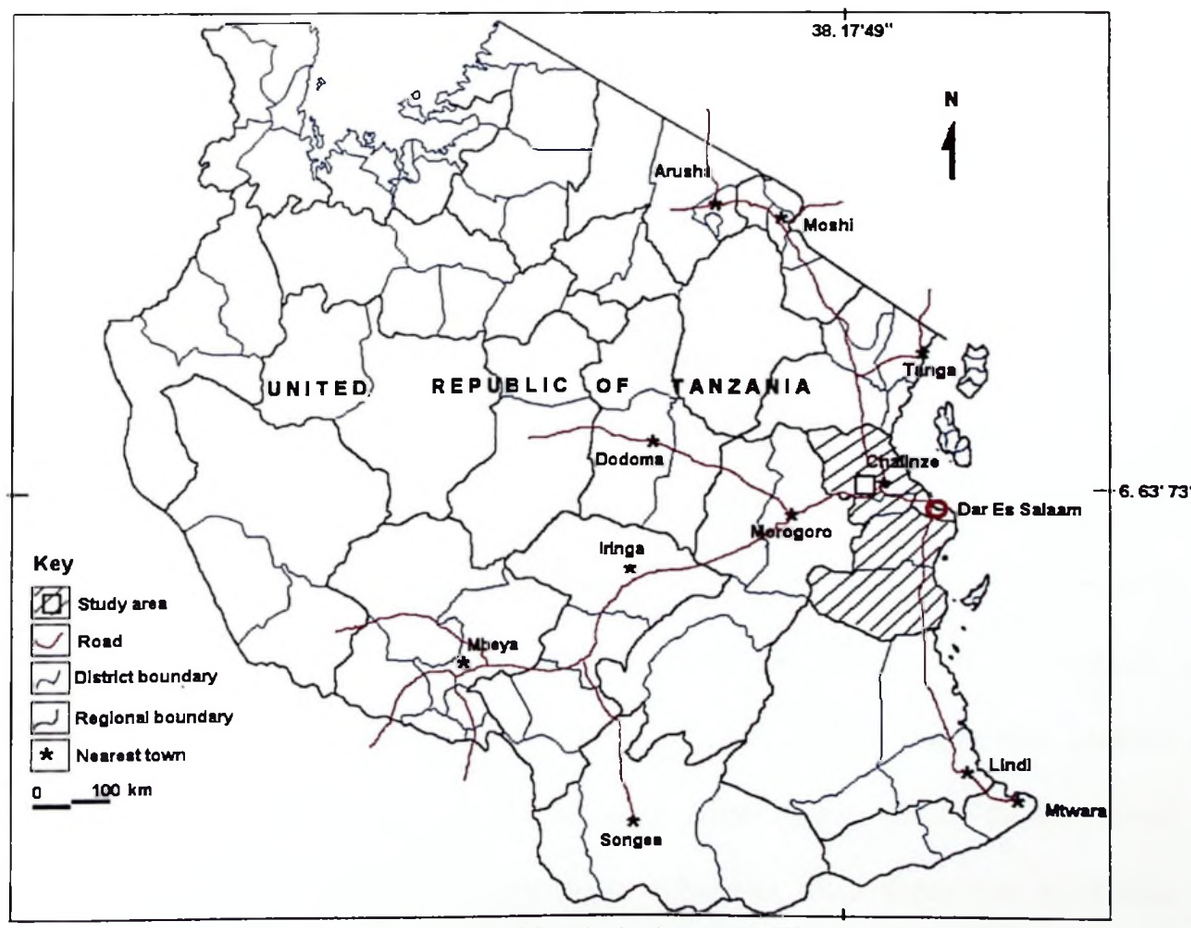

Figure 3.1: Study area Highland sisal estate (Ubena zomozi)

3.2.1 Materials

As the sisal-juice-Inulin was squeezed from freshly collected healthy matured 10-year-old *Agave* H 11648 plants, *A. niger* strains were isolated from *Agave* H 11648 plants' bole rot chunks.

3.2.2 Facilities and analytical instruments

All facilities and analytical instruments were provided by the College of Engineering and Technology (CoET)-Department of Chemical and Mining (CME)-University of Dar es Salaam-(UDSM), unless otherwise stated. Proximate analyses, hydrolysis and citric acid fermentation experiments were conducted at CME-CoET (Protocols specific to the methods were supplies by the respective suppliers and standard methods by Pirt, 1975; AOAC, 1995; Collins *et al.,* 1998 and were used throughout). Viscous Inulin liquids were stored at -20±5°C in a deep freezer unless otherwise stated. All reagents and water used were of the analytical grade whereas analytical grade and HPLC grade chemicals were obtained from Sigma Aldrich.

Microbial culture media used were; Nutrient Agar (NA), Sabouraud Dextrose Agar (SB) CM0041, Oxytetracycline Yeast Extract Agar (OGYE) 66481 HiCrome™ (Sigma Aldrich and Fluka, 2005), Czapek yeast autolysate agar (CYA) (Tran *et al.,* 1998). Others were Petri dishes containing solidified acidified (with 10% tartaric acid) potato dextrose agar (PDA), sisal juice from *Agave* plants as the basal fermentation media, NaOH, phenolphthalein, Whatman filter paper No. 4; While Millipore filtered sterile water, (MillQ-H_2O) was from the Department of Geology at

the University of Dar Es Salaam. Laboratory glassware's and consumables were available at CME-CoET Laboratories.

Isolation recovery screening and confirmation of indigenous *A. niger* from sisal bole rot was conducted at the CME-UDSM-CoET and at the Kungliga Tekniska Högskolan (The Royal Institute of Technology)-Environmental Biotechnology Department (KTH-Bio) Stockholm Sweden. Representative samples were sent for evaluation to a sequencing company named Nadicom (Nadicom, 2006)-The Gesellschaft Für Anngewandte Microbiology MBH, GmBh Pflanzogarten 10, D-35043, and Marburg-Bauerbach, Germany, for the consistence with the morphologic identities done before.

Major instruments that were used are: Varian Atomic Absorption Spectrophotometer (AAS) model AA240 with Graphite Tube Atomizer model GTA120; Mettler Toledo SG7-SevenGo pro™ conductivity, SG68-SevenGo Duo pro™ pH /Ion/Dissolved Oxygen; Milton Roy Spectronic 1001 UV/VIS Spectrophotometer, Perkin Elmer HPLC system consisting of a binary LC pump 250. The Olympus BX-51 fluorescence research microscope which was located at KTH-Bio Laboratory was used for morphological observations. It has special feature that it incorporates the advanced contrasting (Nomarski DIC system) and the Sony DXC 960 MD 3 chip CCD-video camera system. Autoclave SANYO MAC 1200, oven, pressure cooker, Juicer (SANYO blender capacity 5L), Ika Magnetic Stirrer with Heating IKAMAG® safety control-Identity No. 3188800; Stuart scientific oven-incubator, sterile petridishes; Cetromat WR B Braun, Biotechnical International-thermostat controlled

water bath-shaker; Carbolite muffler furnace; platinum striking loop, spirit lamp, Bunsen burner and Labcaire horizontal laminar flow cabinet /microbe free working bench; both were accessed at CME-Laboratory.

3.2.3 Extraction of sisal bole juice

Matured sisal boles were randomly collected, un-dusted and chopped by using either a motorized chopper or electrical saw at the TDTC-CoET. Sisal bole pieces were expressed manually by two methods; the first one was the rapid squeezing using liquid sugar press mill followed by filtration using cheesecloth. The second method involved blending using SANYO blender followed by filter pressing using a locally fabricated special steel filter press machine. Each batch of juice was subjected to physical-chemical characterization and average values were subsequently used and reported. Cellulose rich press residues-briquettes were, oven dried and then tested for calorific value using bomb calorimeter.

3.2.4 Isolation of *Aspergillus niger* from sisal plants

The serial tube dilution method for viable cells counting on agar plates by Collins *et al.,* (1998), was used under strict aseptic conditions. Isolation and recovery of indigenous *A. niger* from sisal boles rots, involved culturing of diluted viable cells/spores at 10^{-1} to 10^{-7} times dilutions, on plates containing one of the following media: NA, PDA, and CYA (Domsch *et al.,* 1980; Tran *et al.,* 1998; Collins *et al.,* 1998; Oxoid, 2006). Samples were incubated at 25, 30 and 45°C; and results were recorded after 24 and 48 hours, while studying the micro and macro morphological

features of CFU under Olympus microscope (Domsch *et al.*, 1980; Tran *et al.*, 1998; Collins *et al.*, 1998).

Systematic identification of bacteria was done using a standardized API 20 NE system for the identification of non-fastidious, non-enteric Gram-negative rods which combine 8 conventional tests, 12 assimilation tests and a database (API®strips, 2006). Fungal and moulds genomes were first identified morphologically using taxonomical keys by Pitt (1993). The fungal genome was then confirmed by the molecular methods, notably polymerase chain reaction (PCR) using sequence of oligonucleotides primers "SOP" AD-02, having the following internal transcribed sequences;

ITS 1	5`-TCCGTAGGTGAACCTGCGG-3`
ITS 4	5`-TCCTCCGCTTATTGATATGC-3`

Universal primer (ITS1) and (ITS4) were chosen because ITS region is known to allow selective amplification of fungal DNA sequences. The region is typical useful tool for molecular systematics at the species level, and even within species (Higuchi *et al,* 1992; Gardes and Bruns, 1993; Mullis, 2007; Gardes and Bruns, 2008).

3.2.5 Selection of strains and screening for enzymatic activities

Spores of pure *A. niger* colonies were harvested from either sisal isolates or industrial DMSZ 8 strain; they were purified by sub sampling from the 10 times diluted samples, using sterile distilled water and shaken for 0.5 minutes; then

inoculated on PDA surfaces. After five days of incubation at 30° C, fungi were isolated from plates that contained no more than 25-250 well-separated pure colonies, and re-cultured aseptically into fresh PDA plates until sporulation (Pirt, 1975; Frisvad *et al.,* 1990; Collins *et al.,* 1998).

Agar plates (2%, 20 ml) were prepared for enzyme screening. The procedure involved suspending 2% agar into a medium, composed of sisal Inulin extract, 10; starch, 10; citric acid, 1.0; KI, 1.0; $MgSO_4$, 0.5; $NaNO_3$, 2.0; KH_2PO_4, 2.0; sodium deoxycholate, 0.2; and glucose oxidase (in g/l): (100,000 U/mg *A. niger,* Sigma), 0.1 in McIlvaine buffer pH 5 (Collins *et al.,* 1998; Fiedurek *et al.,* 2000). Enzyme was added to the hot agar medium when its temperature was just lowered from 100 to 50±5°C, but was still a liquid (to prevent heat inactivation of the glucose oxidase).

Calibrated suspensions of 0.05µl from 12 hr old cultures, the amount that corresponded to an average of single pellets inoculants were drawn by using sterile Eppendroff pipette tips. Inoculation was done at the surface of solidified Inulin starch agar medium plates (Collins *et al.,* 1998; Fiedurek *et al.,* 2000). Agar plates were incubated at 30°C for six hours, and then were checked for the enzyme diffusions.

The violet-blue enzymatic zones resulted after, reaction between starch and iodine which was released from KI by glucose oxidase. A translucent zone around a colony in agar medium plates indicated enzyme diffusions hence Inulin assimilation otherwise a persistent blue inferred no activity (Fiedurek *et al.,* 2000; Zhengyu *et al.,* 2005; Skowronek and Fiedurek, 2006).

3.2.6 Crude enzyme assay

Crude enzymes were extracted using acetone after 24 hours of culturing at pH 5 (Skowronek and Fiedurek, 2006; Zhengyu *et al.,* 2005). A modified AOAC Method 999.03/ K-FRUCHK 03/05 Megazymes Ireland for enzymatic hydrolysis was used, that involved the addition of 0.8 ml of crude enzyme sample into 0.2 ml of standard Inulin (0.2g/100 ml). Incubation was done at pH 5 and temperature value of 40°C. Sampling was done at 0, 30, 60 and 90 minutes. In order to inactivate the enzyme before sugar determinations sample mixtures were heated at 80°C for 5 minutes. HPLC instrument was used to determine concentration of residual Inulin and subsequent fructose produced. Values were calculated and presented as percentages (w/V) of residual Inulin, hydrolysate sugars and fructose (Prosky and Hoebregs, 1999; FRUCTAN HK, 2004; Ngonyani *et al.,* 2006).

Strains showing the greatest enzymatic activity were further cultivated into 500 ml conical flasks with 200 ml of sterile medium that composed of 0.5% yeast extract and 10% sisal bole juice. The samples were shaken on a rotary shaker at 220 rev/min, and incubated for 72 hours at 30°C, after which *A. niger* fungi pellets of desired smooth sturdy morphology were aseptically harvested and re-cultured on fresh PDA plates and, maintenance protocols repeated for the subsequently fermentation experiments.

3.2.7 Performances of *A. niger* fungi isolate and commercial strains

A. niger fungi isolates from sisal bole rot and commercial strains DSMZ 8 were separately cultured in two replicates, into 1000 ml conical-shake flasks containing 500 ml hydrolyzed sisal bole juice with either 40% or 80% (juice: water ratio). This composition was equivalent to fructose 10.18±0.38 and 20.37±0.75g/100ml respectively. The media were further fortified with sterile portion of nutritional additives stocks with equivalent concentrations of 3.1 g/l $NH_4 NO_3$, 0.15 g/l $KH_2 PO_4$, 0.15 g/l NaCl, 1.1 g/l $MgSO_4$, 6.6 mg/l $ZnSO_4.7H_2O$, 0.1 mg/l $FeCl_3$, pH value was adjusted to pH 5.0±0.5 (Datta and Bergemann, 1996).

Citric acid measurements were taken for the first 12 hours up to 24 hours of incubation time; afterwards readings were taken six hourly for the first 12 hours for the first 24 hours, then after 24 hours for the rest of fermentation time. Similarly, control basal media was made of 10g/100ml (10%) sucrose fortified with sterile portion of same amount of nutritional additives. Testing method involved measurements for the biomass, residual sugars and citric acid produced up to 144 hours.

3.3 Hydrolysis of Sisal Inulin

Hydrolysis experiments were based on both the comparative and screening designs to identify the suitability of hydrolysis method while observing interactions effects between two initial conditions (pH and temperature) on fructose yield, by combined pH and either oven or autoclave cooking (NIST/SEMATECH, 2006).

3.3.1 Full factorial design of hydrolysis experiments

Levels and the centre point for FFD were determined by setting the range to be tested. From this range, FFD sets of the real values (Y_i) were tested for each variable parameter and coded them as $X_{i=(-1,0,+1)}$ according to Equation [21].

$$Xi = \frac{(Zi - Zcp)}{\Delta Zi} \qquad [21]$$

Symbol X_i was defined as = the dimensionless value of an independent variable. Z_i = the real value of an independent variable in original units, Z_{cp} = the value of Z_i at the centre point level in the original units, ΔZ_i = a step change in original units (Ooijkaas *et al.*, 1998; Ambati and Ayyanna, 2001).

The response of tested variables were used for prediction using a quadratic polynomial Equation [22] (a two -variable model), which had two main interaction effects, two one-factor interactions, two square one-factor interactions and one-two-factor interaction (NIST/SEMATECH, 2006; Minitab, V 15).

The coefficients ($\alpha_{i = 1, 2,}$) was computed and used for the estimation of the summation of (Y_1), the total hydrolysates (sum of sucrose glucose and fructose) at any time, with a tolerable error ($\xi \sim 0$).

$$Y_i = \alpha_0 + \alpha_1 X_1 + \alpha_2 X_2 + \alpha_{11} X_1^2 + \alpha_2 X_2^2 + \alpha_{12} X_1 X_2 + \xi \qquad [22]$$

Whereas Y = the predicted response; α_0 = the intercept; α_1, α_2, = linear coefficients; α_{11}, α_{22}, = squared coefficients; α_{12}, = interaction coefficients; X_i and X_j = the coded level of variable X_i and X_j (Chowdary *et al.*, 2002; Vohra and Satyanarayana, 2002). The software Sigma plot 10 was used to create graphics and surface plots, while statistical package MINITAB V 15 was utilised to generate a regression model predicting effect of combined parameters on responses fructose yield and residual Inulin. The same interaction model (Y_2) was used in estimating residual Inulin sugars. A quadratic polynomial equation [22], predicted response variables and are thereby presented in Table 3.1.

Table 3.1: Double replicate 2^2 designs for hydrolysis experiments.

S/ Ord	R/ Ord	C/ Pt	Blocks	pH	Temperature±5°C
10	1	0	1	3	110
3	2	1	1	2	132
7	3	1	1	2	132
1	4	1	1	2	80
4	5	1	1	4	132
6	6	1	1	4	80
5	7	1	1	2	80
8	8	1	1	4	132
9	9	0	1	3	110
2	10	1	1	4	80

S/ Ord = Std Order R/ Ord = Run Order C/ Pt = Center Pt

3.3.2 Statistical modelling of the effects of initial hydrolysis conditions

Eighty percent sisal bole Inulin (w/V) which corresponded to concentration 203 g/l Inulin was hydrolysed for 120 minutes, at pH and temperature range of 2-4 and 80-132±5°C respectively. Statistical modelling of the effects of temperature and pH-for

sisal Inulin was done to fructose yields after 120 minutes of hydrolysis (Table 3.2).

Table 3.2: Independent variables in full factorial design

		coded and actual values		
variables	parameters	-1	0	1
X_1	pH	2	3	4
X_2	Temperature (°C)	80±5	110±5	132±5

3.3.3 Optimization of hydrolysis conditions

The Minitab V15-FFD optimization tests method was used to determine the optimum initial conditions for sisal Inulin hydrolysis, within a range of coded levels from -1 to 1 up to 120 minutes, based on constraints listed in Table 3.3.

Table 3.3: Constraints for optimization of fructose yield from initial Sisal Bole Juice-Inulin (SBJ-I) medium composition up to 120 minutes

Variables	Goal	Lower limit	Upper limit
pH	-	2	4
Temperature (°C)	-	80±5	132±5
Fructose yield (g/l)	maximize	16.70	84.96

3.3.4 Oven cooking hydrolysis of sisal bole juice Inulin

Oven cooking was done at temperature values of 30, 60, 70 and 80°C at normal atmospheric pressure, whereas pH values were adjusted to values of 2, 3, 4 and 5. Determinations of sugar concentrations were done at 30 min intervals by using HPLC method. Values were recorded in percentage (w/v), then were reported in (g/l) gram per volume of sisal bole juice, and analysed by statistical methods. The resulted FFD interaction effects from temperature and pH (2 main interaction effects, and 1

two-factor interactions) were determined, while coefficients ($\alpha_{i = 1, 2,}$) were computed and used for the estimation of the effects with a tolerable error ($\xi \sim 0$). After 120 minutes of sisal juice hydrolysis, the response surface model, a polynomial equation was fitted to the data using multiple regressions by MINITAB 15. The responses ($Y_{i = 1}$) of tested variables i.e. Fructose yield were shown by a polynomial equation [22]. The same equation was able to predict other responses e.g. total hydrolysates and residual Inulin (Scheffe, 1959; Yates and Mather, 1963; Neter *et al.*, 1993; NIST/SEMATECH, 2006).

3.3.5 Steam cooking hydrolysis of sisal bole juice Inulin

As the autoclaves, allow good pressure and temperature control thus enabling a homogeneous and economic cooking. Steam was injected at 30 min intervals and monitored at different steam temperatures and pressure 115±5°C (1.6 bar), 121±5 (2.2bar) and 132±5°C (2.9bar). Values for pH were adjusted to 2, 3.5 and 5. The careful control of cooking time, temperature and steam pressure was maintained to prevent overcooking or burning of the sisal Inulin sugars. Measurements were taken at 30 min intervals and sugar kinetics determined by HPLC. Response values were in percentage (w/v) and then reported in (g/l) gram per volume of sisal bole juice. Statistical analysis was performed as presented in section 3.3.4. Equation [22] was used to show the responses model ($Y_{i = 1}$); representing the amount of total fructose produced until 120 minutes. The same equation was able to represent other responses e.g. total hydrolysates and residual Inulin (Fisher, 1926; Scheffe, 1959; Yates and Mather, 1963; Neter *et al.*, 1993; NIST/SEMATECH, 2006).

3.3.6 Determination of hydrolysates sugars

Sisal juice and hydrolysates sugars were analyzed by a modified AOAC Method 999.03; and subsequently quantified using HPLC, with standards prepared from analytical grade reagents. Residual Inulin, total hydrolysates and fructose were analysed using Perkin Elmer HPLC system consisting of a binary LC pump 250 that was set isocratic. Coupled with the Merck Hitachi AS 2000A auto sampler system; Merck Hitachi T-6300 column thermostat which was set at 80°C and column used, was Hamilton HC-7 Ca^{++} Cation Exchange Column; size 305X7.8mm (part number 79436). De-ionized water with traces of Na_2N_3 ($1X10^{-6}$ N; sodium azide) was used as an eluent at flow rate of 0.6 ml/min; injection volume 20 µl and detection done by Perkin Elmer LC-30 refractive index. Time lapse between two injections was 20 minutes. Standards were from Sigma Aldrich. Yield calculations were by referring equations [23] and [24].

$$\text{Fructose yield}\,(\%) = 100 \times \frac{\text{Fructose produced at time t (g/l sisal bole juice)}}{\text{Total sugar (g/l sisal bole juice)}} \qquad [23]$$

$$\text{Residual inulin}\,(\%) = 100 \times \frac{\text{Remaining inulin at time t (g/l sisal bole juice)}}{\text{Total sugars (g/l sisal bole juice)}} \qquad [24]$$

3.4 Pilot Scale Fermentation

Fermentation experiments were also based on both the comparative and screening designs. This was done to investigate the interaction effects of three initial fermentation conditions (pH, initial sugars concentrations and nutrients additives). Fermentation run conditions had a variation made such that one set had a whole nutrients concoction added, while another had no nutrients added and central point had half nutrients concoction added (NIST/SEMATECH, 2006).

3.4.1 Full factorial design for pilot scale fermentation experiments

Levels and the centre point for fermentation experiments FFD were determined by setting the range to be tested. From this range FFD sets of the real values (Y_i) were tested for each variable parameter and coded them as $Xi_{(-1,\ 0,\ +1)}$ according to Equation [21], which was described in section 3.3.1.

Coded term X_i was defined as = the dimensionless value of an independent variable to test, Zi = the real value of an independent variable in original units, Z_{cp} = the value of Z_i at the centre point level in the original units, ΔZ_i = a step change in original units (Ooijkaas *et al.*, 1998; Ambati and Ayyanna, 2001).

The response of tested variables were used for prediction using a quadratic polynomial Equation [25] (a three-variable model), which had 3 main interaction

effects, 3 two-factor interactions and 1- three-factor interaction (NIST/SEMATECH, 2006; Minitab V 15).

Coefficients ($\beta_{i = 1, 2, 3}$) were calculated and used for the estimation of the amount of citric acid produced at a time (Y_1) with a tolerable error ($\xi \sim 0$). The same interaction model was adapted for biomass production (Y_2) and residual fructose sugar (Y_3).

$$Y_i = \begin{pmatrix} \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_2 X_2^2 + \beta_3 X_3^2 + \\ \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 + \xi \end{pmatrix} \quad [25]$$

Whereas Y_i = the predicted response; β_0 = the intercept; β_1, β_2, β_3, = linear coefficients; β_{11}, β_{22}, β_{33}, = squared coefficients; β_{12}, β_{13}, β_{23}, two-way interaction coefficients β_{123} = three way interaction coefficients. The X_i and X_j = the coded level of variable X_i and X_j (Chowdary *et al.*, 2002; Vohra and Satyanarayana, 2002).

The software Sigma plot V 10 was utilised to create graphics and surface plots, while Statistical package Minitab V 15 generated a regression model, which predicted effect of combined parameters on responses (citric acid yield, residual fructose and biomass formed). The response variables were predicted using the quadratic polynomial equation [25] and presented in Table 3.4.

Table 3.4: replicate 2^3 design for fermentation experiment

S/Ord	R/Ord	C/Pt	Blocks	Int. pH	Int. Fructose	Int. Nutrients
8	1	1	1	5	203	1
3	2	1	1	2	203	-1
16	3	1	1	5	203	1
11	4	1	1	2	203	-1
10	5	1	1	5	102	-1
18	6	0	1	3.5	152.5	0
9	7	1	1	2	102	-1
2	8	1	1	5	102	-1
4	9	1	1	5	203	-1
6	10	1	1	5	102	1
1	11	1	1	2	102	-1
12	12	1	1	5	203	-1
14	13	1	1	5	102	1
15	14	1	1	2	203	1
17	15	0	1	3.5	152.5	0
13	16	1	1	2	102	1
7	17	1	1	2	203	1
5	18	1	1	2	102	1

S/ Ord=Std Order; R/ Ord=Run Order; C/ Pt=Center Pt; Int=initial concentration

3.4.2 Statistical modelling of initial fermentation conditions

Statistical modelling of effects of initial fermentation conditions after 168 hours of fermentation processing was done at pH range of 2-5; and initial fructose concentrations from 102-203 g/l, Nutrient additive (g/l fructose) were coded between -1 and 1 (Table 3.5).

Table 3.5: Independent variables in full factorial design

		Coded and actual values		
Variables	Parameters	-1	0	1
X_1	pH	2	3.5	5
X_2	Initial fructose (g/l)	102	153	203
X_3	Nutrient solution (g/l)	-1	0	1

3.4.3 Optimization of fermentation processing

The Minitab V15-FFD optimization tests method was used to determine the optimum initial conditions for citric acid fermentation processing, within the range of un-coded levels from -1 to 1 at 168 hours based on the constraints listed in Table 3.6.

Table 3.6: Constraints for optimization of citric acid yield from initial Sisal Bole Juice- Fructose (SBJ-F) medium composition up to 144 hours

variables	Goal	Lower limit (g/l SBJ-F)	Upper limit (g/l SBJ-F)
pH	-	2	5
Initial fructose (g/l)	-	102	203
Nutrient solution (g/l)	-	-1	1
Citric acid yield (g/l)	maximize	16.70	84.96

3.4.4 Fermentation vessel assembling and specifications

Assembling of pilot scale fermenter involved the Belco Micro carrier spinner flask with capacity 15L (1965-015000). This large spinning bottle was converted into a versatile manually operated bioreactor which accommodated 10L broth (Photo 3.1)

The fermenter had three ports for mounting sparger, pH, and temperature and dissolved oxygen probes. Stirring was done using a Teflon magnetic driven impeller and temperature was set to closer to ambient temperature 30±1°C (Figure 3.2).

Photo 3.1: Submerged Fermentation with Belco-Micro carrier Spinner flasks.

Stirring speed was set at 8 (RPM), working volume (10,000ml), impeller shaft 43 cm, paddle diameter 8.5 cm "slight bent so as to obtain optimum impeller diameter (D)/Tank diameter ratio (tons) = 0.3" (Figure 3.2).

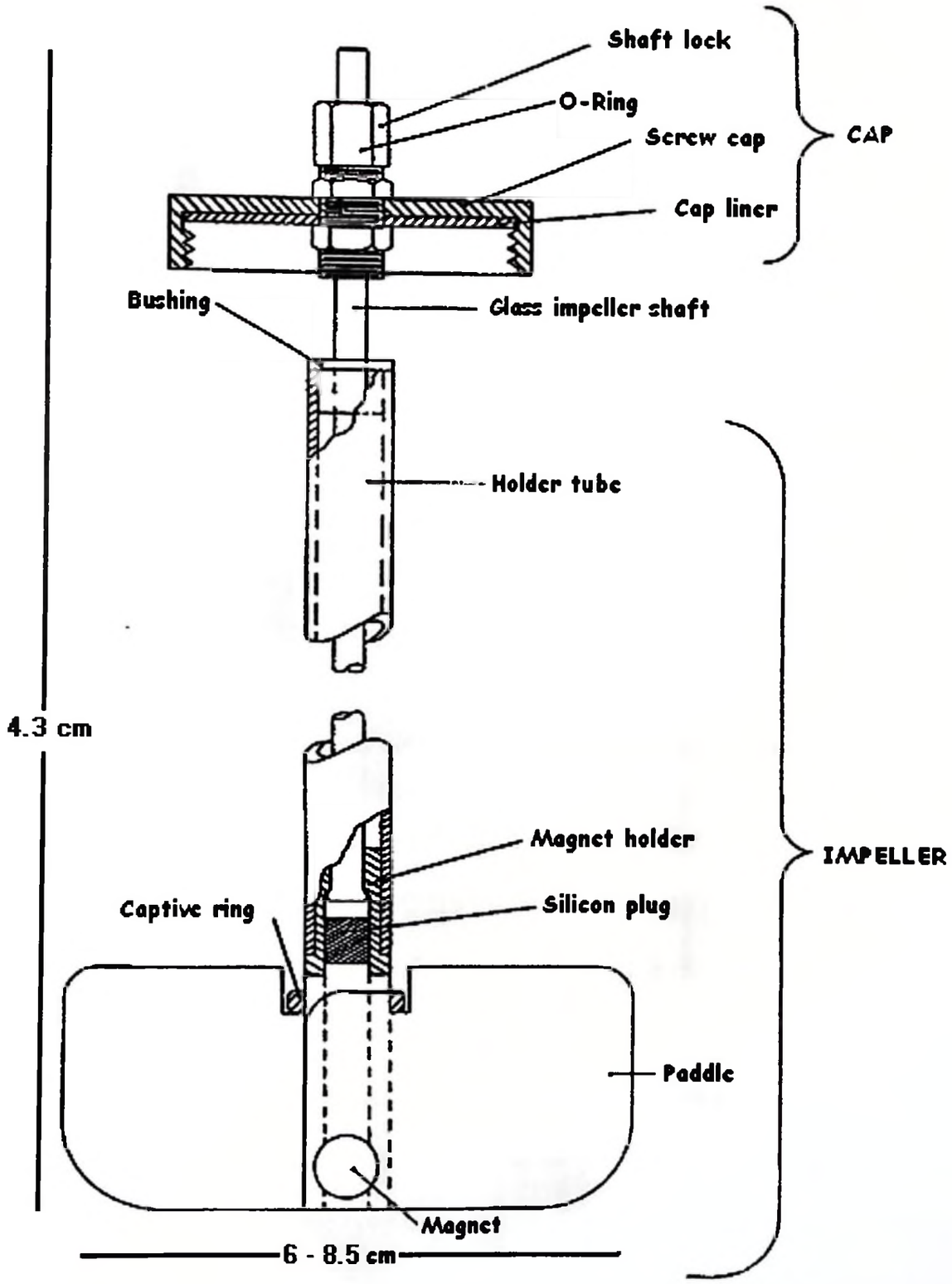

Figure 3.2: Sketch of Belco spinner assembly (capacity 15 L (1965-015000))

Specifications	Values
Model	1565-15000
Size (ml)	15000
Centre neck screw cap(cm)	10
Side arm screw cap(cm)	4.50
Outside diameter = Tank diameter ratio T (cm)	29.40
Approx height(cm)	46
Stirring speed = N (RPM)	8
Impeller shaft (cm)	43
Paddle diameter D (cm)	8.50
Impeller diameter (Paddle diameter)/Tank diameter ratio (D/T)	0.30
Liquid height at 10000 ml = Z (cm)	14.72
Impeller height W (cm)	1.46
Bottom to impeller height C (cm)	2.77

3.4.5 Sterility of a fermenter air supply, temperature and pH control

The fermenter vessel and parts were thoroughly pre washed and dried at 100°C. Then assemblage was autoclaved at 121°C (15 lbs/inch^2 pressure) for 30 minutes before and after each cycle (Shuler and Kargi, 2001; Shuler and Kargi, 2006).

Air supply was provided by using an aquarium pump, and nasal gastro tubes used as sparger and the flow controlled by using a hospital blood transfusion regulator at a flow rate of 1.5±0.3cc/minute (vvm). The initial pH was controlled manually using 1N H_2SO_4. Dissolved oxygen (DO_2) and pH probes were inserted via a side arm (Lee and Yun, 1999).

3.4.6 Rheological behaviour considerations in bio-reactor assemblage

The broth apparent viscosity, (μ_{ap}) was compensated by altering operating conditions. Slightly adjustments of both the impeller agitation speed (N) and aeration (Q) conditions to maintain adequate k_La values was cautiously done, as there was a tendency for the shear zones occurrence close to the impeller that was feared to inflict stress to fungi cells (Gavrilescu *et al.*, 1993; Badino *et al.*, 2001; Kusai *et al.*, 2002; Campesi *et al.*, 2009).

The oxygen transfer was presented by relationship, relating C_L and C^*, i.e. the dissolved oxygen concentration in the broth at the steady state and saturation respectively. These values were recorded using DO probe, and estimated by equation [16] derived from the method proposed by Schumpe *et al.*, (1983); Schumpe, (1983).

$$k_L a = \ln (DOT'\text{-}DOT_o) - \frac{\ln(DOT'\text{-}DOT)}{(T - T_o)} \qquad [26]$$

Equation [16] was used to calculate values for k_La, DOT' and T are the measurement of dissolved oxygen and time (seconds) at the steady state: where as DOT_o and T_o are values for dissolved oxygen and time (seconds) at the OFF. Rearrangement of Equation [16] gives Equation [27], which presents the oxygen uptake rate (*OUR*) values after turning of O_2 supply for <5 min and then turning back to the original settings (steady state).

$$OUR = Q_{O_2} X = k_L a(C^* - C_L) \qquad [27]$$

For reproducibility, the modified Belco micro carrier spinner flask with capacity 15L that was used in this study had to be manipulated so as to maintain equal distribution of both heat and air. In this case a magnetic hot plate stirrer was set at 31± 0.5°C and spinning was maintained at 8 rpm using a magnet driven impeller, thus simultaneously maintaining uniform fermenter aeration rate at 1.0-4.0 l/l/min and temperature of 30°C (Figure 3.2).

In view of the fact that Shuler and Kargi, (2001), reported that *OUR* values, in large-scale cultures falls within 40 to 200 (mmol O_2/l-h), and were also supported by Gavrilescu *et al.*, (1993); Badino *et al.*, (2001); Kusai *et al.*, (2002). It was therefore, expected that *OUR* values for the adopted fermenter not to fall in short of that range.

3.4.7 Preparations of inoculums and culture maintenance

Inoculums were prepared aseptically and freshly inoculated into PDA plates every two months. Spores of *A. niger* strains were used in subsequent fermentation experiments (BfK-KAT and BYF-KAT). Wild *A. niger* strain (BYF-KAT) after comparison to DMSZ 8, was grown on Petri dishes containing PDA (pH 5.5). Vegetative conidia inoculums of 10^6-10^7 spores/ml was prepared in a sterile 500 ml cotton wool plugged replicated Erlenmeyer flasks containing 200 ml of 20% fructose and yeast extract 2g/l at pH 5.0. Shaking was done on a rotary shaker at 140rev/min for 12 hours at 30°C, as per modified procedures involving glucose (Torres *et al.*, 1998; Ruijter *et al.*, 2002).

3.4.8 Vegetative inoculum transfer procedure and fermentation technique

Culturing was done by aseptically harvesting and transferring of 5% (v/v) of the vegetative mycelia pellets of approximately 1-2 mm diameters into the main pilot scale production fermenter which contained fresh sterile broths of desired initial pH, nutrients additive and sugar concentrations as described in section 3.4.9. The incubation temperature was kept at 30 ± 1°C throughout the fermentation period of 168 hours after inoculation. Air sparging and agitation were done by using a magnetic driven spinning impeller. Fermentation conditions were maintained throughout for 168 hours after inoculation (Datta and Bergemann, 1996; Ruijter *et al.*, 2002).

3.4.9 Preparations of fermentation media and nutrients additives

Fructose rich-Inulin hydrolysates were obtained by autoclave cooking of sisal bole juices at 115±5°C, and pH adjusted to value of 3.5 for 180 minutes. The calculated total hydrolysate sugars had average value of 25.07±0.21% (v/w).

Nutrients additives concoction was prepared using salt solution as per modified method by Datta and Bergemann, (1996). Therefore 100% concoction of nutrients additives per one litre feedstock was composed of the following: 3.1 g/l $NH_4 NO_3$, 0.15 g/l $KH_2 PO_4$, 0.15 g/l NaCl, 1.1 g/l $MgSO_4$, 6.6 mg/l $ZnSO_4$. 7 H_2O, 0.1 mg/l $FeCl_3$ (Table 3.7).

Table 3.7: Fermentation broth compounding

	SBJ(%w/v)	Fru (g/l)	pH	YE (g/l)	*Nutrients (%)	SBJ (ml)	Vol (ml)
High	80	200	5.0	2	100	8000	10000
Central	60	150	3.5	2	50	6000	10000
Low	40	100	2.0	2	0	4000	10000

* = levels for concoction of nutrients additives (1 g/l) = 100% (0 g/l) = 0%

3.5 Production of Citric Acid from Sisal Bole Juice

The conditions for fermentation were adopted from several works e.g. (Datta and Bergemann, 1996; Alvarez-Vasquez *et al.*, 2000; Ikram-Ul *et al.*, 2001; Ali, *et al.*, 2001) and were fixed at optimum levels and maintained throughout, for instance oxygen tension was maintained at value of (1.5-2±0.2 l/l/min) and fermentation temperature (30±1°C).

Since on average, sisal boles are composed of 70-80% (w/v) juice, pure sisal boles juice 100% (w/w) consists of hydrolysable sugars corresponding to fructose 25.07±0.21%, (about 250.70g/l). The initial sisal bole juice concentrations were 40, 60 and 80% (sisal bole juice: water ratio), these corresponded to total hydrolysates sugars equivalent to 11.66±1.15, 17.49±1.72 and 23.32±2.30 g/100ml (fructose 10.18±0.38, 15.27±0.57 and 20.37±0.75 g/100ml sisal bole juice inulin respectively); and each were fortified with 2 g/l yeast extract.

Adding 0%, 50% or 100% levels of nutrients media concoction studied the effect of

nutrients. The initial pH values were also adjusted by additions of dilute H_2SO_4 to (2.0, 3.5, and 5.0±0.2), and fermentation monitored for 168 hours. Biomass formation, sugar consumptions and citric acid production were also measured (Table 3.7). Sterilization unless otherwise stated was done at 121°C (2.2 bar) for 20 minutes.

3.5.1 Recoveries sludge treatment and determination of citric acid

The citric acid rich broths were filtered by pressing through cheesecloth to separate pellets from citric acid rich broths. Clear filtrate was subjected to purifications protocols and product presented in two formulations the industrial liquid and crystalline citric acid (Collins and Mention, 1962).

The residual biomasses/sludge in form of mycelia pellets, were easy to desiccate by filter pressing-off the excess water. The mycelial-pellets mass of *Aspergillus niger* was then analysed gravimetrically (Alvarez-Vasquez *et al.*, 2000; Ikram-Ul *et al.*, (2001). Filter press cakes were to be utilized as animal feed supplement or bio fertilizer, after pre-treatment. However, this step was skipped in this research, because it demanded Agronomic inputs and experimental animals.

Citric acid was analyzed using Perkin Elmer HPLC system set at the ambient temperatures. Column used was Hamilton reverse phase pH stable, PRP X 300-ion exclusion (part number 79465). Eluent used was made of 1.0 mN sulphuric acid, with flow rate 1.00 ml/min. Injection volume was set at 20 ml and detection was done by UV-Visible L-4250 Merck Hitachi detector at a wavelength 210 nm. Time

lapse between two injections was 15 minutes and standards used were from Sigma Aldrich. The yield of citric acid was calculated by equation [28].

$$\text{Citric acid yield}(\%) = 100 \times \frac{\text{citric acid produced at t (g/l sisal bole juice)}}{\text{hydrolysate sugar utilized at t (g/l sisal bole juice)}} \qquad [28]$$

3.5.2 Quantification of residual sugar and biomass yield

Residual Sugars were analysed using Perkin Elmer HPLC system consisting of a binary LC Pump 250 that was set isocratic, as the Merck Hitachi AS 2000A auto sampler was used. The Merck Hitachi T-6300 Column thermostat was set at 80°C. Column used was Hamilton HC-75Ca^{++} cation exchange column, size 305X7.8mm (part number 79436). The eluent used was de-ionized water with traces of Na_2N_3 ($1X10^{-6}$ N; sodium azide), at a flow rate 0.6 ml/min. Aliquots (20 µL) were used and detection was done by a Perkin Elmer LC-30 refractive index. Time lapses between two injections were 20 minutes and Standards used were from Sigma Aldrich. The total residual sugars were calculated by equation [29].

$$\text{Residual sugar}(\%) = 100 \times \frac{\text{Residual sugar at time t (g/l sisal bole juice)}}{\text{Total sugars (g/l sisal bole juice)}} \qquad [29]$$

The biomass analysis was done, gravimetrically after washing the mycelial mass with distilled water and drying in an oven at 80°C to constant weight. The percentage values calculations were done as per equation [30].

$$\text{Biomass Yield } (\%) = 100 \times \frac{\text{Dry pellets recovered at time t (g/l sisal bole juice)}}{\text{Total sugars (g/l sisal bole juice)}} \qquad [30]$$

3.5.3 Statistical treatment of results and data

Statistical tests of the treatment effects were done and tested by the Duncan multiple ranges in the form of (< p >) probability values (Duncan, 1975; Dunnett, 1980; Snedecor and Cochran, 1980; Tukey, 1993; Berthouex and Brown, 1994; Jones and Tukey, 2000; Kim, 2004; Lehmann and Romano, 2005; NIST/SEMATECH, 2006; McCloskey, 2008). Computations were conducted using computer software's; such as the Microsoft word 2003; Excel 2003; Sigma plot V10; Super-Pro Designer V4.3; Penlab V 601; Dax V7.1 and Minitab V15. Results were presented graphically in the form of HPLC chromatograms, response surface curves and graph showing trends for substrate consumption were also produced.

3.5.4 Statistical modelling of the effects of initial fermentation conditions

Sisal bole juice inulin hydrolysate-fructose (SBJ-I) (w/V) was used in fermentation experiments using indigenous *A. niger* strain (BYF-KT). Modeling effects of initial fructose concentration, pH and nutrients additives-for fermentation of sisal inulin hydrolysate fructose after 168 hours was done at optimum conditions of fructose concentration (102-203g/l), pH range of (2-5) and nutrient concoction coded at (-1, 1) as shown in (Table 3.8).

Table 3.8: Independent variables in full factorial design

		coded and actual values		
variables	parameters	-1	0	1
X_1	pH	2	3.5	5
X_2	fructose conc. (g/l)	102	153	203
X_3	Nutrient additive	-1	0	1

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4. RESULTS

In view of the fact that data collection was made in replications, therefore thus results presented are the mean values. The results for isolation identification and determinations of the best performing *A. niger* isolates were analysed and presented using tables and graphics. the results for Hydrolysis and fermentation were studied , analysed and tested statistically using ANOVA. The statistical models were created, discussed and presented.

4.1 Physical chemical characteristics of sisal bole juice extracts

Two juices the darker liquid (SBJ A) was extracted by using sugar press mill and a much clearer liquid (SBJ B) was recovered from the conventional blending of sisal bole chunks (Photo 4.1 A-E). Table 4.1 thereby presents physical chemical characteristics results of respective juices.

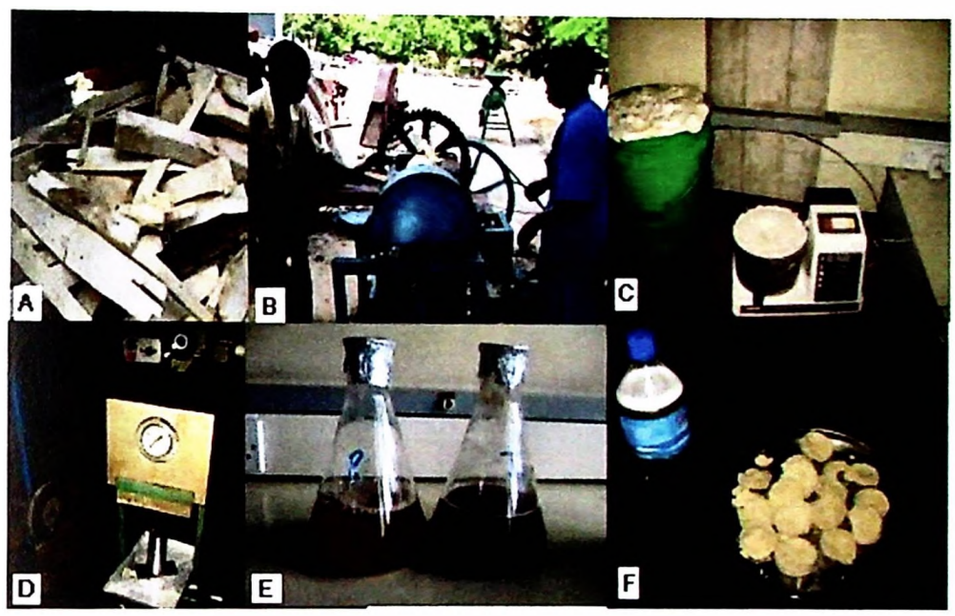

Photo 4.1: Sisal bole juice extraction pictures; (A) = Sisal bole chunks, (B) = Sugar cane press mill, (C) = Blender and sisal paste, (D) = filter press machine, (E) = Sisal bole inulin from two extraction methods, (F) = Filter press cakes (cellulose residues)

HPLC chromatograms (Figure 4.5) revealed that both dark and clear sisal bole juices had the same retention-time value of 8.3±0.2 minutes (APPENDIX XIII) and sugar concentration values of 26.9±0.31 and 26.4±0.24 g/100ml respectively (Table 4.1).

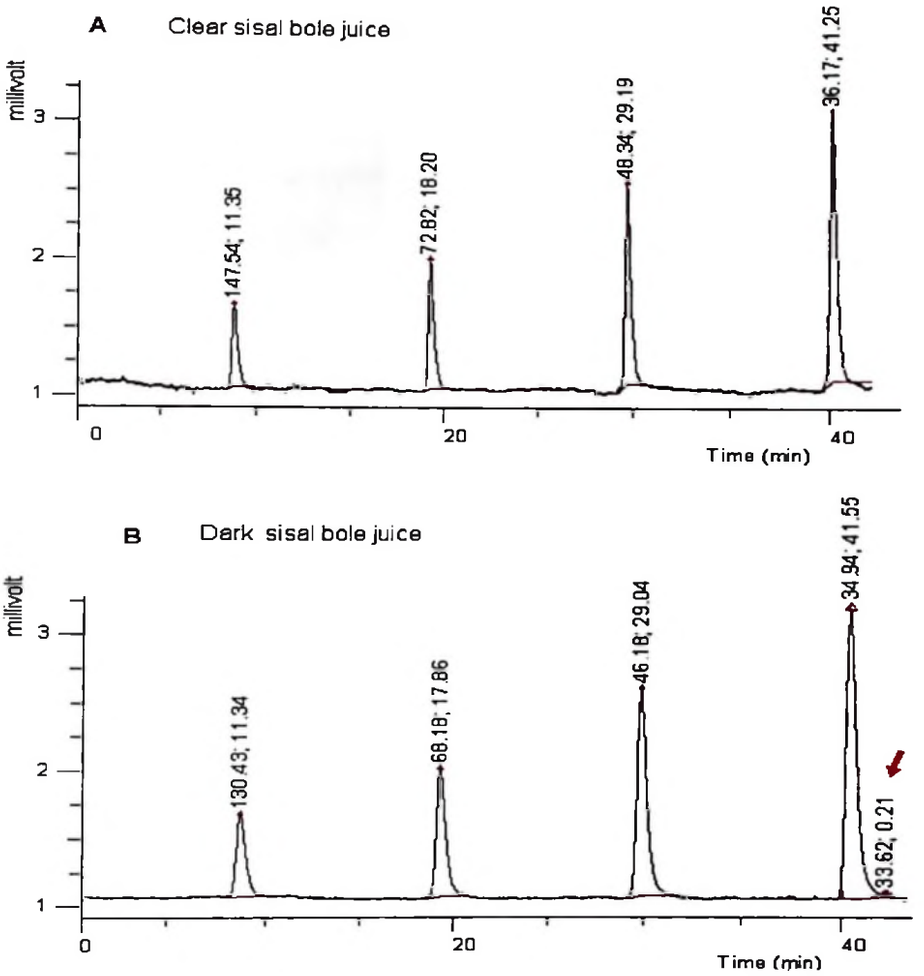

Figure 4.1: Sisal bole juice inulin chromatograms; (A) = for clearer liquid (SBJ B) and (B) = for darker liquid (SBJ A) at concentration values 0.1, 0.2, 0.3 and 0.4g/ml.

The last peak in the HPLC chromatogram Figure 4.2 (B) revealed an emerging fructose peak, which signified catalytic assisted hydrolysis by metallic species such as Fe^{2+}, Fe^{3+}, Mn^{2+} and Mn^{4+}. This suggested the possibility of hydrolysing sisal inulin by using conventional industrial catalysts (Abasaeed and Lee, 1995).

Mechanism employed in that is based on the Brønsted-Lowry acid-base theory whereby positively charged metal ions in contact with protonated water lower pH of the substrates (Kyle, 1992; Moran and Shapiro, 2000; Lindhorst, 2007).

Table 4.1: Physical chemical properties of sisal bole juices

Sisal bole composition				
Parameters	Clear extract	Dark extract	Recommended parameters	levels
pH	5.22	5.24	-	-
viscosity	2.488 (mPaS)	2.556 (mPaS)	-	-
density	1.044	1.065 (g/cc)	-	-
color	opaque	amber	-	-
Refractive index	1.348	1.356	-	-
% TOT Volatiles	2.58 (%w/v)	2.25 (%w/v)	-	-
% TOT fibers	13.73 (%w/v)	12.98 (%w/v)	-	-
% TOT Juice	83.69 (%w/v)	85.47 (%w/v)	-	-
Juice composition			-	-
% water in juice	71.26 (%w/v)	71.02 (%w/v)	-	-
%Total Sugars			-	-
mean	26.40 (%w/v)	26.90 (%w/v)	-	-
max	29.10 (%w/v)	27.90 (%w/v)	-	-
min	24.10 (%w/v)	24.70 (%w/v)	-	-
stdev	0.02 (%w/v)	0.03 (%w/v)	-	-
%Equivalent Fructose				14-20
mean	24.85 (%w/v)	25.56 (%w/v)	[1]Glucose	20 (%w/v)
max	29.79 (%w/v)	31.20 (%w/v)	-	-
min	22.19 (%w/v)	21.87 (%w/v)	-	14 (%w/v)
stdev	1.79 (%w/v)	2.92 (%w/v)	-	-
% Fructose yield	94.12 (%w/v)	95 (%w/v)		
% Inorganic salts	0.14 (%w/v)	0.80 (%w/v)	-	-
% Crude fibers	1.37 (%w/v)	1.37 (%w/v)	N.A	N.A
Inorganic salts	1.417 (mg/l)	8.00 (mg/l)	-	-
TS	0.47 (mg/l)	0.52 (mg/l)	-	-
TN	21.16 (mg/l)	23.80 (mg/l)	[2]$NH_4 NO_3$	2 (g/l)
TP	0.15 (mg/l)	0.15 (mg/l)	[2]$KH_2 PO_4$	0.5-1 (g/l)
K	27.70 (mg/l)	14457.50 (mg/l)	[2]KCL	0.5 (g/l)
Na	17.89 (mg/l)	0.25 (mg/l)	2NaCl	0.15 (g/l)
Fe	4.54 (mg/l)	29.20 (mg/l)	[2]$FeSO_4 \cdot 7H_2O$	0.01 (g/l)
Mn	0.07 (mg/l)	7.85 (mg/l)	$2Mn^{+2}$	~3-10 (ppb)
Mg	4.85 (mg/l)	31.90 (mg/l)	[1]$MgSO_4 \cdot 7H_2O$	0.5 (g/l)
Ca	85.96 (mg/l)	525.01 (mg/l)		
Zn	0.39 (mg/l)	3.55 (mg/l)	$ZnSO_4.7H_2O$	6.6 (mg/l)
Cu	0.25 (mg/l)	0.25 (mg/l)		

[1](Shu and Johnson, 1948)
[2](Papagianni *et al.*, 1999a, b; Pazouki *et al.*, 2000)

Physical chemical characteristics of sisal bole juices presented in Table 4.1 indicated no significant molecular difference between dark and clear sisal inulin samples. However, mineralogical determinations revealed that values for Fe and Mn differed significantly ($p = 0.239$ and 0.533 for iron and manganese respectively) with actual values higher in darker inulin (7.85 and 29.2 mg/l for manganese and iron respectively) while clear inulin had much lower values (0.07 and 4.54 mg/l for manganese and iron respectively). Total Nitrogen values were between 52.89 (3.77 NH_4NO_3) mg/l and 47.60 (3.41 NH_4NO_3) mg/l, phosphorous levels were found to be 0.15 mg/l in both liquids (Table 4.1). Levels of macro and micro nutrients (minerals) specifically high Mn and , suggested pre-treatment of hydrolysates fructose prior fermentation processing as excessive amounts are undesirable as explained in section 2.8.12 and Table 2.4, also it is supported by Soccol *et al.*, (2006) on **Chemical factors affecting citric acid production (Soccol *et al.*, 2006)**

HPLC chromatograms revealed no significant difference in terms of inulin molecular structure between dark and clear sisal inulin samples. This suggested that sisal boles could be either milled or blended to produce juice without affecting the molecular structure of inulin. A clearer sisal juice (SBJ A) had fewer inorganic salts than darker sisal juice (SBJ B). Signalling that in order to recover a purer form of inulin, the milling machine should be made of as an inert material as possible for example wood or special non-reactive steel.

Detergent and acid washing of filter press residues produced cellulose rich briquette materials (crude fibres briquettes) which were composed of 0.48±0.2% moisture. The

combustion of dry residues had energy content of 1.65-1.71x10^4 kJ/Kg (Table 4.1). The residues recovered after filter pressing, are combustible and therefore are potential alternative energy source, thus could cut production costs.

4.2 Isolation Screening and Identification of Filamentous Fungi

In principle the microbiology of sisal bole rots seems to be limited to essentially some fungi yeast and bacteria, species those which are tolerant to harsh environmental conditions characterized by comparative low pHs' of sisal saps, hard lignified stem-bark and a cuticle layer that covers plant leaves. In actual fact, most of the microorganisms seemed to form spores or a symbiotic alliance i.e. waits for others to break huge molecules for them to assimilate for the survival (Taiz and Zeiger, 1998). Fungal isolation experiments were carried out in three replicates, and results are presented as mean values (Table 4.2 and 4.3).

Table 4.2: Microbial counts in sisal bole rot chunks (grab samples N = 10).

Dilution range	CFU -count range	Average counts x10^5 (CFU)					
		Mould	Bacteria	Fungi	Yeast	Others	Total
10^{-2}	80 and above	ND	ND	ND	ND	ND	ND
10^{-3}	36-42	2.00	4.00	5.50	1.98	0.67	13.48
10^{-4}	30-40	2.08	3.31	6.60	2.43	0.58	14.42
10^{-5}	28-32	1.80	1.00	7.40	1.62	0.86	11.82
10^{-6}	25-30	3.60	4.30	6.80	5.09	0.78	19.79
10^{-7}	15 and less	ND	ND	ND	ND	ND	ND
Total counts		9.48	12.61	26.3	11.12	2.89	62.40
	Min	1.80	1.00	5.50	1.62	0.58	10.50
	Max	3.60	4.30	7.40	5.09	0.86	21.25
	Average	2.37	3.15	6.58	2.78	0.72	15.60
	Stdev	0.83	1.49	0.79	1.58	0.12	4.81
	Average (%)	15.19	20.21	42.15	17.82	4.63	100

ND = not determined

Four major groups of fungi and moulds were isolated. Individual group isolated were named and averaged in order of abundances; *Aspergilla* (36±0.8)%, *Penicillin* (28±0.1)%, *Yeast* (15±1.6)% and *Fusarium* (10±1.2)%. Remaining fraction included various spore forming bacteria species, of which the dominant were *bacilli* and *coccid.* Also seen were coma shaped but were not considered in this study (Table 4.2 and 4.3).

Table 4.3: Relative abundances of Fungi in sisal bole rot grab samples (N = 10)

Reference	Morphological Identification	CFU/(g) sisal bole rot	CFU/(100g) sisal bole rot	Remarks
OR5	Yeast budding	$11.12x10^5$	(15±1.6)	
OR5 KT	Yeast budding			For PCR
OR5 NA	Yeast budding			For PCR
4a	Yeast single			
4a KT	Yeast single			For PCR
Wfus.	*Fusarium solani*	$7.42x10^5$	(10±0.12)	For PCR
Wfus KT	*Fusarium solani*			
BYF KT	*Aspergillus niger wild*	$26.7x10^5$	(36±0.8)	For PCR& (citric acid)
BYF	*Aspergillus niger wild*			(citric acid)
BWf KT	*Aspergillus tamarii*			For PCR& (citric acid)
BWf	*Aspergillus tamarii*			
BfK	*Aspergillus nidulans*			For PCR
Yf_1	*Aspergillus nidulans*			For PCR
Yf_2	*Aspergillus nidulans*			
Pe Green	*Penicillin*	$2.08 x10^5$	(28±0.1)	
Pe Gray	*Penicillin*			
White-cream	Endospore coccid	$5.19x10^5$	(7±1.5)	API 20 NE TESTS
Off white	Endospore rods	$4.45x10^5$	(6±1.5)	API 20 NE TESTS
Shiny white	Coma motile species	$2.97x10^5$	(4±1.5)	API 20 NE TESTS
Others		2.89	(4.63±1.5)	

4.2.1 API 20 NE results for non-enteric Gram (-) ve bacteria after 24-48 hours.

A combination of standard biochemical tests and assimilations tests, with the system for API 20 NE identification for non-enteric Gram (-) rods after 24 and 48 hours, was adapted. The system was able to characterize bacteria that are acclimatized and grows with minimal requirements (non-fastidious) under harsh sisal environments (APPENDIX XIV). Reliability of tests was guaranteed and achieved by the application of standardized inoculums of low bacterial concentrations (Johnson and Case, 2004). Bacteria sampling was done from freshly refined cultures using a sterile (0.5 µl) micro pipette tip, a techinique that eliminated contaminants from mixed cultures and subcultures.

The frequently the motile and endo spore forming non enteric-non facidous bacteria were common bacteria isolates. These were identified by using the API 20 NE TESTS (APPENDIX XIV), and hence were named as the *Brevundimonas diminuta sp, Shewanella putrefaciens sp, Brevundimonas vesicularis sp* and *Pasteurella sp* (Johnson and Case, 2004; Ngonyani, 2006). In addition to that, encountered co-existence of moulds and bacteria was probably due to the inherent symbiosis.

In general both microorganisms assimilated raw sisal inulin and used it for metabolism. Symbiosis was felt whenever inulin was used as the sole carbon source indicating that the fungi and mould species were good in processing raw sisal juice-inulin, whereas bicteria devoured processed inulin and metabolites afterwards. The identified bacteria were able to assimilate starch and were resistant to common

antibiotics. Starch assimilation tests suggested that bacteria strains preferred complex form of carbohydrate, which could also suggest exploration of more metabolites from mixed cultures grown in various specific media which are fortified with inulin (APPENDIX XIV; Johnson and Case, 2004).

Occurrence of high fungi biodiversity, suggest the existence of very stable microbial communities acclimatized to sisal stems throughout its life cycle. Exploration of diversified species for example *Fusarium, Penicillin, A. nidulans* and *A. tamari* should be the next endover as these microbial communities are of great use in future industrial processing of sisal inulin (Hosea, 1996; Massala, 2003).

4.2.2 Morphological differences of *A. niger* from other filamentous fungi

The observation and studies of Macro and micro-morphologic differences between *A. niger* and other filamentous fungi such as *Fusarium* and *Penicillin* species were done under the Olympus BX-51 fluorescence research microscope (Domsch *et al.*, 1980; Samson and Pitt, 1990; Pitt, 1993; Hosea 1996; De Hoog, 2000; Samson and Pitt, 2000).

The dominant *Aspergilla* species encountered were *A. nidulans*, *A. tamari* and *A. niger* in ratios of (3:2:2) respectively. Macroscopically *A. niger*, colonies on surface of Czapeck agar at 30°C initially were white, and then blackened with conidial production. On reverse side, colonies were pale yellow and grew radial (Raper and Fennell, 1965; Domsch *et al.*, 1980; Samson and Pitt, 1990; Pitt, 1993). Five days

old, *A. niger* hyphae became distinctive, septet and their respective conidia were long and smooth. The hyaline was measured at length of (500 to 800 µm).

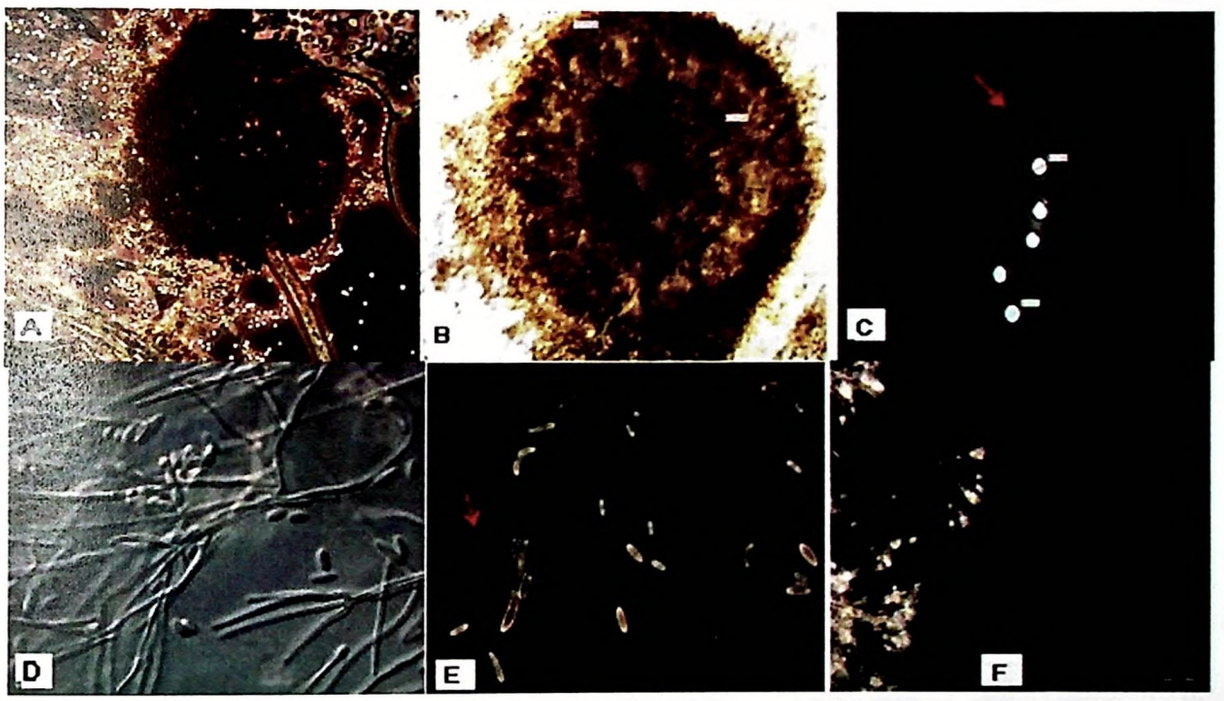

Photo 4.2: Some microorganisms of industrial potential from sisal bole rot stems; A = *A. niger* conidia, B = transect of *A. niger* conidia, C = *A. niger* spores, D = *Fusarium sp*, E = banana spores *Fusarium solani* and F = Septet-conidiophores of *penicillin sp.*

A typical biseriate characteristic of *Aspergilla* was, evidently seen under the light microscope. Presence of a metulae and their corresponding *conidiogenous phialides* covered the entire vesicle to form a rough fruiting conidia head whose diameter measured at 132.78±0.02 µm (A) and (B) in Photo 4.2. Conidiophores head, metulae and phialide diameters measurements were between 3.77 and 3.89±0.02 µm (Raper and Fennell, 1965; Domsch *et al.*, 1980; Samson and Pitt, 1990; Pitt, 1993; De Hoog, 2000). Free *A. niger* spores are also shown in Photo 4.2 plate (C). Henceforth, *A. niger* was distinguished from group members and used in subsequently experiments.

On the contrary, macroscopic morphologies of *Fusarium sp* colonies after culturing in Czapek medium revealed a characteristic rapid growing cream-white cottony colonies with aerial protruding mycelium and cream reverse typical to *Fusarium sp*. The moist cream-colored sporodochia (were viewed with the naked eye as clusters of conidiogenous cell areas were raised). Microscopic morphologies of *Fusarium* showed that the hyphae were septate and hyaline. Conidiophores were simple (non-branched) or sometimes few branched monophialides (phialides with a single opening) were spotted. Seen in (Photo 4.2 plates (D) and (E)), *Fusarium sp* colonies displayed characteristic banana like spores (macro conidia) common to *F. solani*. These were moderately curved, stout, thick-walled, usually 3-5 septate, measuring 4 to 65 μm long, and were borne on short conidiophores that immediately formed sporodochia (Samson and Pitt, 1990; Pitt, 1993; De Hoog, 2000). Microconidia were borne from long monophialides, and were one to three-celled (2-5 x 8-16 μm long), and looked like false heads (i.e. in clusters of conidia at the tip of a phialide).

Macroscopic features of growing colonies of *Penicillium* displayed rapid growing filamentous velvety texture and flat colonies. The dominant colonies were initially white and become gray-green and some few were olive-gray. The plate reverses were pale-yellow with dark orange pigment diffusing into the surrounding agar. Microscopic features typical for *penicillin sp* that is the septate hyaline hyphae (1.5 to 5 μm in diameter) were vividly seen. Also some *Penicillin sp* showed their distinct septet conidiophores with respective branched conidiophores, metulae, phialides, and conidia typical to *P. purpurogenum* (Samson and Pitt, 1990; Pitt, 1993; Domsch *et al.*, 1980). Metulae were secondary branches that were formed on conidiophores and

carried flask-shaped phialides (the brush-like clusters which often are referred to as *"penicilli"*). The conidia (2.5-5µm in diameter) were round-unicellular, and visualized as unbranched chains at the tips of the phialides.

4.2.3 Molecular biology identification using (PCR) and genomic results

Photo 4.3 and Table 4.4, presented results after molecular biology identification techniques by polymerase chain reaction (PCR) sequencing using primers "SOP" AD-02 (Higuchi *et al,* 1992; Gardes and Bruns, 1993; Peterson, 2000; Nadicom, 2006). PCR results showed high consistence with the systematic macroscopic identities done using taxonomical keys discussed in section 4.2.2, (Samson and Pitt, 1990; Pitt, 1993; De Hoog, 2000). Concurrently, the genomic DNA results for *Aspergilla* fungi shown in Photo 4.3 indicated that specimens 449.7-(BYF KT) *A. niger wild*, 449.9-(BfK) *A. nidulans* and 449.10-(Yf_1) *A. nidulans* seemed to be very close strains. Nevertheless, sample 449.8-(BWf KT) *A. tamarii* showed a different pattern. This suggested a need for a more intensive identification work at species level using a larger sample population.

Therefore molecular biology identification techniques of moulds and fungi (Higuchi *et al,* 1992; Gardes and Bruns, 1993; Peterson, 2000; Nadicom, 2006) confirmed the morphologic identintifications using protocols by Pitt, (2003). This suggested that the *Agave* hybrid H 11648 bole rot sustains very stable high bio-diversitified microbial communities naturally acclimatized to sisal stems-inulin rich environment, including various fermenting moulds and fungi.

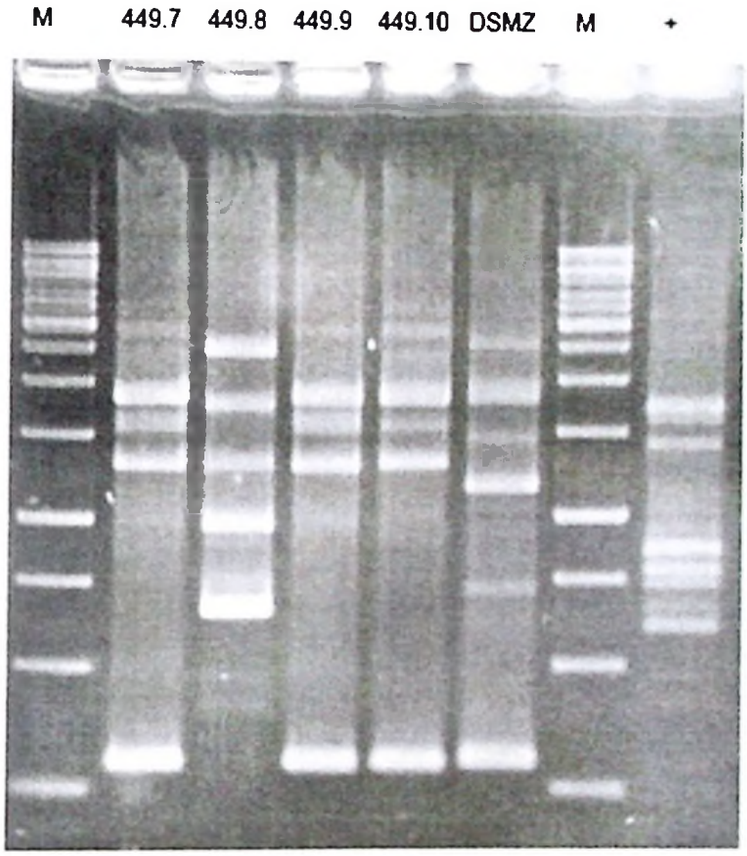

Photo 4.3: Genomic DNA results for *Aspergilli* species from sisal (H 11648)

Four major groups of fungi and moulds isolates were established and identified as *Aspergilla* (36±0.8) %, *Penicillin* (28±0.1) %, *Yeast* (15±1.6) % and *Fusarium* (10±1.2) %. The fungi BYF KT was inferred as *A. niger* endemic to sisal *Agave* hybrid H 11648 stems. The identified four major groups of fungi and yeasts suggested further tests on their versatile use in selective fermentation processing of useful metabolites. Genomic DNA results for *Aspergilli* fungi sample 449.8 had very distinct characteristics from other Aspergilla which suggested further characterizations.

Table 4.4: Genomic PCR sequencing summary of Fungi and moulds

SNo nadicom	Reference	Identified as	Remarks
449.1	OR5 KT	*Pichia membranifaciens*	
449.2	4a KT	*Clavispora lusitaniae*	
449.3	OR5 NA	*Pichia membranifaciens*	
449.6	Wfus	*Fusarium solani*	
449.7	BYF KT	*Aspergillus niger*	for CA production
449.8	BWf KT	*Aspergillus tamarii*	for CA production
449.9	BfK	*Aspergillus nidulans*	
449.10	Yf_1	*Aspergillus nidulans*	
API 20 NE	Endospore coccid		
API 20 NE	Endospore rods		
API 20 NE	Motile species		

4.8 Screening of Strains with Industrial Potential

All representatives from four fungi groups showed significant enzyme activity zones on starch inulin agar plates (Photo 4.4 and Table 4.5), which suggested the assimilation of the complex inulin-carbohydrate from sisal bole, and consequently their potentiality in selective industrial processing of inulin (Collins *et al.*, 1998; Fiedurek, 2000). Among the isolates, fusarium strain showed excellent enzyme magnitude of 10.4 mm, which made it a mould of future interest in enzymology (Fiedurek, 2000; Collins *et al.*, 1998).

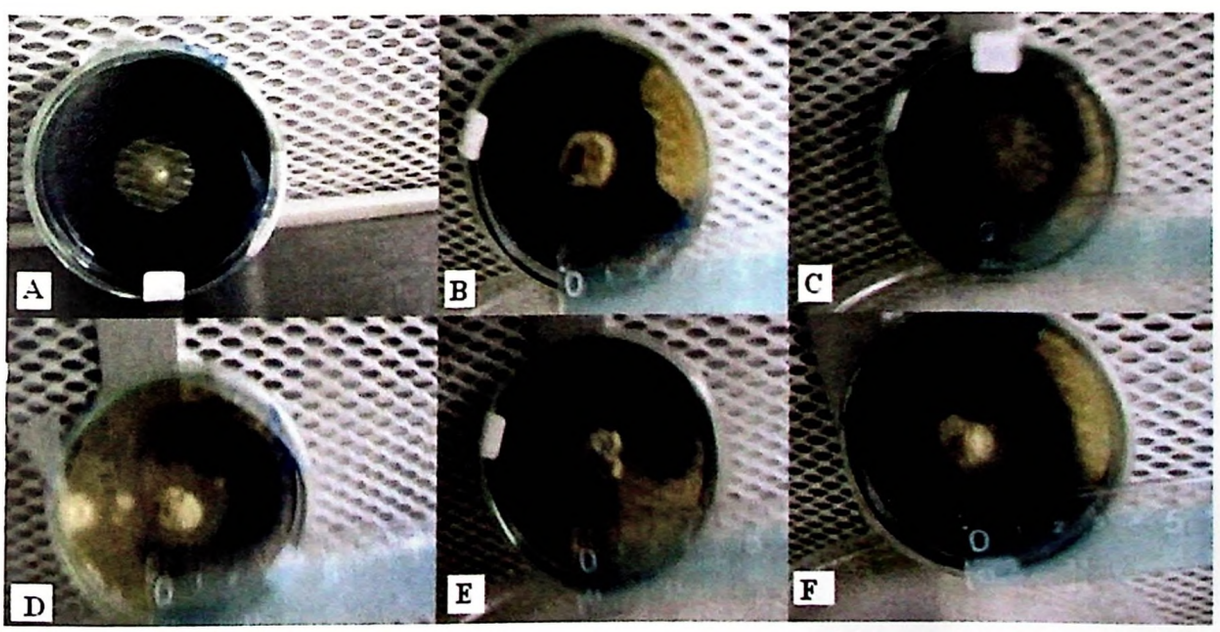

Photo 4.4: Inulin starch agar plates, showing translucent-enzyme activity zones on as follows: A = *Fusarium sp* (10.4mm), B = *Aspergillus* BfK (8.4mm), C = *A. niger* BYF-KT (14mm), D = *Penicillin sp* (15 mm), E = *A. niger* BYF (10.2 mm) and F = *A. niger* DSMZ 8 (10mm).

Table 4.5 presents average diameters of enzyme diffusion zones from selected fungal strains shown in Photo 4.4. The *Aspergilli sp* had diffused enzymes diameters values

between 10.1±0.1 and 12±2 mm. *Pichia membranifaciens* showed higher activity among yeast strains with diameter value of 11.5±1.5 mm. As *Fusarium solani* had average diameter of 11.2±0.8mm, penicillin showed highest diameter with value of 14±1mm. Standard DMSZ8 *Aspergillus* strain had average value of 9.5±0.5mm.

Table 4.5: Enzyme diffusion zones from selected fungal strains

Species	Identity	Genera	N	CFU	Diameters enzyme activity (mm)	
					Range	Average
Yeast (15±2)%	OR5 KT*	*Pichia membranifaciens*	3	5	9-12	9.5±0.5
	4a KT*	*Clavispora lusitaniae*	3	4	8-11	9.5±1.5
	OR5 NA*	*P. membranifaciens*	3	6	10-13	11.5±1.5
Fusarium (10±2)%.	Wfus.*	*Fusarium solani*	3	10	10-10.4	10.4±0.8
Aspergilli (36±2)%,	BYF*	*Aspergillus niger* wild 1	3	8	10-10.2	10.1±0.1
	BYF KT*	*Aspergillus niger* wild 2	3	10	10-14	12±2
	BWf KT*	*Aspergillus tamarii*	3	12	11-12	11.5±0.5
	BfK*	*Aspergillus nidulans*	3	7	8-14	11±3
	Yf_1*	*A. nidulans*	3	11	9-13	11±2
Penicillin (28±2)%	YGPe*	*Penicillin sp*	3	4	13-15	14±1
Standard	DSMZ 8#	*A. niger* STD	3	1	9-10	9.5±0.5

* = Sisal bore rot isolate # = Standard *Aspergillus niger* strain

Therefore, extracellular enzyme production by selected isolates inferred the likelihood production of useful metabolites (Collins et al., 1998; Fiedurek, 2000). The shown extents of enzyme secreted into agar medium by fungi BfK-KT (*A. niger* wild 1) and BYF-KT (*A. niger* wild 2), when compared with standard strain DSMZ 8

(diameter of 10 mm), showed excellent assimilation preferences to inulin, which suggested the ability to produce metabolites including citric acid (Table 4.6).

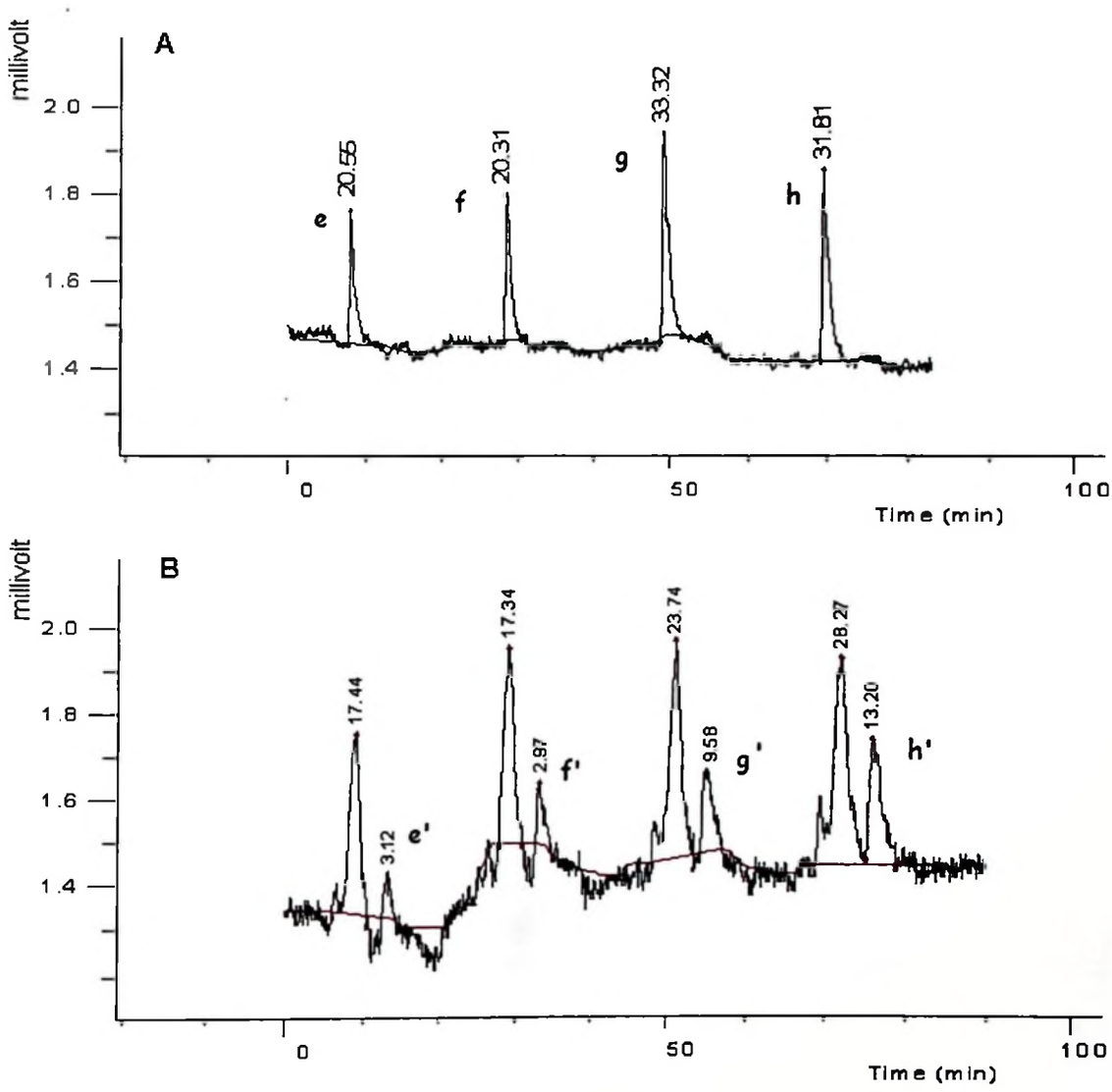

Figure 4.3: Sisal bole juice inulin chromatograms; (A) = for clearer liquid (SBJ B) and (B) = for darker liquid (SBJ A) at concentration values 0.1, 0.2, 0.3 and 0.4g/ml.

HPLC chromatograms in Figure 4.3 show crude enzyme activities after acetone extraction from 24 hours fermentation broths. Emergence of fructose peaks confirmed enzyme activities by indigenous isolates (Collins *et al.,* 1998; Fiedurek, 2000; Wang, 2008). The activities measurements are presented by two chromatograms; (A) = chromatogram show single inulin peaks at (0 minute), while

(B) = show the appearance of fructose peaks at (30 minutes). Identities chromatogram peaks of fungi are presented by; (e & e^1) = *Fusarium*, (f & f^1) = *Penicillin*, (g & g^1) = *Aspergillus* BfK and (h & h^1) = *A. niger* BYF-KT.

Results presented in Table 4.6 indicated that after 90 minutes of incubation, fructose yields from strains *Aspergillus* BfK and *A. niger* BYF-KT were 80.79 and 78.59%, respectively. While *Fusarium sp* produced 75.00%, and *Penicillin* strain produced 71.10% fructose. These results supports that, the tested strains showed high competence in production of inulin hydrolytic enzymes.

Table 4.6: Crude extracellular enzyme activities

	Activity			Time(min)			
				0	30	60	90
Fusarium sp	++++	R-inulin	(g/100ml)	20.56	17.44	8.13	2.30
		Fructose	(g/100ml)	0.00	3.12	12.39	15.42
		yield	(%)	0.00	15.18	60.26	**75.00**
Penicillin sp	++++	R-inulin	(g/100ml)	20.31	17.34	6.80	3.09
		Fructose	(g/100ml)	0.00	2.97	12.63	14.44
		yield	(%)	0.00	14.62	62.19	**71.10**
A. nidulans BfK	+++++	R-inulin	(g/100ml)	33.32	23.74	10.20	2.25
		Fructose	(g/100ml)	0.00	9.58	20.81	26.92
		yield	(%)	0.00	28.75	62.45	**80.79**
A. niger BYF-KT	+++++	R-inulin	(g/100ml)	31.81	28.27	10.90	3.14
		Fructose	(g/100ml)	0.00	13.20	20.00	25.00
		yield	(%)	0.00	41.50	62.87	**78.59**

R-inulin = residual inulin Activity = enzyme activity ranking

4.4 Selection of Strains with Citric Acid Production Potential

Morphological development of *A. niger* was taken as criteria for selecting best performing strain in submerged citric acid fermentation. As fungal pellet sizes, represented specific growth rate, they increased with the decrease in initial hydrolysates-fructose concentrations (Papagianni and Mattey, 2006). This tendency suggested that the specific production rate decreased in favor of the specific growth rate that is the available carbon source was being utilized solemnly for cell growth leaving no excess for citric acid accumulations (Papagianni *et al.*, 1999b; Žnidaršić and Pavko, 2001; Papagianni and Mattey, 2005).

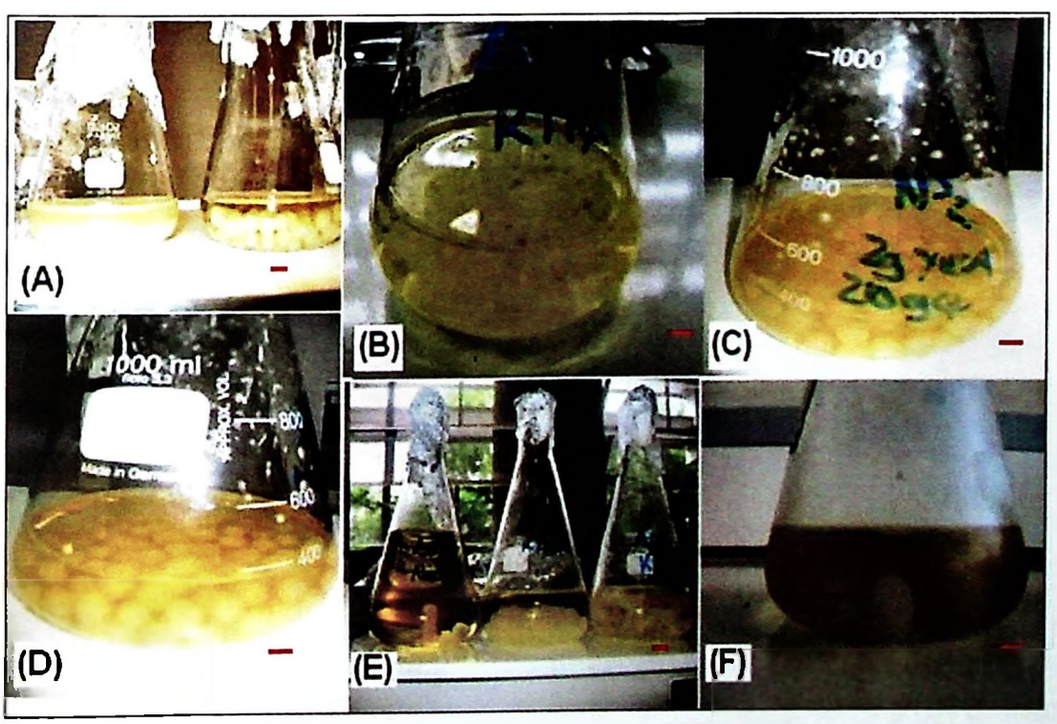

Photo 4.5: Pellet morphologies of 24 hours old *A. niger* strains relative to 2.5 mm red bar as follows; flask labelled A = *A. niger* (BYF); B = *A. niger* (BYF-KT); C = *A. niger* (DSMZ 8); D = *A. niger* (BYF); E = *A. niger* (DSMZ 8), (BYF-KT 1) and (BYF-KT 2); F = *A. niger* (DSMZ 8) mega pellets.

Shown in Photo 4.5, are the variations in pellet-morphologies with time and initial sugar concentration for various *A. niger* strains. The comparison was made relative to 2.5mm red bar and reported as follows; flask labelled (A) = 24 hours old *A. niger* BYF having diameters measurements of (2.5±0.05mm) grown in medium initial sugar (concentration of 150g/l). Flask labelled (B) = 24 hours old *A. niger* BYF-KT with diameter measurements of (1.5±0.05 mm) in high initial sugar (concentration of 200g/l); (C) = 24 hours old *A. niger* DSMZ 8 (2.5±0.05 mm diameter) grown in medium initial sugar (concentration of 150g/l); (D) = 24 hours old *A. niger* BYF in high initial sugar (concentration of 150g/l). Meanwhile flasks shown in (E) = 24 hours old *A. niger* DSMZ 8 (An_1 Nadi); BYF-KT 1 (An_2 KT_A) and BYF-KT 2 (An_2 KT_B) had average diameter measurements of (1.5±0.05, 1.0±0.05 and mixed 0.5-1.5±0.05mm respectively). These were both cultured in high initial sugar (concentration of 200g/l) but spore inoculum sizes were different i.e. 10^3, 10^5 and 10^6 spores/ml respectivelly. Flask labelled (F) = shows mixed mega pellets with diameter measurements between (2.5 and 20±0.05mm). These were obtained after over growing *A. niger* DSMZ 8 in low initial sugar (concentration of 50g/l) for 72 hours.

The morphology studies indicated that fungal strains BYF (*A. niger* wild1) and BYF KT (*A. niger* wild 2) displayed sturdy smooth pellets morphologies with average diameters of 2±0.5 mm. These values were closer and compared well with standard (DSMZ 8) which measured at the average diameters value of 3±1mm, thus suggested the suitability of *A. niger* strains generic to sisal boles in citric acid production potential tests (Photo 4.5 B and E respectively).

4.4.1 Effectiveness of indigenous *A. niger* in Citric acid fermentation

After 144 hours of fermentation experiments, the tested *A. niger* BYF-KT strain produced the amount of citric acid measuring at 37±0.43 and 68±0.5 g/l, from initial inulin hydrolysates-fructose with concentrations values 40% (102g/l) and 80% (203g/l) respectively. In comparison under the same conditions, the amounts of citric acid produced by DMSZ 8 were 28±0.2 and 82±0.4 g/l.

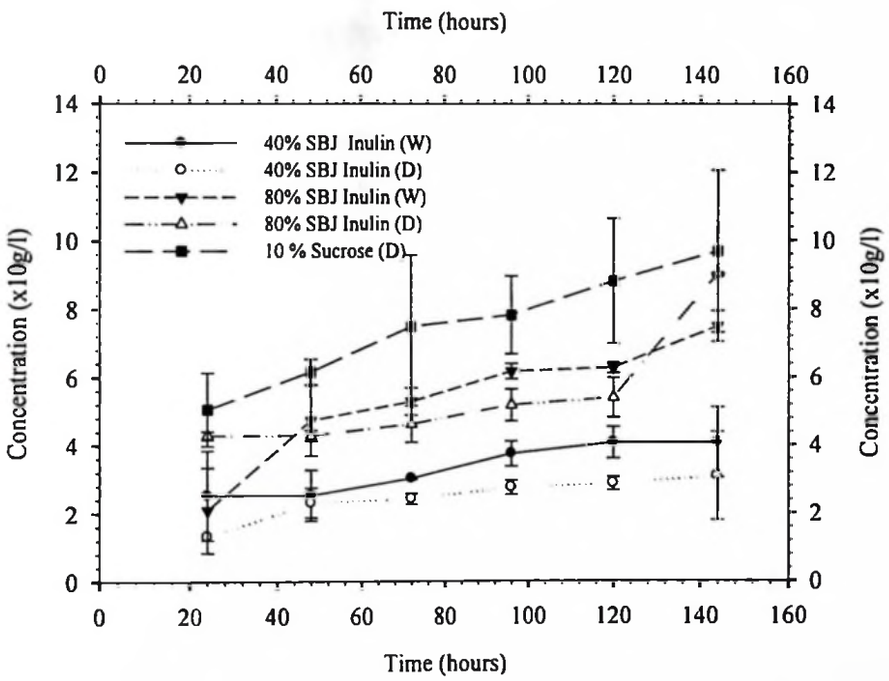

Figure 4.4: Yields comparison for *A. niger* (W) = BYF and (D) = DSMZ 8

The amount of citric acid produced by both BYF *A. niger* wild 1 and DMSZ 8 were remarkably closer to yield value of 88±4 g/l, which was obtained by fermentation of 10% (100 g/l) sucrose using standard strain DMSZ 8. Therefore, *A. niger* (BYF *A. niger* wild1) was thus considered for the subsequent experiments (Figure 4.4).

4.5 Sisal inulin Hydrolysis at Different pH and Temperatures

Shown in Figure 4.5 are typical chromatograms of hydrolysate yields at 0-180 minutes, after autoclaving at 115°C (1.6bar) and different pH; marked by points a-a′ = pH 2, point b-b′ = pH 3, point c-c′ = pH 4 and point d-d′ = pH 5.

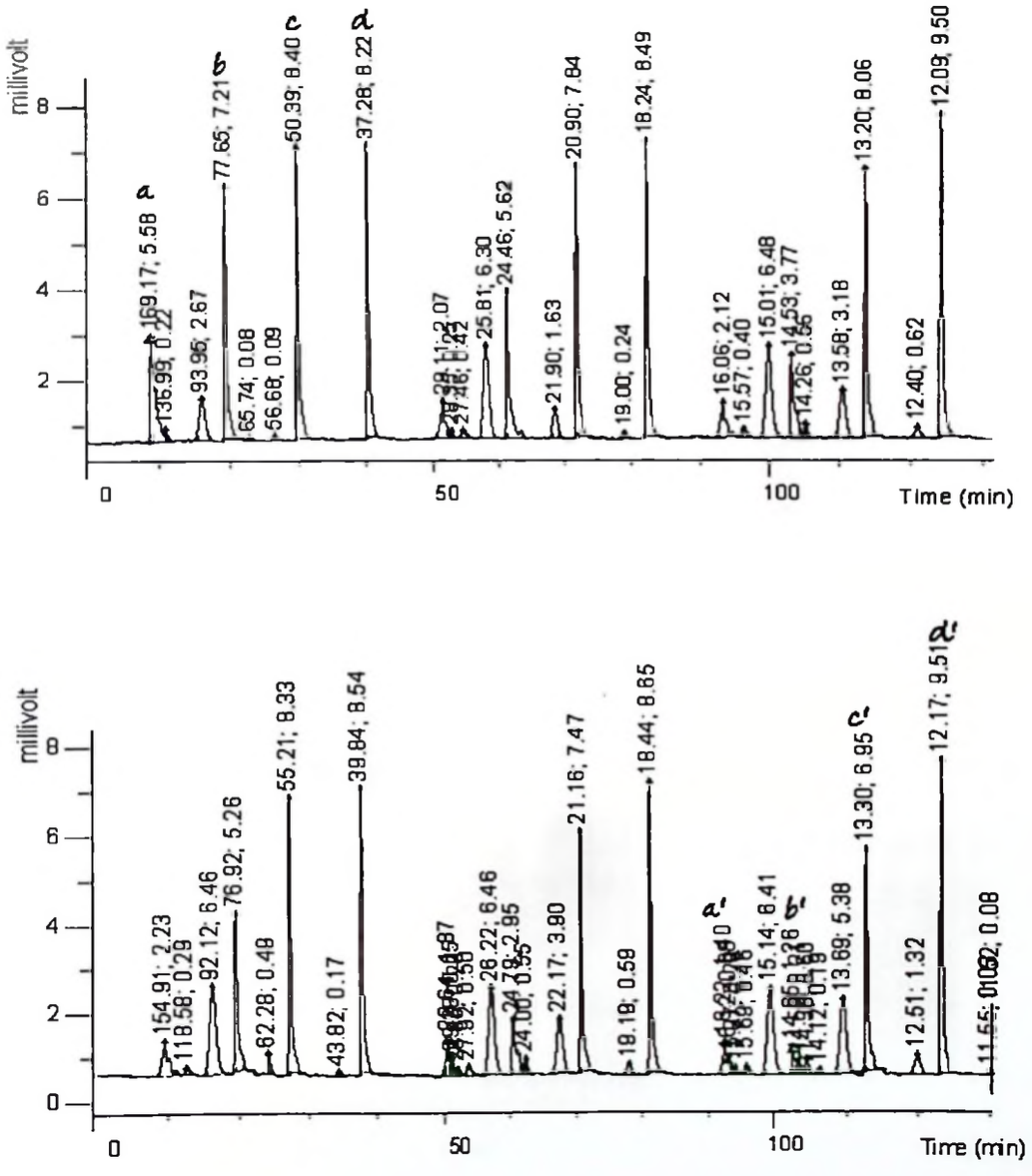

Figure 4.5: Chromatograms showing hydrolysis trends after 30 min intervals.

Point's a-a′, revealed that hydrolysis at pH 2 was complete within 30 minutes, whereas hydrolysis yield at pH values of 4 to 5 increased with time. Prolonged hydrolysis time at temperatures of 115±5°C (1.6 bar), was assumed to facilitated breaking up of complex carbohydrates other than inulin, thus at the end of hydrolysis much more un-accounted for monomer sugars were expected to be incorporated into the total hydrolysates (Abasaeed and Lee, 1996; Abasaeed and Lee, 1995).

Hydrolysis at lower temperatures of 30-70°C and higher pH values 4-5 required prolonged reaction times (Figure 4.5 c & c′ and d & d′). On the other hand, the observed reduction in total sugar yields would be associated with maillard and pyrolysis reactions (Lindhorst, 2007; Kalliat, 2008).

4.5.1 Effect of oven cooking on yields at different temperature and pH

The effect of increasing temperature on Yield is shown in Figure 4.6 to 4.20, after hydrolysis by either cooking at 30, 60, 70 and 80°C and pH range of 2-5; or autoclaving at steam temperatures of 115, 121 and 132°C; and pH range of 2-5.

Hydrolysate sugars obtained after the oven cooking at temperature of 30°C and pH 2, 3, 4 and 5 are shown in Figure 4.6 to 4.8.

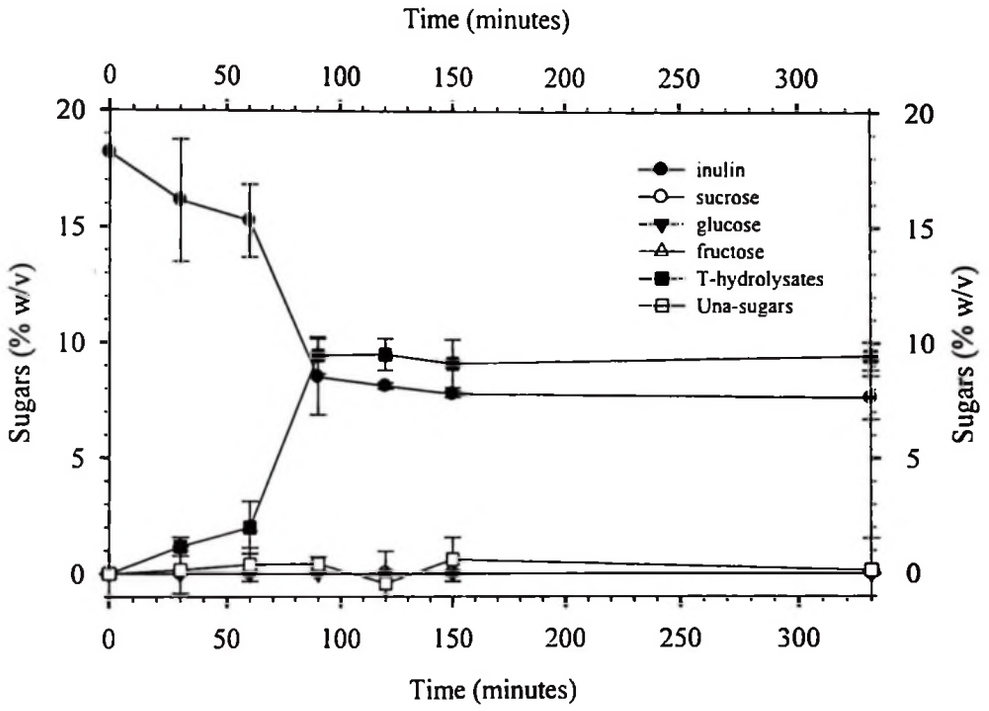

Figure 4.6: Yields of sugars after oven cooking at 30°C and pH 2.

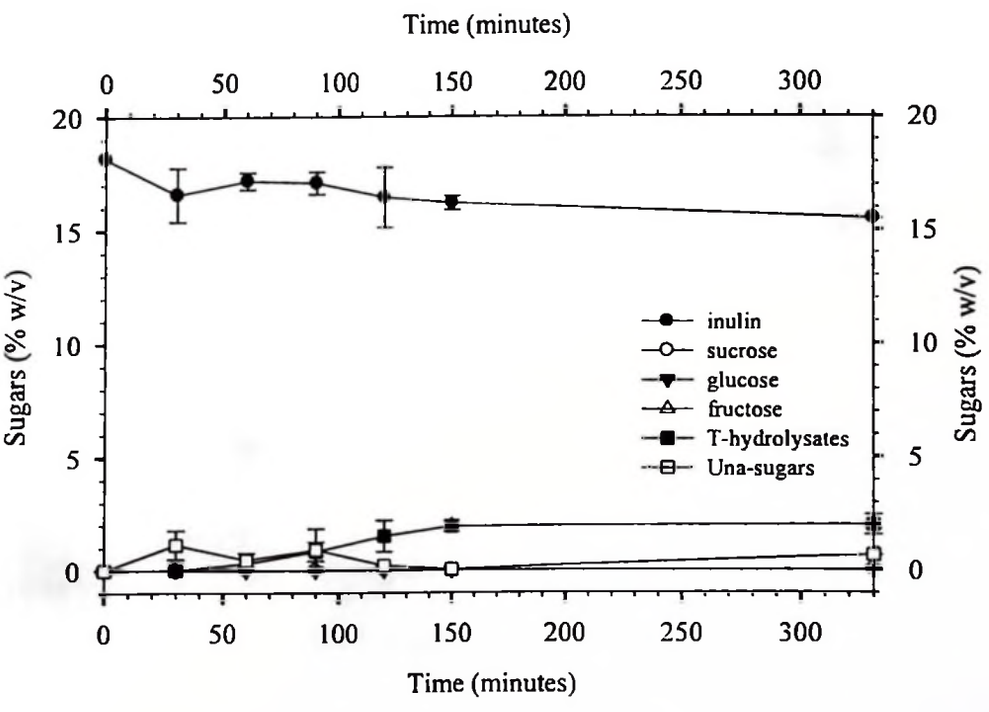

Figure 4.7: Yields of sugars after oven cooking at 30°C and pH 3

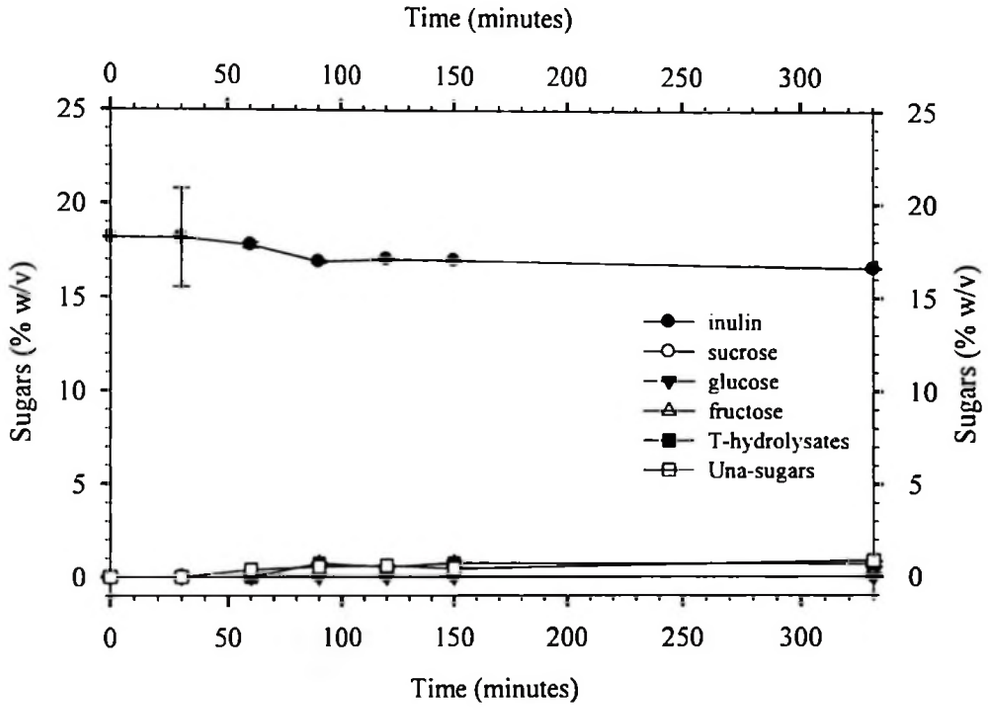

Figure 4.8: Yields of sugars after oven cooking at 30°C and pH 4

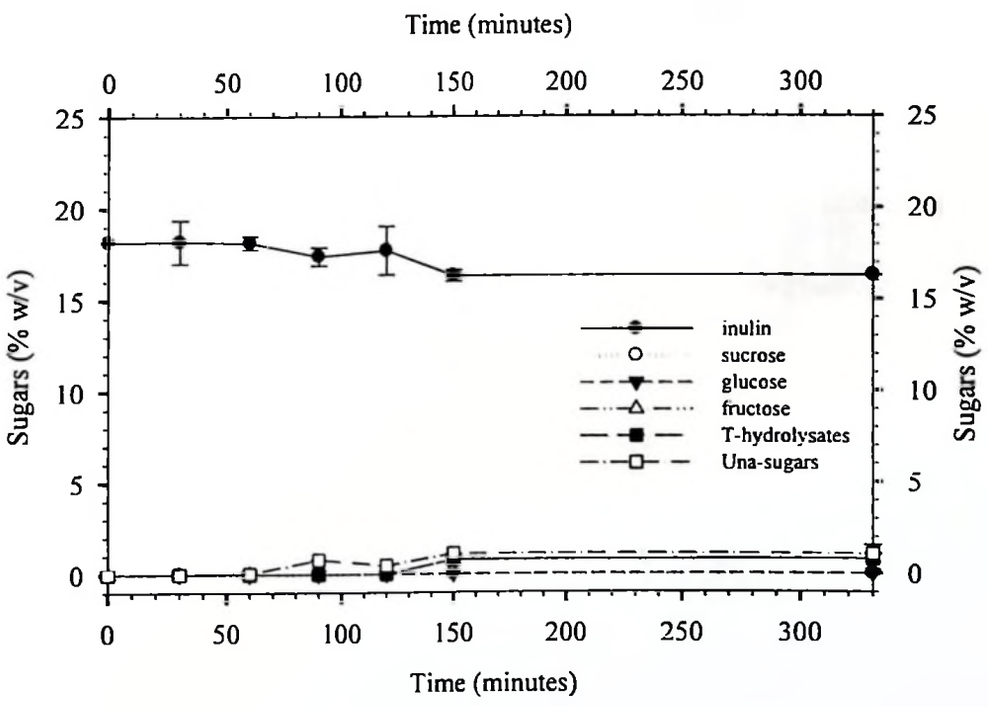

Figure 4.9: Yields of sugars after oven cooking at 30°C and pH 5

The overall results indicated that high hydrolysates yield occurred at pH 2 (Figure 4.6). Increasing reaction time did not necessarily improved yields at pH range of 3 to 5 as was perceived in Figure 4.7 to 4.8. Instead, the lowering of pH, promoted acid catalysed reaction of carbonyls moieties of the fructose. This reaction is mediated by the conjugate acid of the fructosyl (carbonyl) group; which a much better electrophile than the neutral fructosyl (Morrison and Boyd, 1997; Moran and Shapiro, 2000; Lindhorst, 2007; Kalliat, 2008).

Such reactions are triggered by the presence of protons, which in turn converts OH group into a migrating H_2O group, consequently weakening fructosyl bonds and hence inulin hydrolysis. This is contrary to the great stability of sisal bole inulin under the normal field conditions and its physiological functioning as carbohydrate-food reserve (Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

Since the Highland sisal estate and Dar es Salaam ambient temperatures are ($30\pm2^{o}C$), thus hydrolysis optimization at $30^{o}C$ which is closer to field temperatures, required either enzymatic or acid catalysis (Morrison and Boyd, 1997; Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

Shown in Figure 4.10 to 4.12, are hydrolysate sugars obtained after the oven cooking at temperature of $60^{o}C$ and pH 2-5.

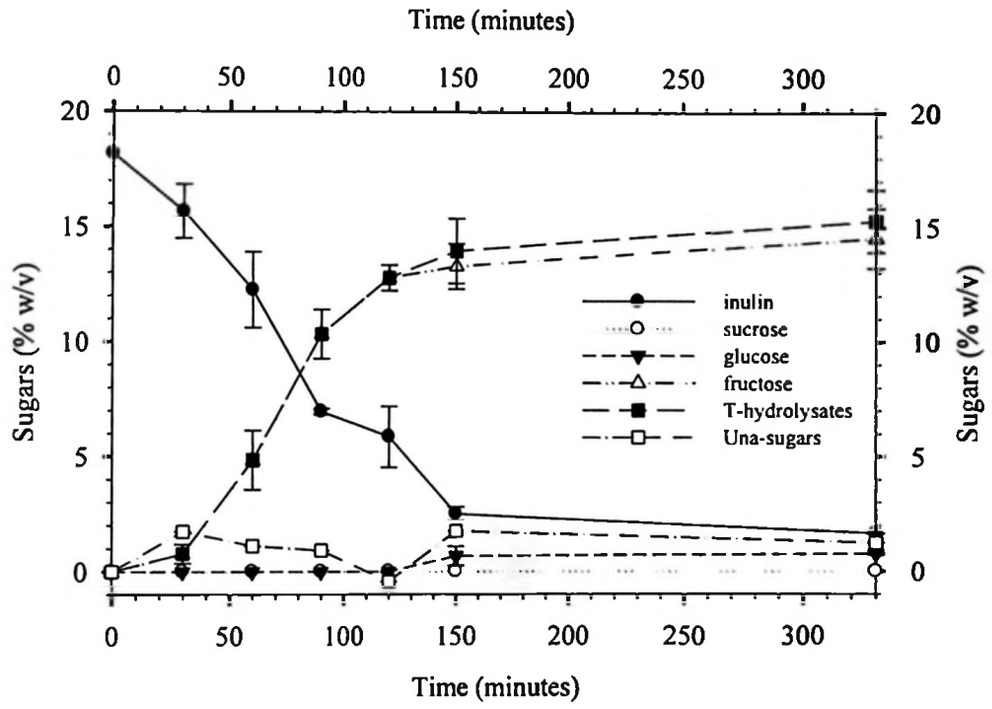

Figure 4.10: Yields of sugars after oven cooking at 60°C and pH 2

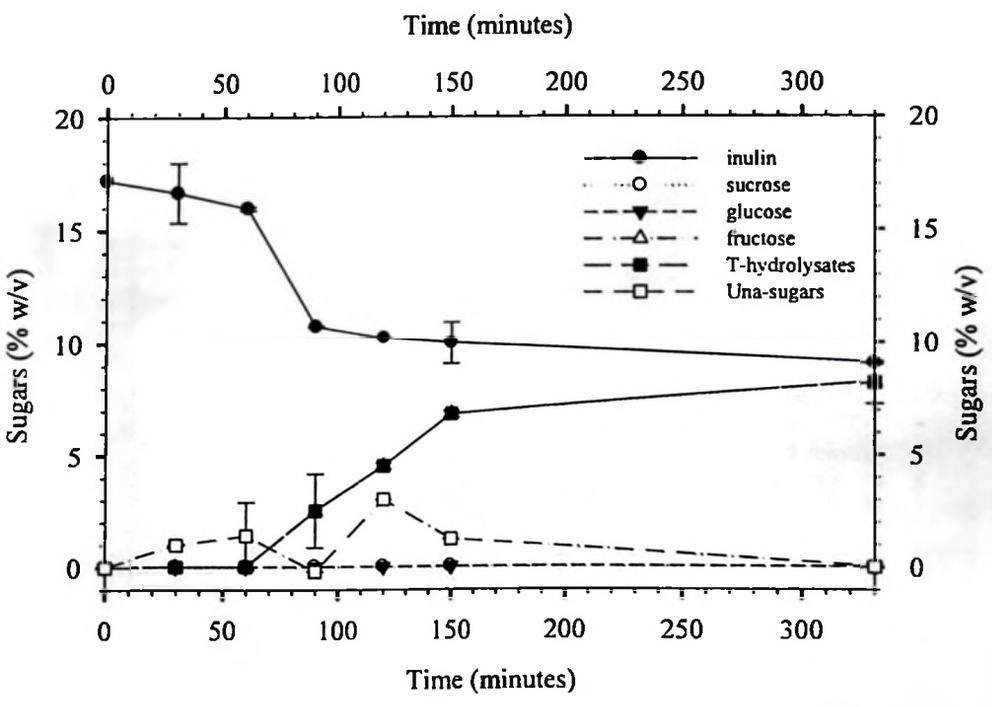

Figure 4.11: Yields of sugars after oven cooking at 60°C and pH 3

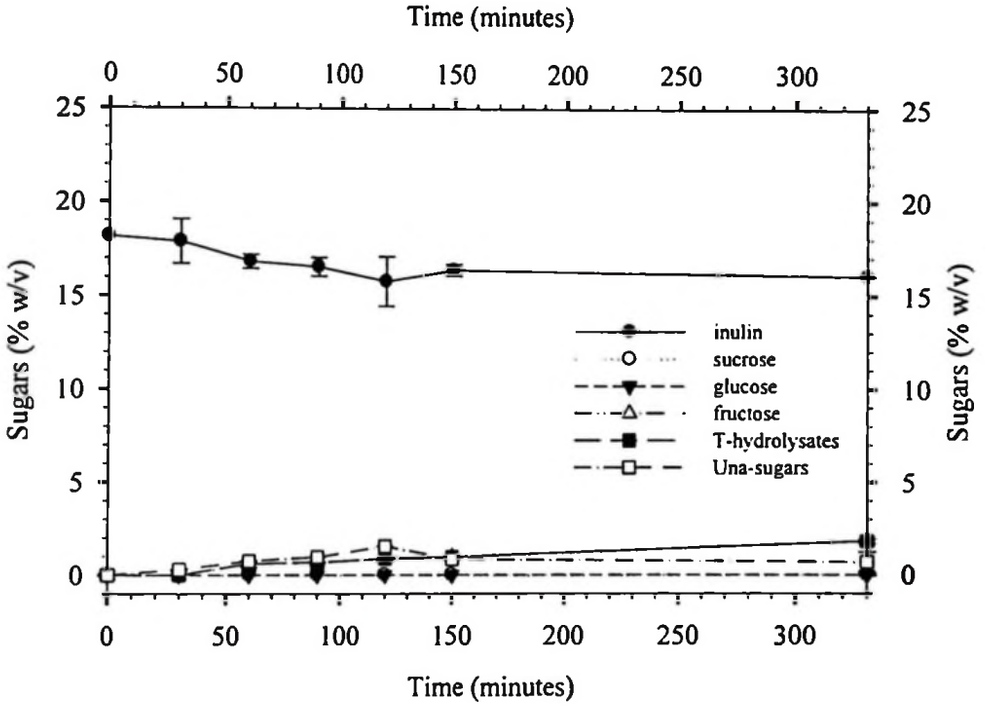

Figure 4.12: Yields of sugars after oven cooking at 60°C and pH 4

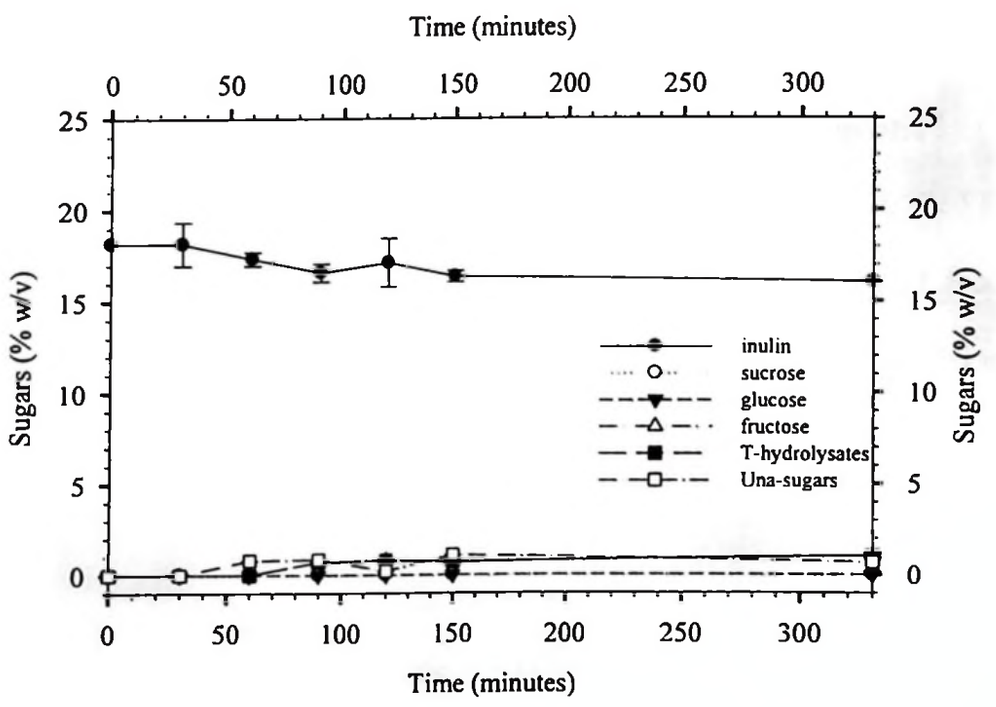

Figure 4.13: Yields of sugars after oven cooking at 60°C and pH 5

Results for oven cooking at temperature of 60°C and pH 2-5 indicated that highest hydrolysates yield occurred at pH 2, and there was an improved yield at pH 3 (Figure 4.10 and 4.10). At pH range of 4-5, no significant yield improvement was achieved as shown in Figure 4.12 and 4.12, probably because of the stability of Inulin molecule at low temperatures of 30-60°C, which is also supported by the activation energies (E_a) values 3.125 and 0.199 $JK^{-1}mol^{-1}$ for pH values of 4 and 5 respectively (Figure 4.48).

This stability may be justified, thermodynamically by the selectivity phenomenon occurring between kinetically controlled chemical reactions i.e. inulin hydrolysis. At low E_a, the kinetically controlled product (GF_{n-1}) is being formed faster than thermodynamically controlled product fructosyl units (F_m), as a result the forward reaction requires catalysis (Kyle, 1992; Moran and Shapiro, 2000; Kalliat, 2008).

As temperature was slightly elevated to 60°C, an improved yield occurred at pH 2 and 3, contrary to the yields at the ambient temperature value of 30°C. Slightly increase in temperature and existence of protons, improved breakage of fructosyl bonds hence facilitated the release of freer fructose molecules (Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007).

The sugars obtained after oven cooking at temperature of 70°C and pH 2, 3, 4 and 5 are shown in Figure 4.14 to 4.16.

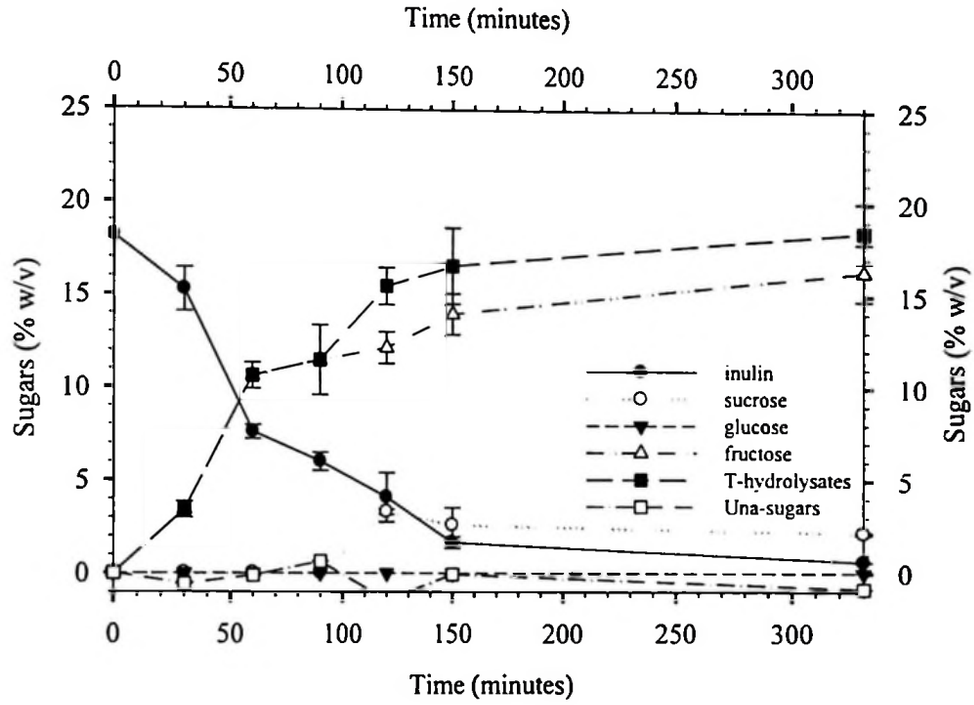

Figure 4.14: Yields of sugars after oven cooking at 70°C and pH 2

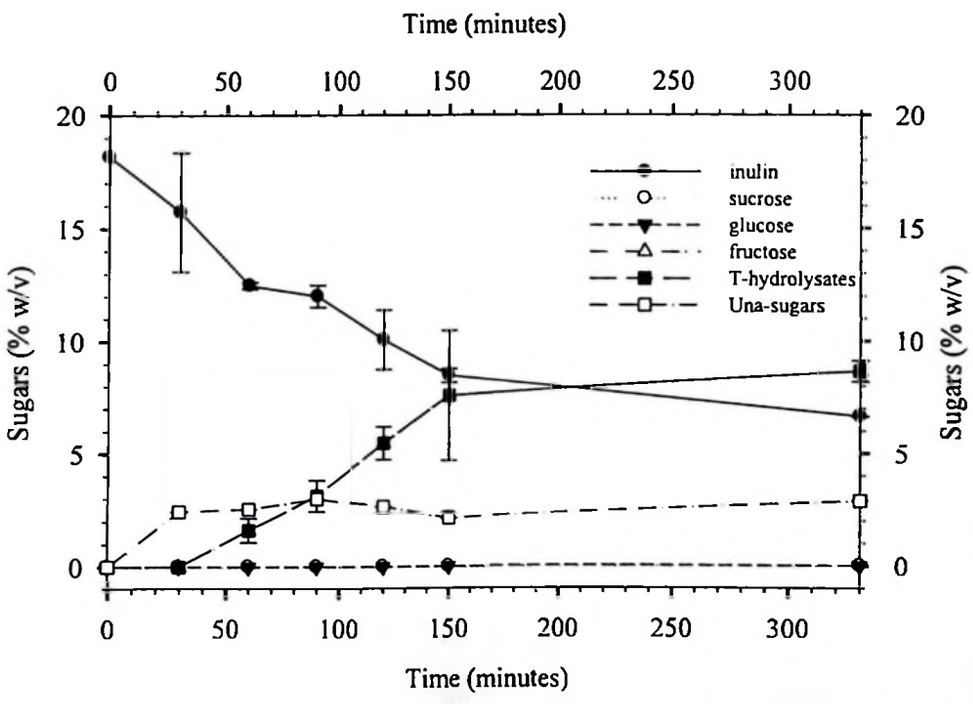

Figure 4.15: Yields of sugars after oven cooking at 70°C and pH 3

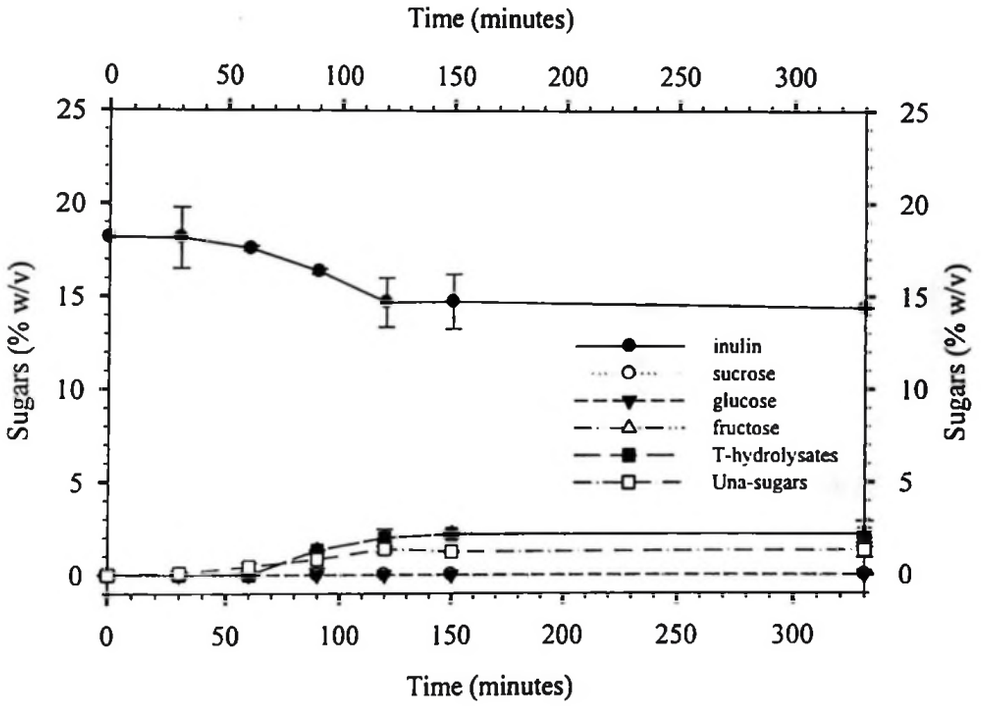

Figure 4.16: Yields of sugars after oven cooking at 70 °C and pH 4

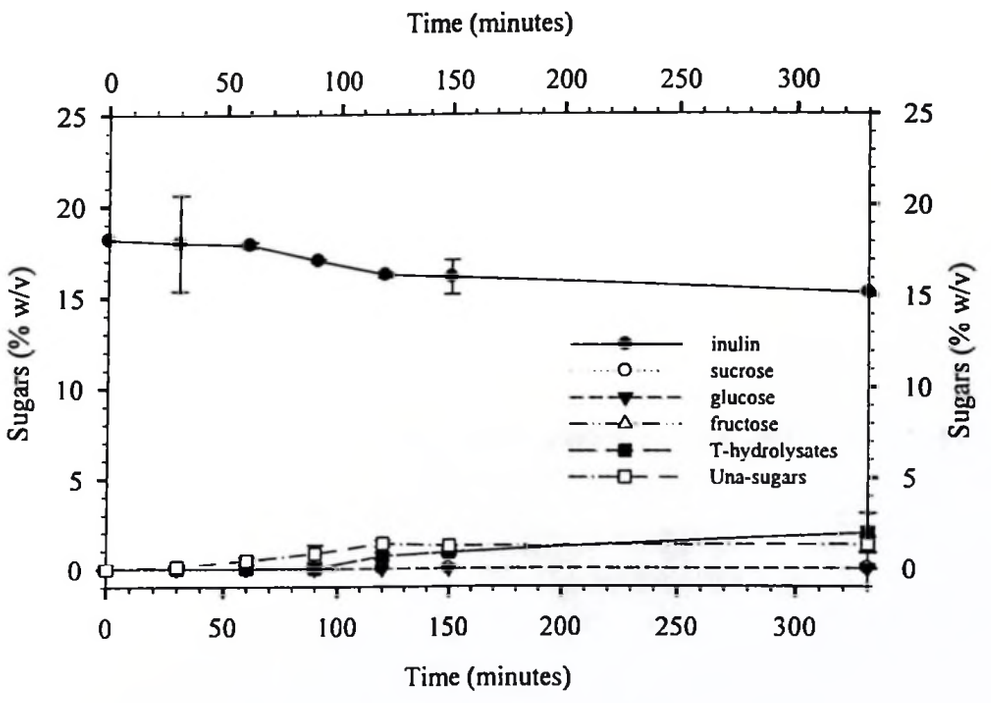

Figure 4.17: Yields of sugars after oven cooking at 70 °C and pH 5

Oven cooking at temperature of 70°C and pH 2-5 indicated that highest hydrolysates yield occurred at pH range of 2 to 3. However, no significant improvements in hydrolysates yield occurred at pH ranges of 4 to 5, specifically after increasing reaction time beyond 150 minutes (Figure 4.16 to 4.16).

The reason could be that lowering of pH to 2 and 3 while elevating temperature prompted the acid catalysed reaction of the carbonyl fructose moieties. However slight increasing temperature and presence of H^+, improved breakage of fructosyl bonds thus releasing more fructose molecules (Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

It was noted that at 70°, pH ranges of 4 to 5, and prolonged reaction time beyond 150 minutes; still did not improve hydrolysates yield. The reason could be that the low (E_a) triggered the formation of the kinetically controlled intermediate products (GF_{n-1}) at fastest rates, as compared to thermodynamically controlled product (F_n) fructose (Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

Shown in Figure 4.18 to 4.20 are hydrolysates sugars obtained after the oven cooking at temperature of 80°C and pH 2, 3, 4 and 5.

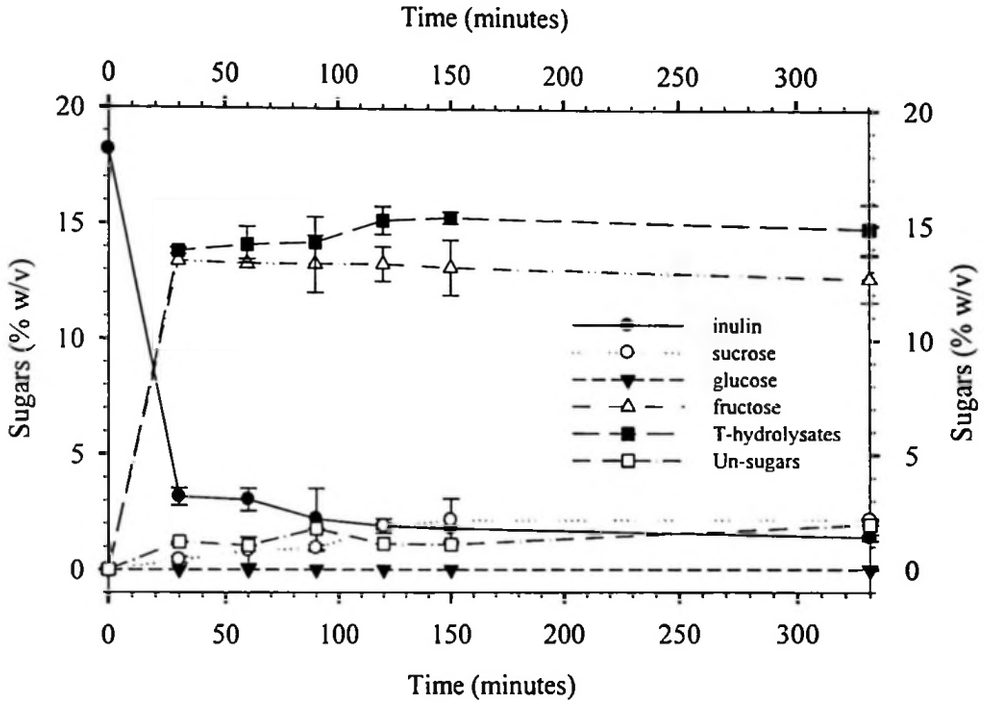

Figure 4.18: Yields of sugars after oven cooking at 80°C and pH 2

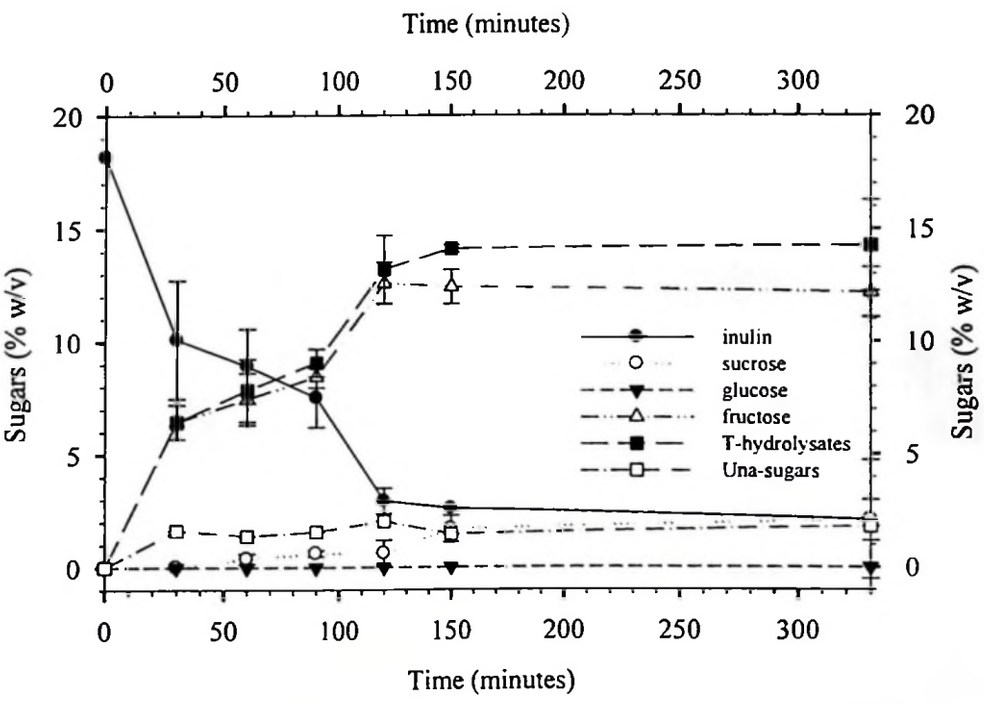

Figure 4.19: Yields of sugars after oven cooking at 80°C and pH 3

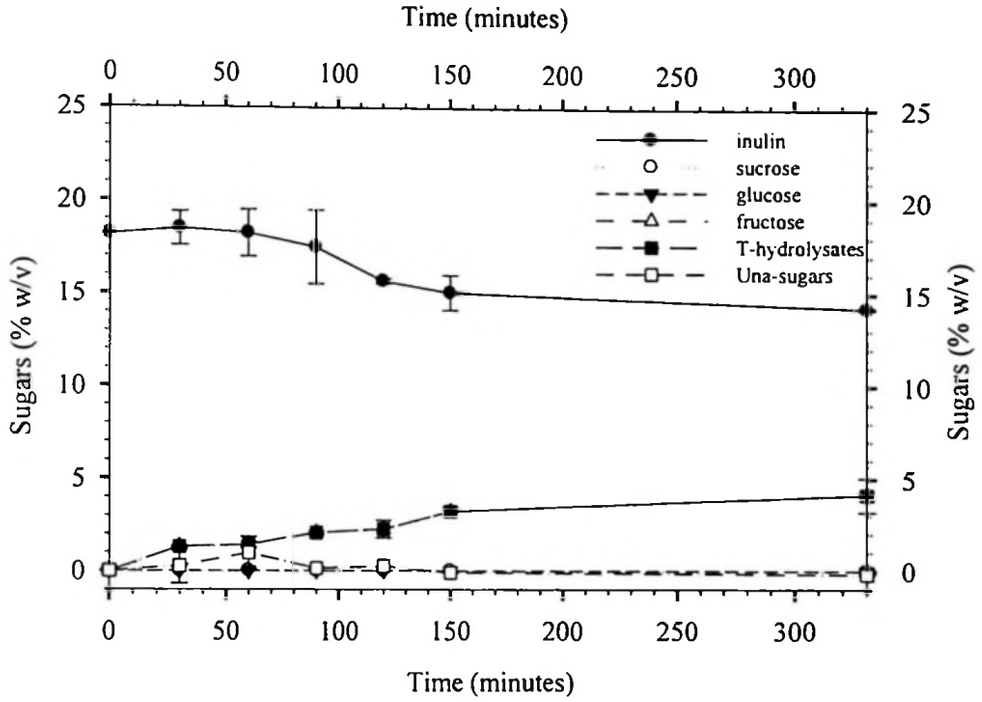

Figure 4.20: Yields of sugars after oven cooking at 80°C and pH 4

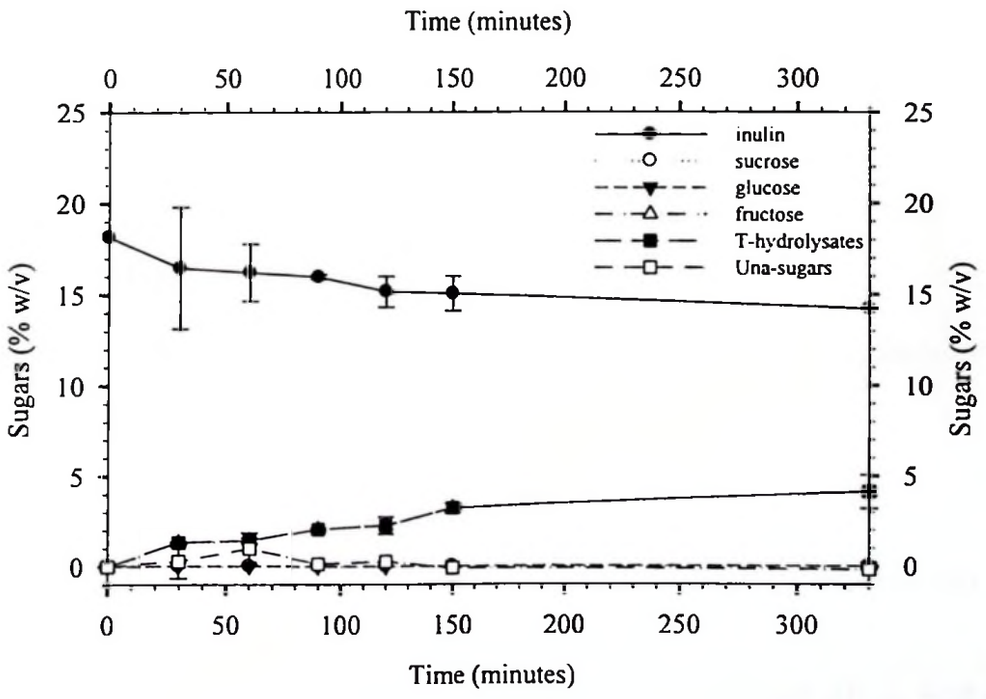

Figure 4.21: Yields of sugars after oven cooking at 80°C and pH 5

Results for oven cooking at 80°C and pH 2-3 produced comparatively higher hydrolysates yield, with the anticipation that yield could increase with an increase in reaction time (Figure 4.18 to 4.16). The concept behind this attribute is slightly the overcoming of the steric hindrance and/or inductive effects among the glycosidic bonds linking 'glucose-fructose-fructosyl moieties' (Morrison and Boyd, 1997).

An increase in n-number of attached fructosyl groups for a huge molecule like inulin may be a contributing factor to the general stability of molecule thus resulting in lower rates of hydrolysis at lower temperatures (Edelman and Jefford, 1968; Lindhorst, 2007; Kalliat, 2008). Oven-cooking hydrolysis at 30 to 60°C and normal pressure demanded the application of catalyst and slight elevation of temperature as supported by Arrhenius relationship (Kalliat, 2008).

Even though the reaction rates proceeded at slower pace at this temperature range, it was expected that increased yield could be achieved by prolonging reaction time probably beyond 150 minutes. The slightly increase in the temperatures specifically to 80°C and pH 4-5, was expected to improve because of acid catalysed enolisation of carbonyl group, enhancing breakage of fructosyl bonds between (GF_{n-1}) intermediates (Lindhorst, 2007; Kalliat, 2008).

4.5.2 Effect of steam cooking on yields at different temperature and pH

Hydrolysate sugar results obtained after autoclaving at 115°C and pH 2, 3, 4 and 5 are shown in Figure 4.22 to 4.24.

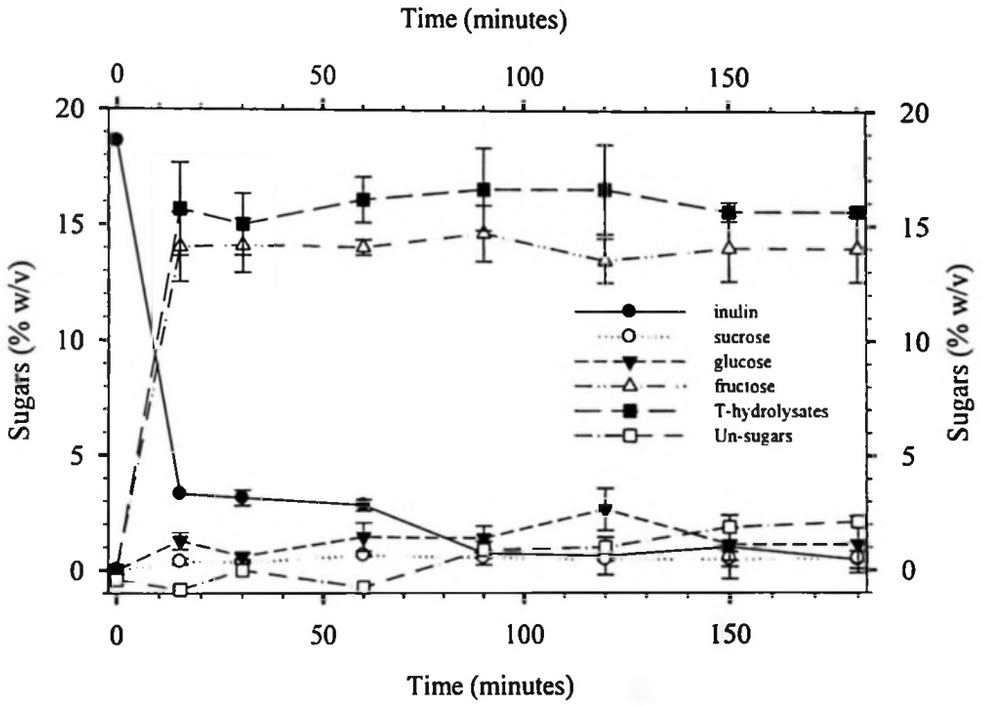

Figure 4.22: Yields of sugars after autoclaving at 115°C and pH 2

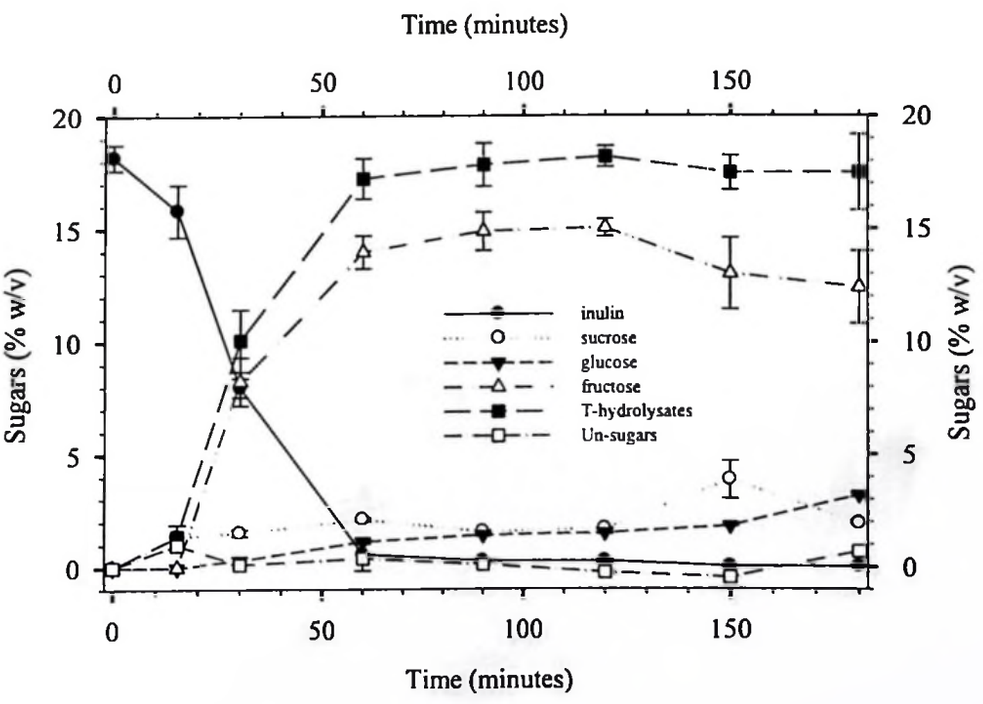

Figure 4.23: Yields of sugars after autoclaving at 115°C and pH 3

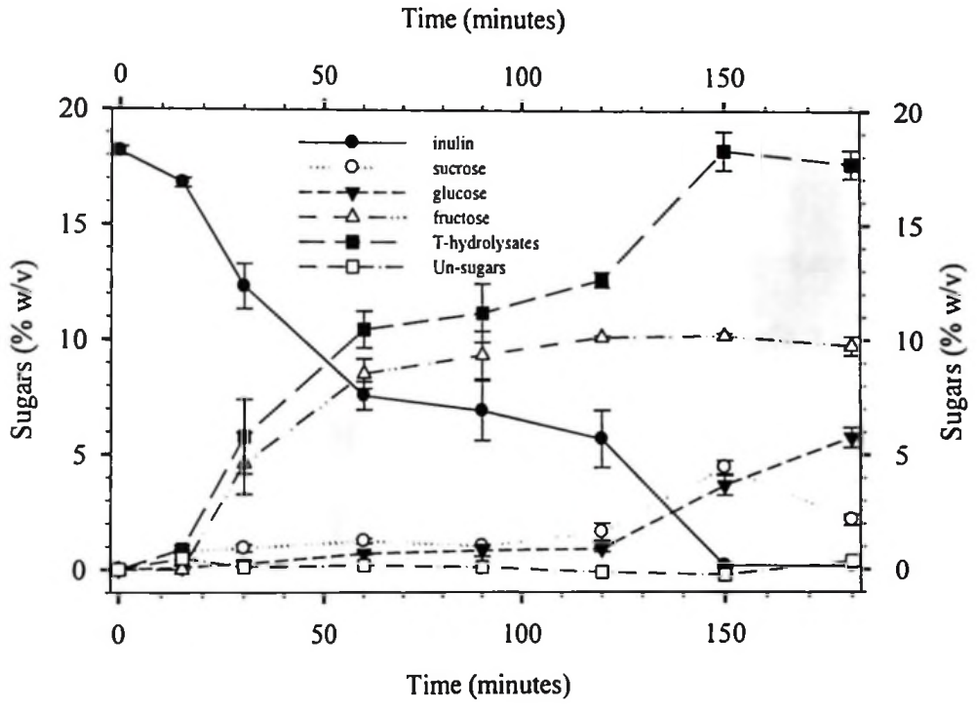

Figure 4.24: Yields of sugars after autoclaving at 115°C and pH 4

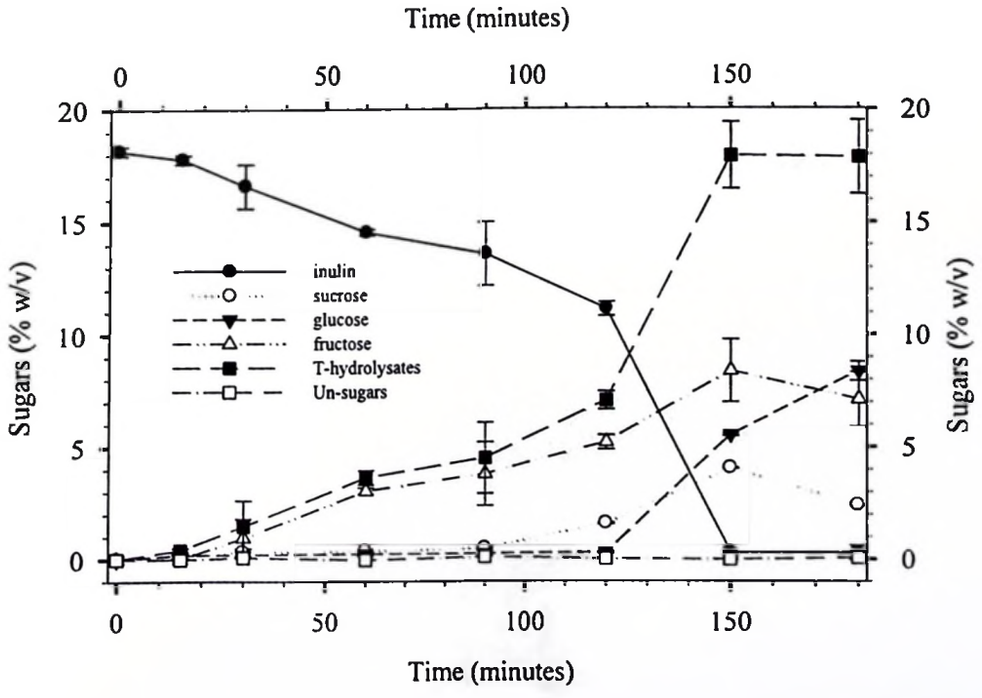

Figure 4.25: Yields of sugars after autoclaving at 115°C and pH 5

Results after autoclaving at 115°C indicated that highest hydrolysate yields occurred at pH 2 and increased in reaction time improved yields at pH values of 3 to 5. These outcomes suggested that hydrolysis by autoclave cooking at temperatures 115°C and pH 2 was quicker (Figure 4.22) and effective; as supported by the activation energy value of 7.223 ($JK^{-1}mol^{-1}$), contrary to hydrolysis rates at pH 3, 4 and 5, and E_a values 6.573, 3.125 and 0.199 ($JK^{-1}mol^{-1}$) respectively (Mohr, *et al* 2008).

Reaction rates preceded at higher pace at this temperature range, though the anticipated increased in yield with time was not fully realised. Slight decrease in hydrolysates yields and browning were observed instead, especially at pH values of 2 and 3 (Figure 4.22 to 4.22). The explanation could be that, by extending autoclaving beyond 150 minutes, the pyrolysis and acid catalyzed maillard's reactions continued to increase, consequently reducing the total hydrolisate yield values (Banerjee, *et al.*, 1981; Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

Results for total sugars after autoclaving at 121°C and pH 2, 3, 4 and 5 are shown in Figure 4.26 to 4.28.

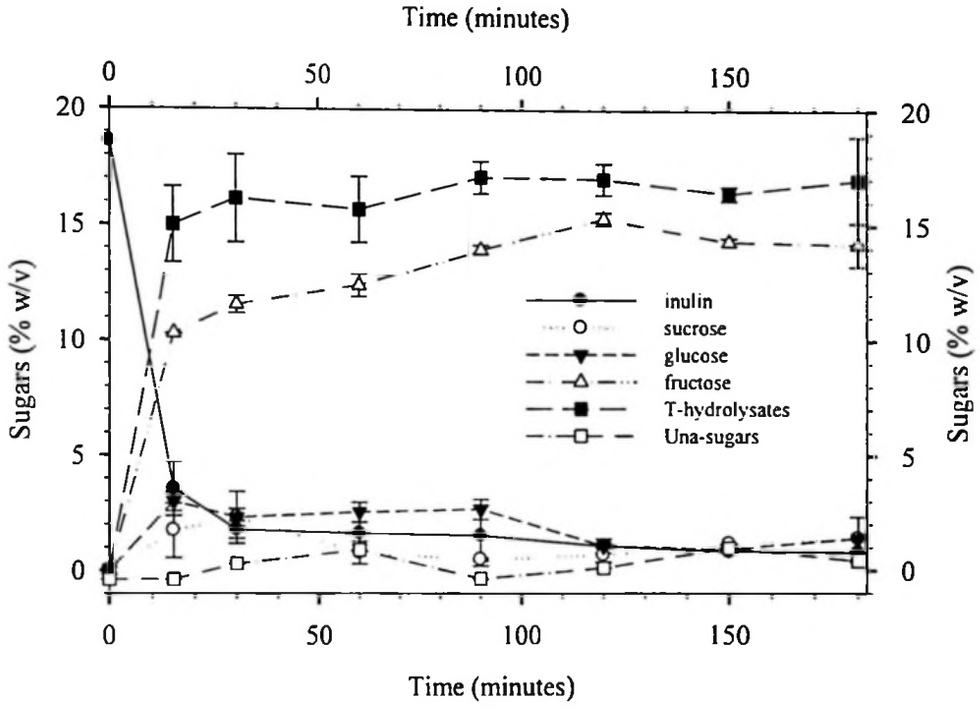

Figure 4.26: Yields of sugars after autoclaving at 121°C and pH 2

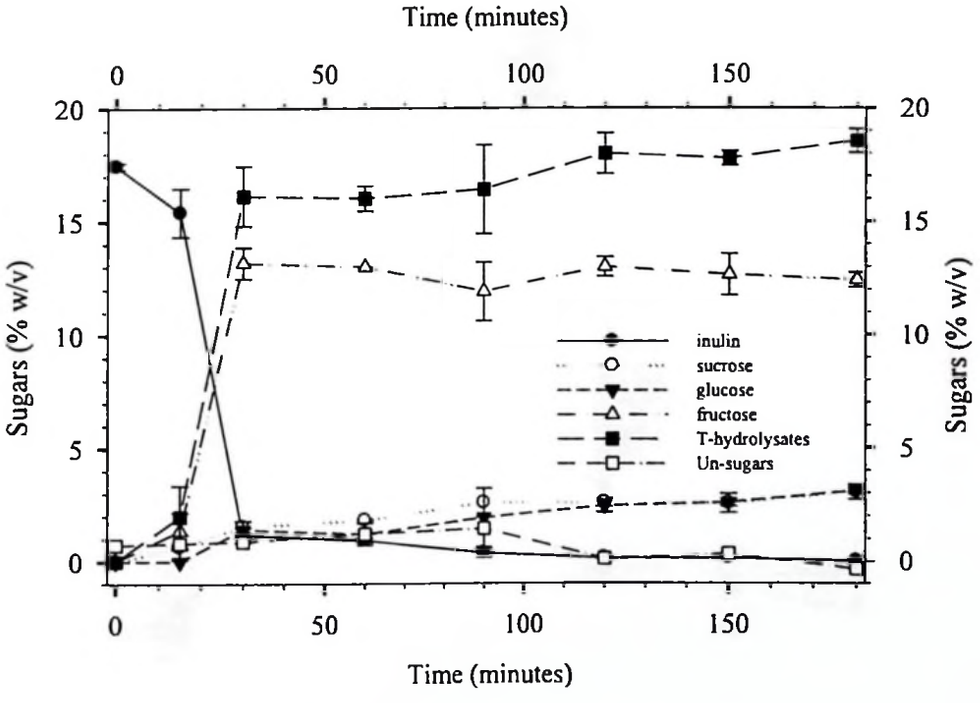

Figure 4.27: Yields of sugars after autoclaving at 121°C and pH 3

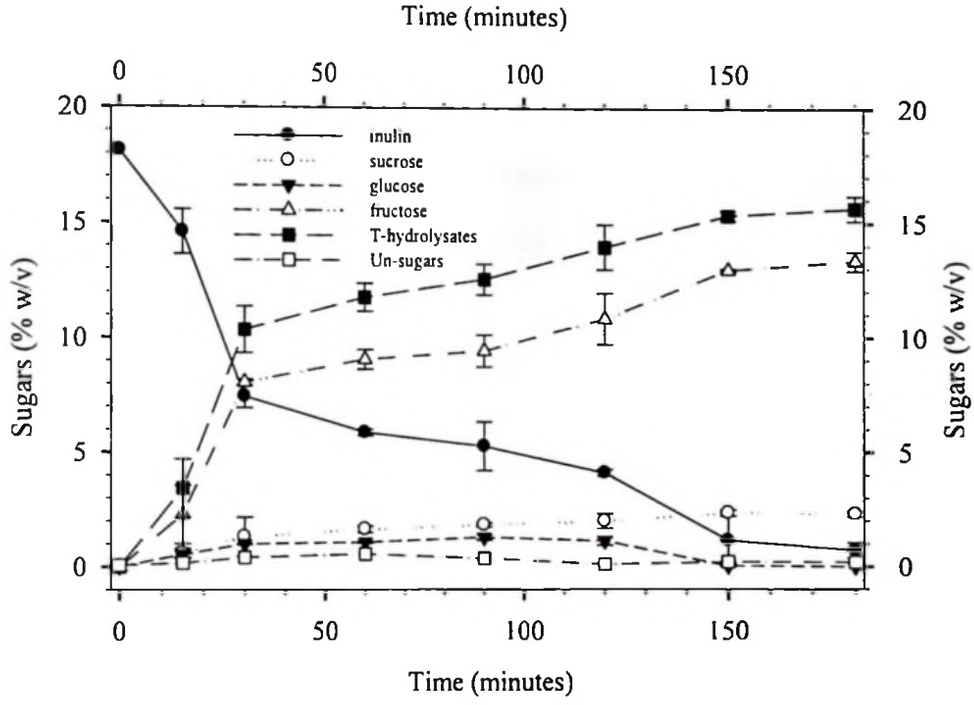

Figure 4.28: Yields of sugars after autoclaving at 121°C and pH 4

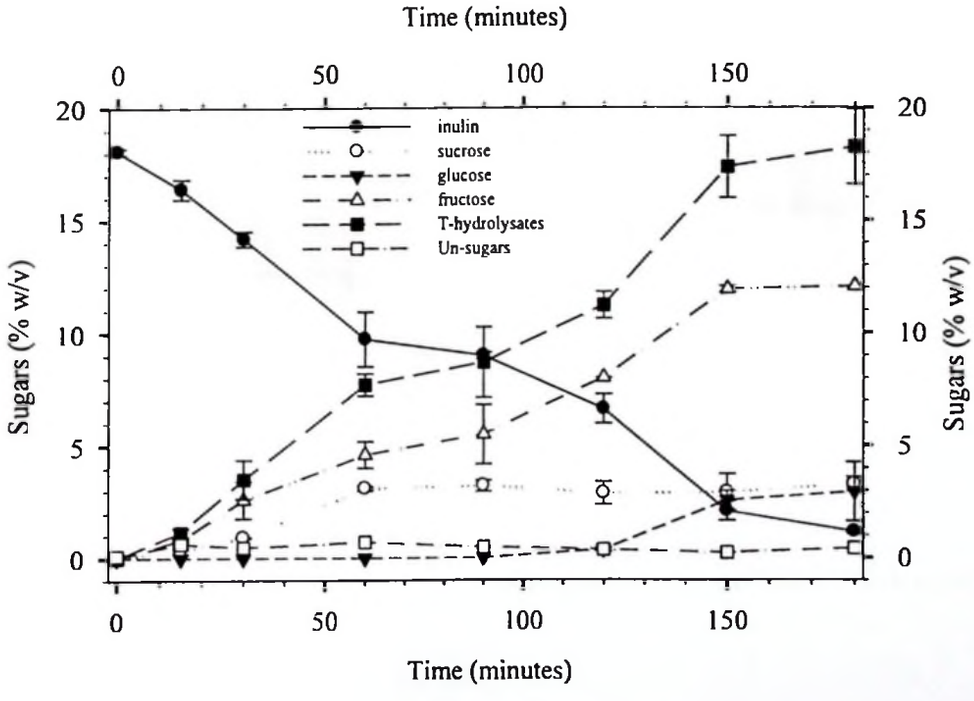

Figure 4.29: Yields of sugars after autoclaving at 121°C and pH 5

The trend for autoclaving at 121°C temperature shows that higher hydrolysates yields occurred at pH 2 and 3, and an increase in reaction time improved hydrolysates yields at pH values of 4 and 5. However, it was demonstrated that hydrolysis rates by autoclave cooking at 121°C was also, enhanced by lowering of pH. However, as total hydrolysates sugars increased in both pH values, fructose yields decreased (Figure 4.26 to 4.28). Most likely, some of fructose molecules were converted into pyrolysis compounds of fructose such as furfurals (Morrison and Boyd, 1997; Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

Hydrolysis by autoclave cooking at 121°C and pH value of 2-3 was quicker and effective as supported by higher activation energy values 7.223 and 6.573 $JK^{-1}mol^{-1}$ respectively shown in Figure 4.48 (Mohr, *et al* 2008). As reaction rates proceeded at very high pace at this temperature range, also the pyrolysis and acid catalyzed maillard's reactions proceeded at a high pace. The fast browning of hydrolysates sugars was vividly seen as total hydrolysate sugars appeared to increase. As a concequence, fructose yields decreased probably due to the pyrolysis of fructose and acid catalyzed maillard reactions especially at pH values of 2 to 5 (Morrison and Boyd, 1997; Kotz and Purcell, 1991; Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

Figure 4.30 to 4.30 presents the hydrolysate sugars obtained after autoclaving at 132°C and pH 2, 3, 4 and 5.

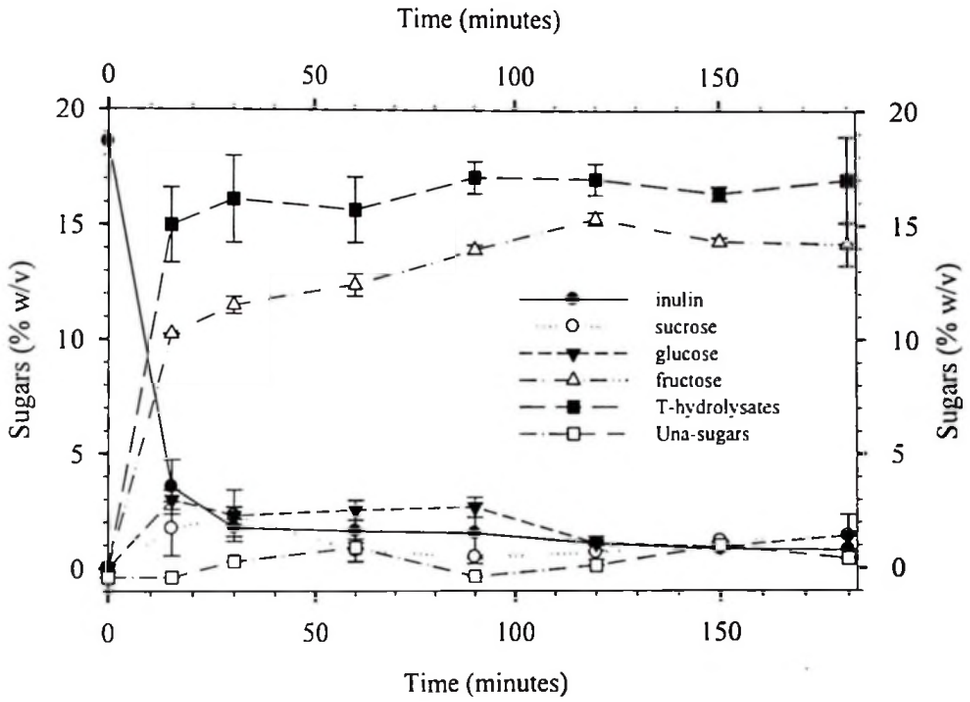

Figure 4.30: Yields of sugars after autoclaving at 132°C and pH 2

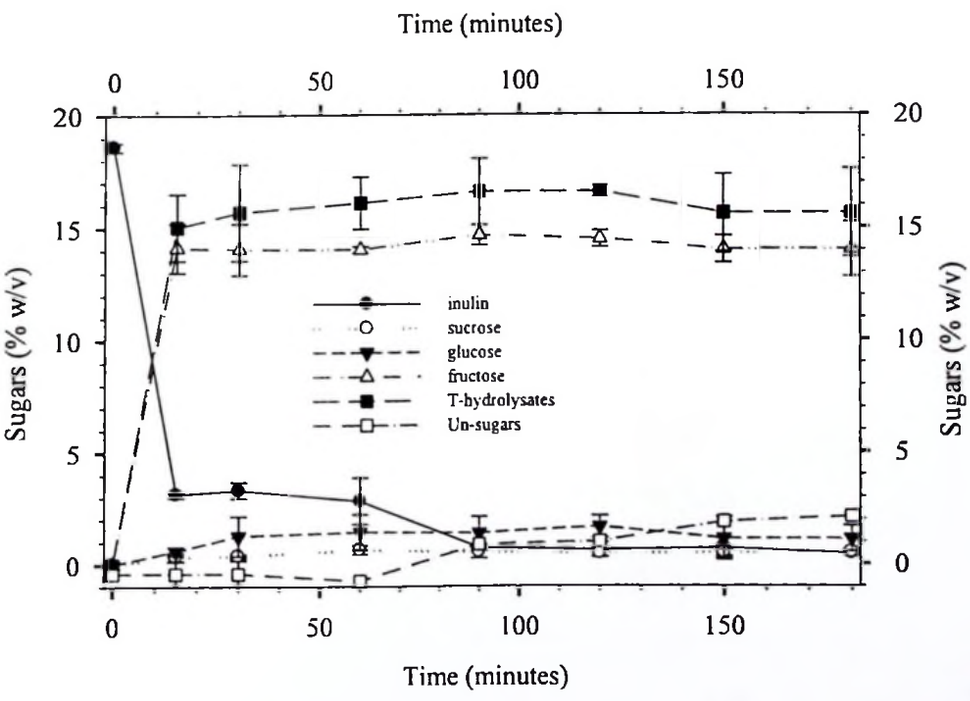

Figure 4.31: Yields of sugars after autoclaving at 132°C and pH 3

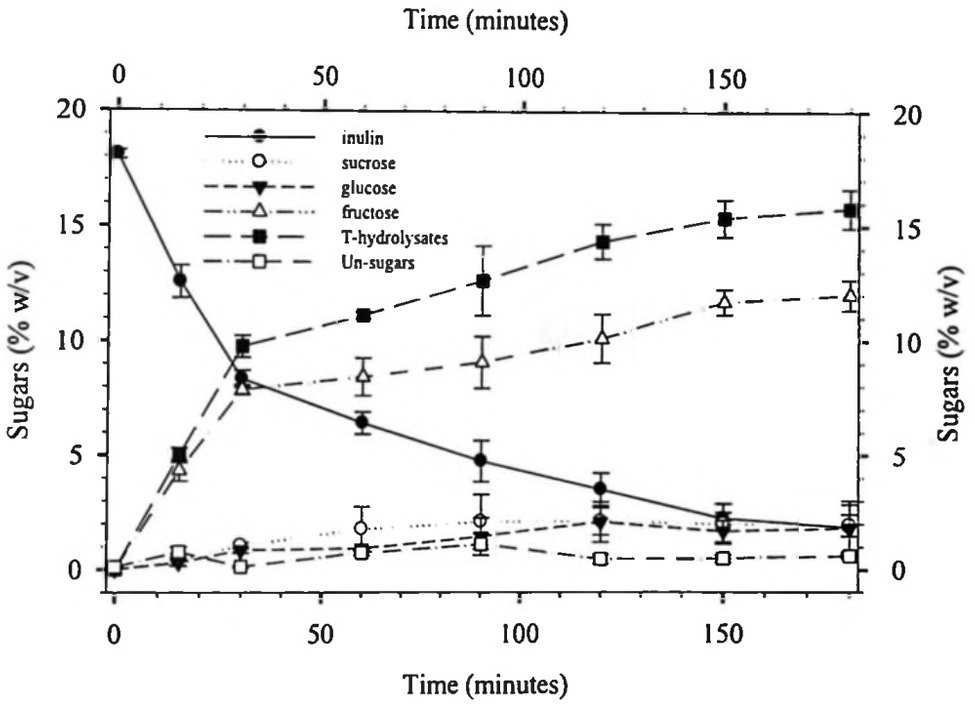

Figure 4.32: Yields of sugars after autoclaving at 132°C and pH 4

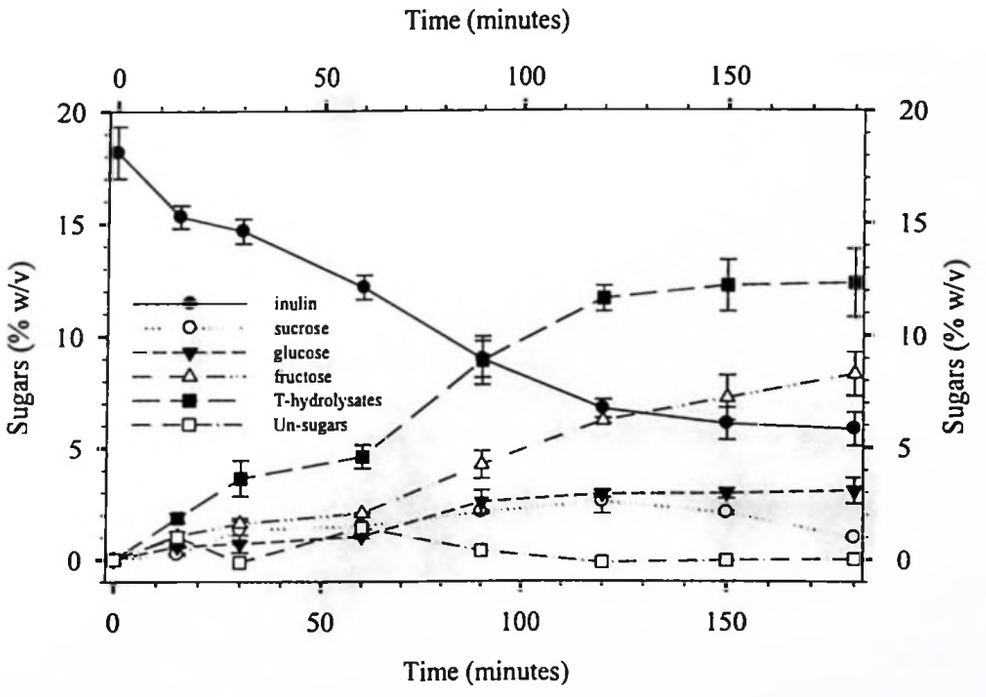

Figure 4.33: Yields of sugars after autoclaving at 132°C and pH 5

The recovery of high hydrolysate yields occurred by autoclave cooking at 132°C and pH 2-4. Probably because, at pH value of 2-3 hydrolysis occur spontaneously because of higher activation energy with values 7.223 and 6.573 ($JK^{-1}mol^{-1}$) respectively (Mohr, *et al* 2008). At pH values of 4-5, hydrolysate yields seemed to decrease, meaning that both total hydrolysates sugars and fructose yields were being reduced by both denaturisation and acid catalyzed maillard reactions (Kyle, 1992; Lindhorst, 2007; Kalliat, 2008).

Revealed by HPLC data, which showed drastic reduction in total sugars (Figure 4.30 to 4.32), the Maillard reaction were strikingly been enhanced by the exposure to high temperatures and prolonged reaction time The reducing sugar-fructose, which is the main constituent of sisal-inulin hydrolysates, has a great reducing power due to its ketonic nature. It is very reactive and unstable when mixed with oxidants such as mineral acids denaturizes to form hard to metabolize brownish furfural compounds (Kotz and Purcell, 1991; Kyle, 1992; Asghari and Yoshida 2006; Lindhorst, 2007; Kalliat, 2008).

In conformity to the Arrhenius theory, total hydrolysates yield increased with an increase in temperature. Lowering of pH and prolonged heat treatments not only broke down complex carbohydrate molecules but also facilitated catalysed side reactions of the resultant fructose monomers sugar (Asghari and Yoshida, 2006).

4.5.3 Effects of pH on fructose yield at temperature 30-132°C

The effect of initial pH on fructose yield during inulin hydrolysis, at temperature 30-132°C, is shown in Figure 4.34.

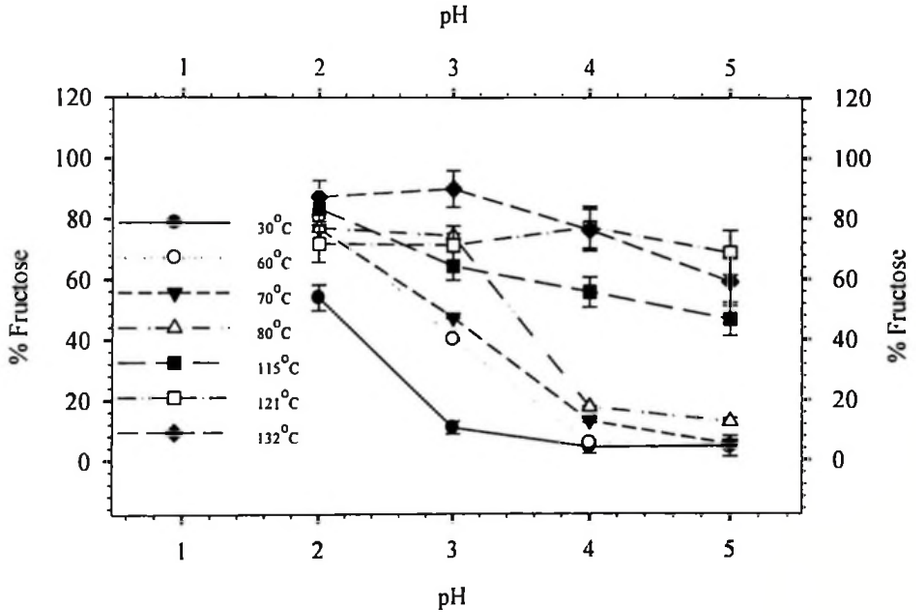

Figure 4.34: Effect of pH on fructose yield

Figure 4.34 to 4.38; show that higher fructose yield was at pH range of 2-3, and temperatures 115-132 °C, while reduced fructose yields are recorded at 30-60 °C. Results indicate that both pH and temperature affected fructose yields, which could be explained kinetically by stability of inulin molecule at low temperatures.

Hydrolysis at pH 2 was quicker and effective as supported by the activation energy value 7.223 ($JK^{-1}mol^{-1}$) and was opposed to values 6.573, 3.125 and 0.199 ($JK^{-1}mol^{-1}$) at pH 3, 4 and 5 respectively (Mohr, *et al* 2008). However, at this range minimization of fructose yield by maillard's reactions was evident.

4.5.4 Effect of temperature on fructose yield at pH 2-5

Figure 4.35 shows the effect of reaction temperature on hydrolysates-fructose yield.

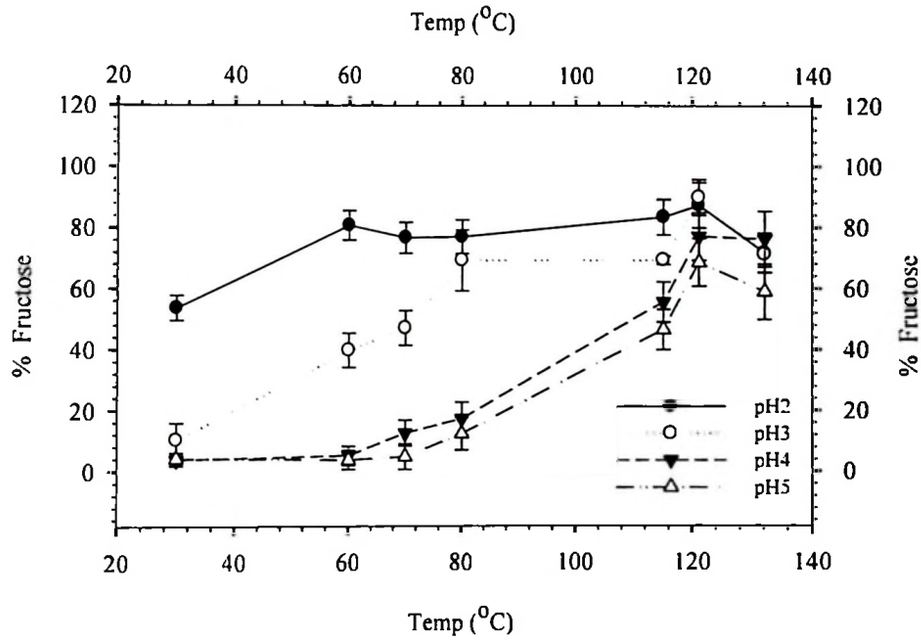

Figure 4.35: Effect of temperature on fructose yield at pH 2-5

Higher hydrolysates-fructose yields occurred at temperature 115°C. Whereby at temperature 132°C, fructose yields were being decreased drastically, probably by both pyrolysis and maillard reactions, despite of the consistence to Arrhenius relation. Fructose yields increased exponentially but slowly at oven temperature of 30-80°C and pH range of 4 to 5. On the other hand, the comparatively high yield was obtained after cooking at oven temperatures of 60 °C and pH 2, possibly, because at pH 2 the activation energy is at its highest rate (7.223 $JK^{-1}mol^{-1}$). The main reason for reduction of fructose yield at temperature value of 132°C (2.9 psi), could be that higher autoclaving temperature contributed to thermal decomposition of both

fructose and inulin, at the expense of the formation of furfural and hydroxymethylfurfural compounds, which are typical compounds of the Maillard reactions (Nirupama *et al.*, 1981; Kim and Lee, 2004).

This recommended the need for a careful monitoring of hydrolysis conditions (low-pH and high-temperature). Seeing that feedstock when treated under such severe conditions results in the accumulation of hydroxymethylfurfural compounds, which in-turn hinders availability of sugars to microorganisms (Nirupama *et al.*, 1981; Kim and Lee, 2004).

4.5.5 Effects of reaction time on fructose yield at pH 2-5

The effect of reaction time on fructose yield at fixed pH 2 during hydrolysis of sisal inulin is shown in Figure 4.40 to 4.46.

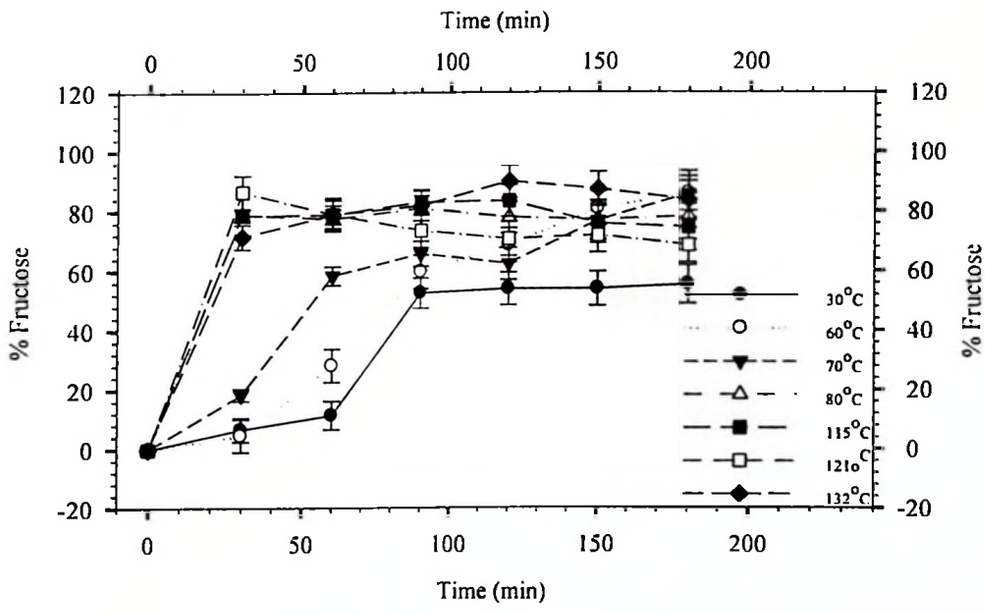

Figure 4.36: Effect of time on fructose yield at pH 2

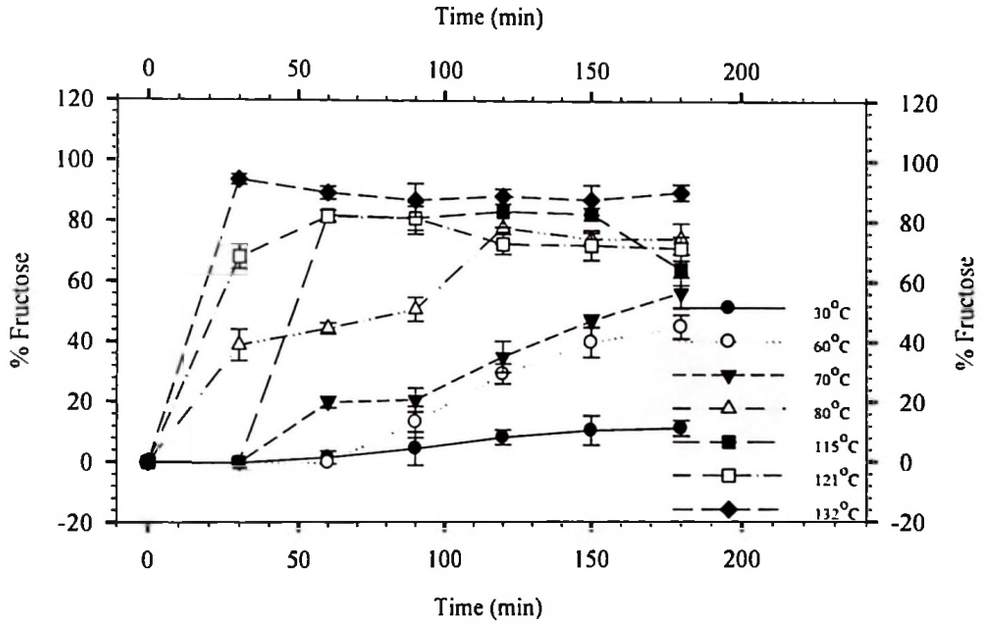

Figure 4.37: Effect of time on fructose yield at pH 3

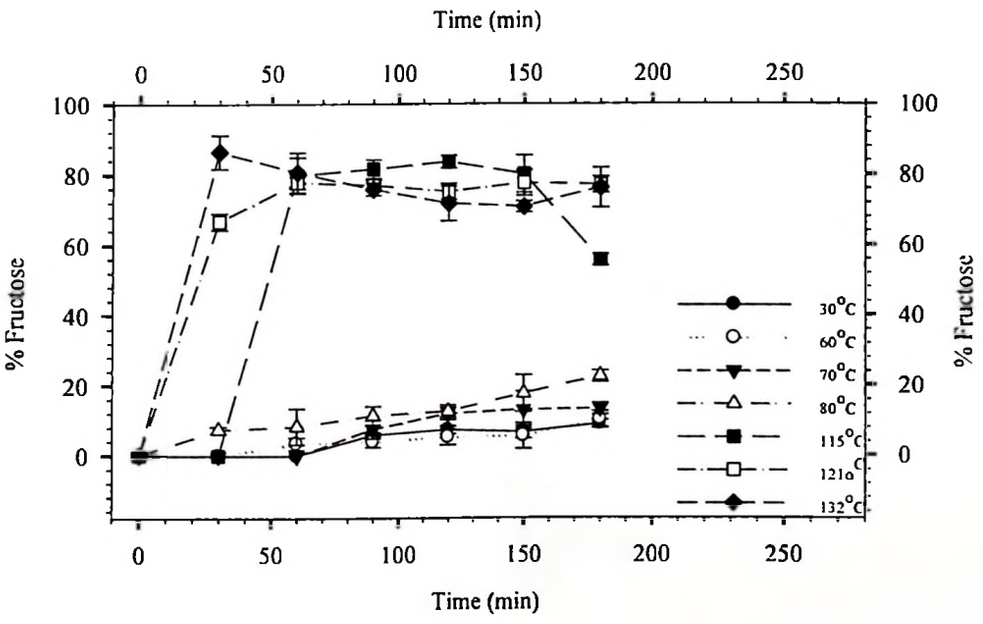

Figure 4.38: Effect of time on fructose yield at pH 4

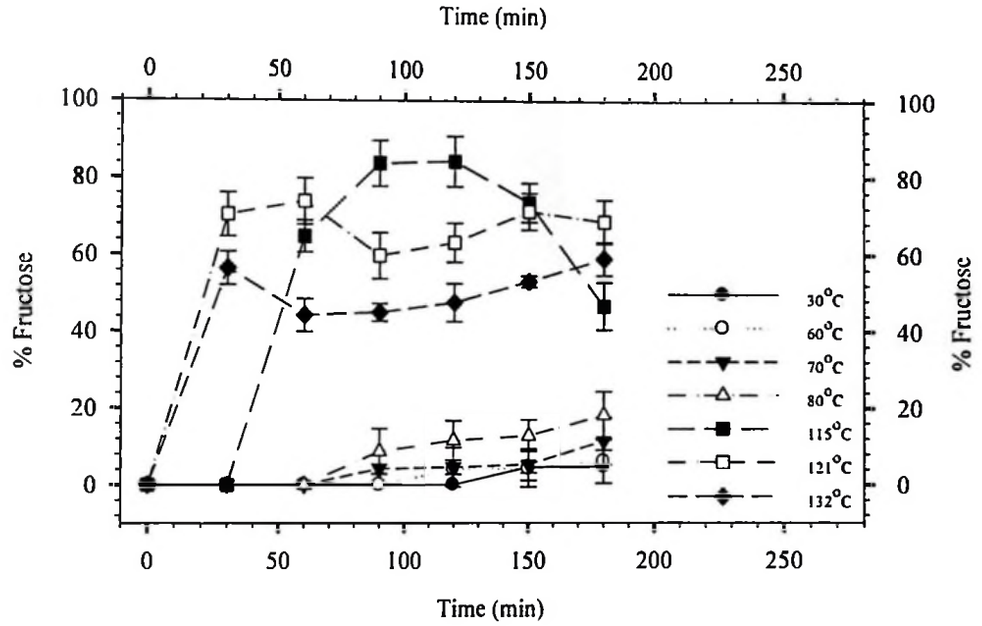

Figure 4.39: Effect of time on fructose yield at pH 5

The general trend showed prolonged reaction time was required for attaining a higher yield at pH values of 4 to 5 and temperatures 30, 60, 70 and 80 °C. This observation is consistent to the Arrhenius relation, which explains that the reaction rate increases with activation energy of reaction (Lindhorst, 2007; Kalliat, 2008; Mohr, *et al* 2008).

4.5.6 Effects of reaction time on fructose yield at temperature 30-132°C

The effect of reaction time on fructose yields at temperature 30-132°C during inulin hydrolysis, is shown in Figure 4.40 to 4.45.

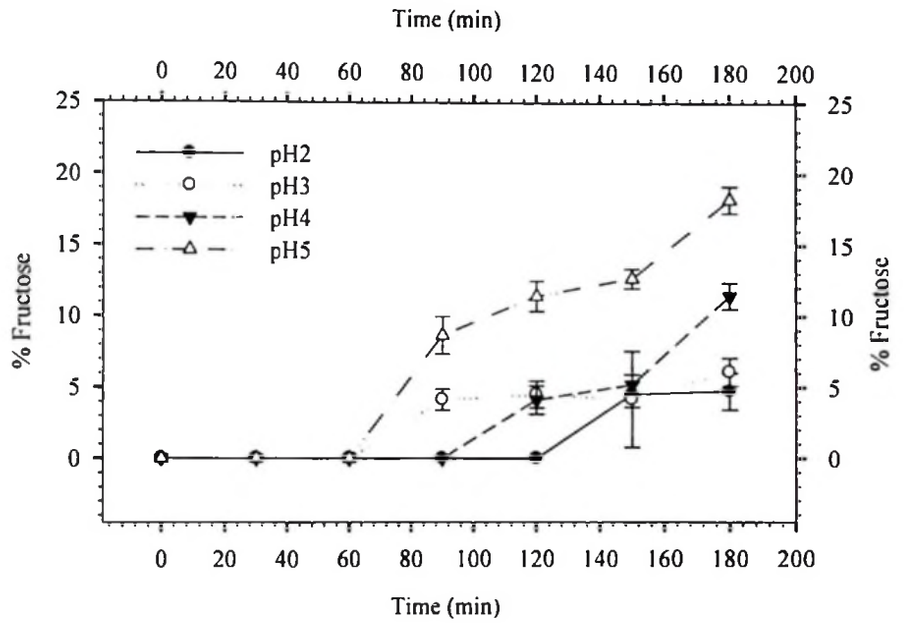

Figure 4.40: Effect of reaction time and pH on fructose yield at 30°C

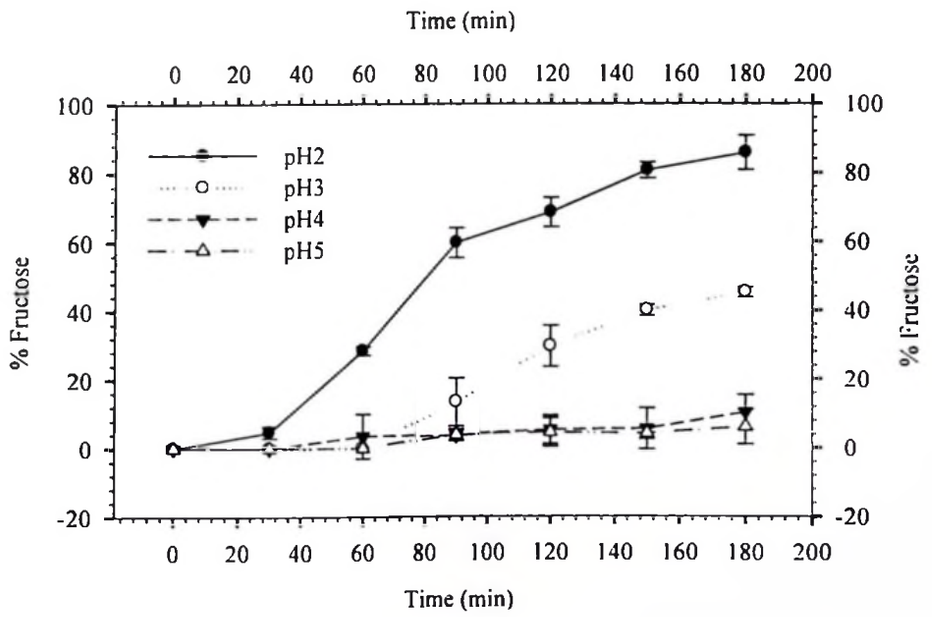

Figure 4.41: Effect of reaction time and pH on fructose yield at 60°C

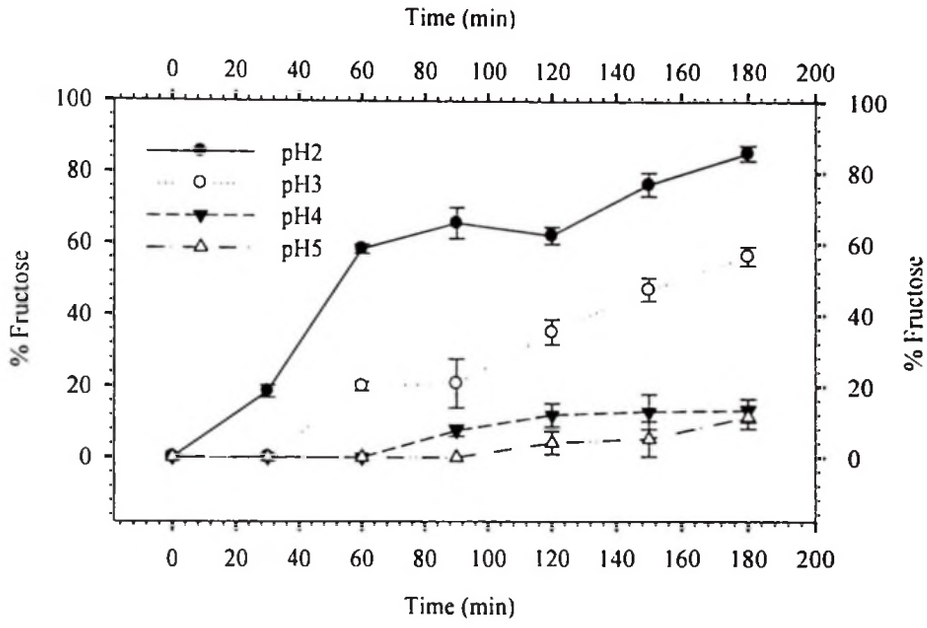

Figure 4.42: Effect of reaction time and pH on fructose yield at 70°C

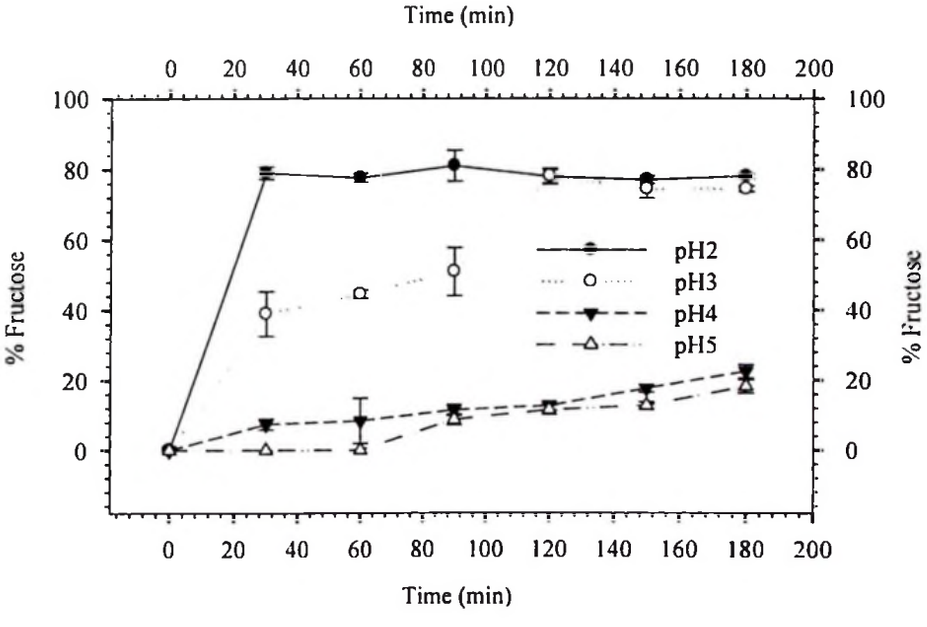

Figure 4.43: Effect of reaction time and pH on fructose yield at 80°C

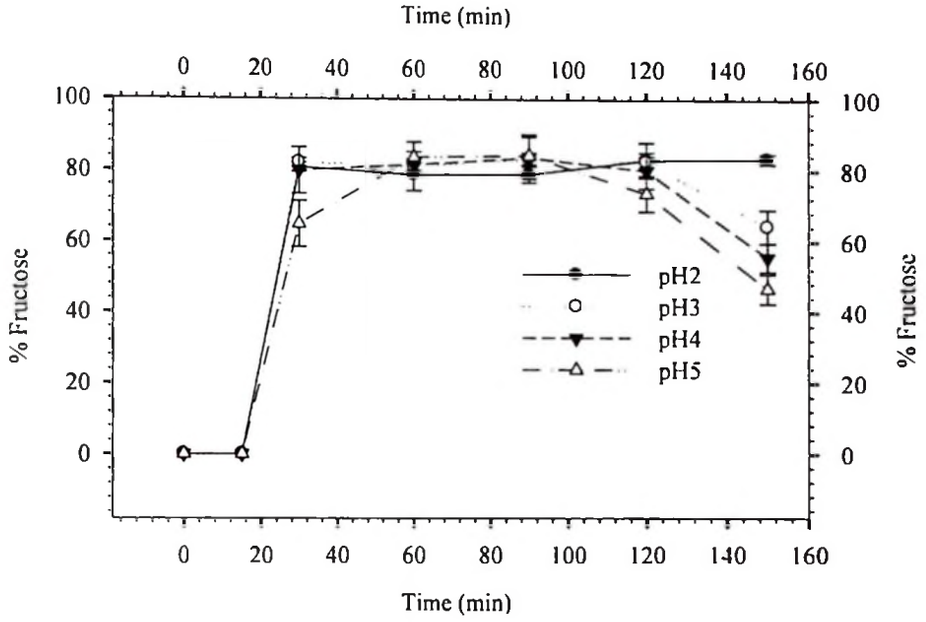

Figure 4.44: Effect of reaction time and pH on fructose yield at 115°C

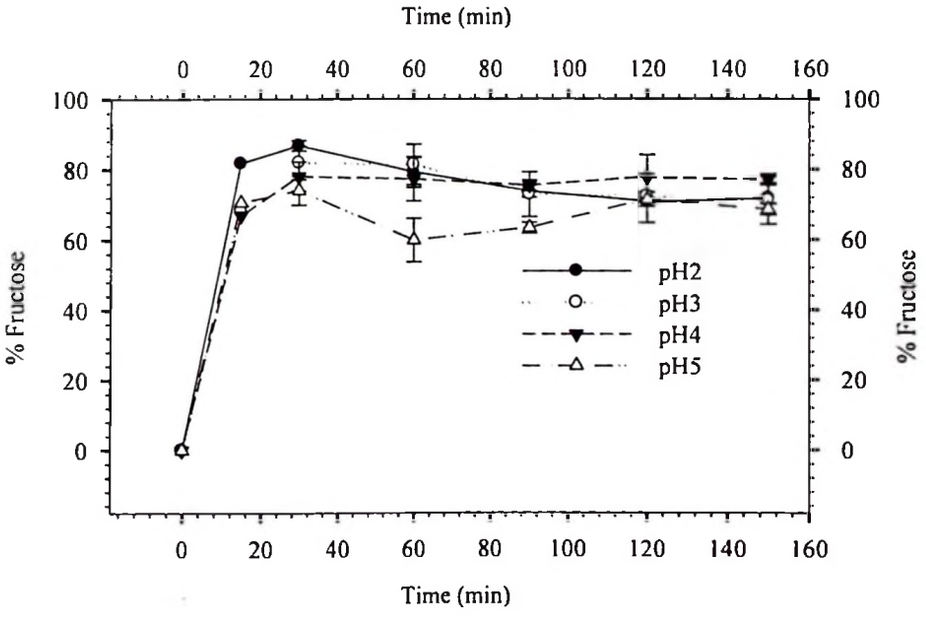

Figure 4.45: Effect of reaction time and pH on fructose yield at 121°C

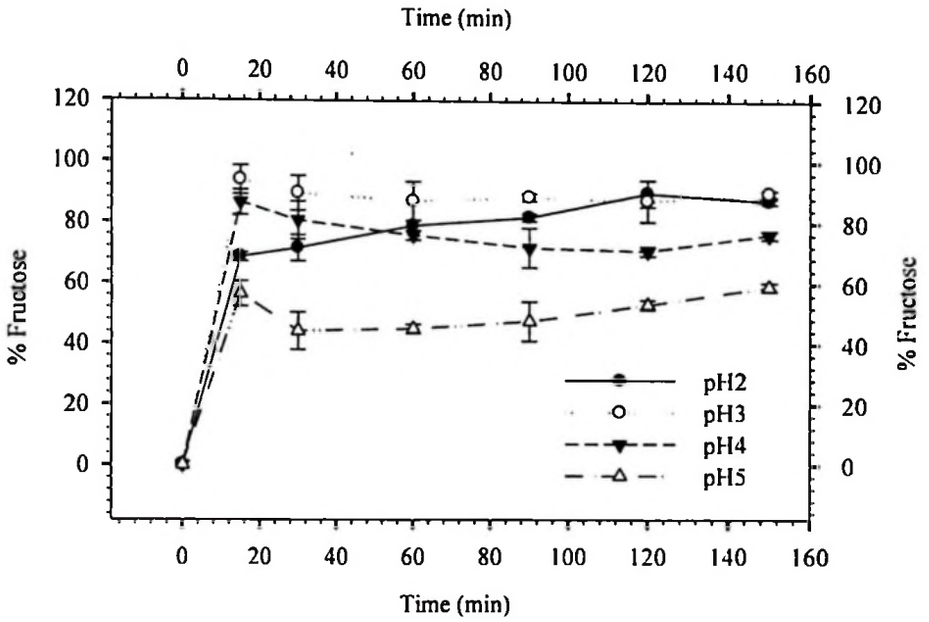

Figure 4.46: Effect of reaction time and pH on fructose yield at 132°C

The results shown in Figure 4.40 to 4.46 indicated that by fixing temperature between 30-80°C, while increasing reaction time and varying pH from 2 to 5 improved fructose yields. On the other hand, results in Figure 4.44 to 4.46 demonstrated that yields did not necessarily increase by increasing reaction time, at higher temperatures between 115-132°C and varying pH values from 3 to 5.

At lower temperatures between 30-80°C and pH 3-5, prolonged reaction time improved yield. The explanations could be that under these conditions, maillard reactions and decomposition of fructose are at the minimum rate (Nirupama *et al.*, 1981; Kim and Lee, 2004; Mohr, *et al* 2008).

4.5.7 Fructose yield at temperature 115±5°C and pH 2-5

Fructose yields after autoclaving at 115±5°C (1.6 bar) are provided by (Figure 4.47).

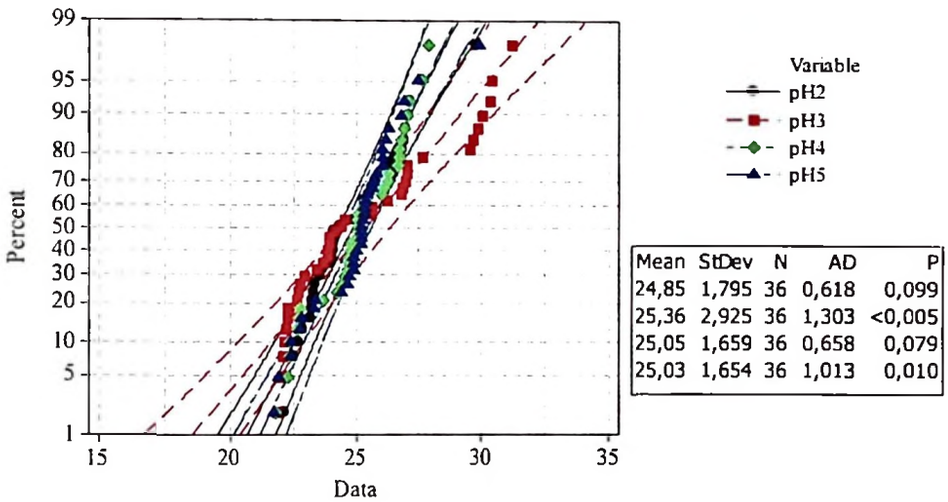

Figure 4.47: Probability plot for fructose yield at 115°C and pH values of 2, 3, 4 and 5 at (95%CI)

Results presented by (Figure 4.47) shows that the lowest mean value of 24.85±0.099 for total fructose occurred at pH 2. The high mean of 25.36±2.925, which was obtained at pH 3; was not conclusive because of higher standard deviation. Therefore, the highest fructose yield values 25.05 ±0.658 and 25.03±1.013 were obtained at pH values of 4 and 5 respectively. This suggested hydrolysis at pH range of 4 to 5 is more viable; as at temperature 115±5°C (1.6 bar) chances of acid catalysed fructose degradation are minimum (Nirupama *et al.*, 1981; Kotz and Purcell, 1991; Kyle, 1992; Kim and Lee, 2004; Lindhorst, 2007; Kalliat, 2008).

4.5.8 Inulin hydrolysis kinetics and activation energy (E_a)

Figure 4.48 shows reaction rate versus temperature, which increased with increase in temperature.

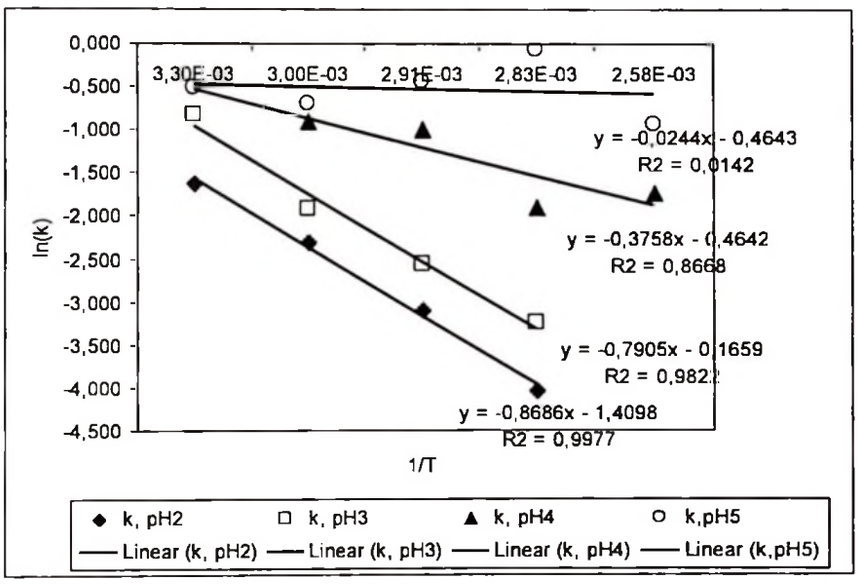

Figure 4.48: A plot of ln (k) Vs 1/T

As was illustrated by equation [12], values of the "y-intercepts" corresponded to ln (*A*); therefore pre exponential factors (A) values were 0.244, 0.847, 0.629 and 0.629 for pH values of 2, 3, 4 and 5 respectively. Calculated values for slopes i.e. (-Ea/R) were -0.8686 (pH 2); -0.7905 (pH 3); -0.3758 (pH 4) and -0.024 (pH 5). Using the universal gas constant (R) = 8.314472(15) $JK^{-1}mol^{-1}[1.8x10^{-6}]$ (Mohr, *et al* 2008), the calculated (E_a) values are therefore 7.223, 6.573, 3.125 and 0.199 ($JK^{-1}mol^{-1}$), for pH values of 2, 3, 4, and 5, respectively.

From this analysis, one can conclude that the lowest (E_a) was observed at pH 5

whereas higher (E_a) was at pH 2, and was consistent to the Arrhenius relation. Supported by the E_a value of 0.199 ($JK^{-1}mol^{-1}$), irregularity behaviour which was observed at pH 5 could be explained thermodynamically using equation [6], and thus suggested the high stability of inulin molecule at pH 5 (Edelman and Jefford, 1968; De Leenheer, 1996; Lindhorst, 2007; Kalliat, 2008; Mohr, *et al* 2008).

4.5.9 Effect of combined initial temperature and pH on yields

The functional relationship between the experimental factors (pH and temperature) and fructose yield are presented by the surface plots **Figure 4.49** to 4.51. Thus effects and optimum operating conditions were established from the surface corners.

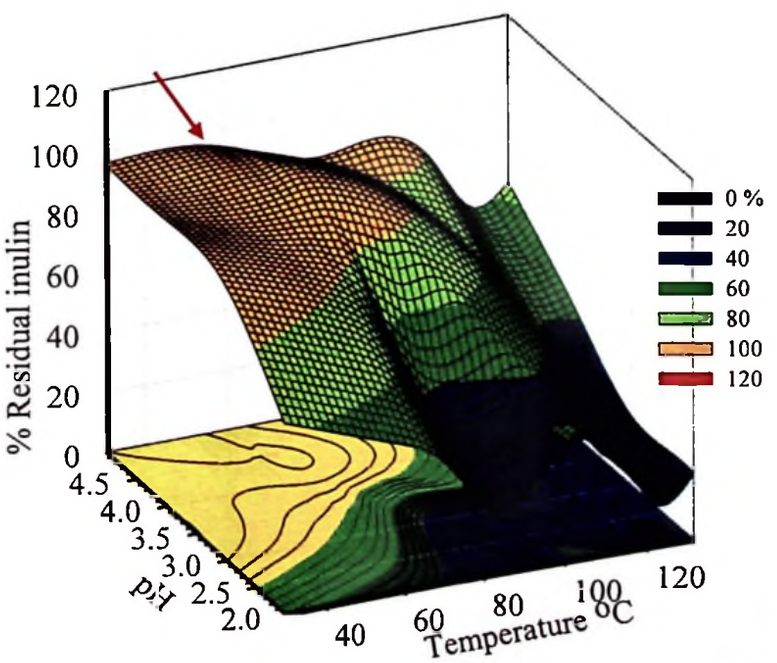

Figure 4.49: Effect of pH and temperature on residual inulin

Results revealed that highest residual inulin was obtained at pH 5 and temperature of 30°C, which corresponded to the minimum fructoses yield. Lowest residual inulin occurred at pH 2 and temperature of 132°C (Figure 4.49), which matched with high fructoses yield region (Figure 4.51).

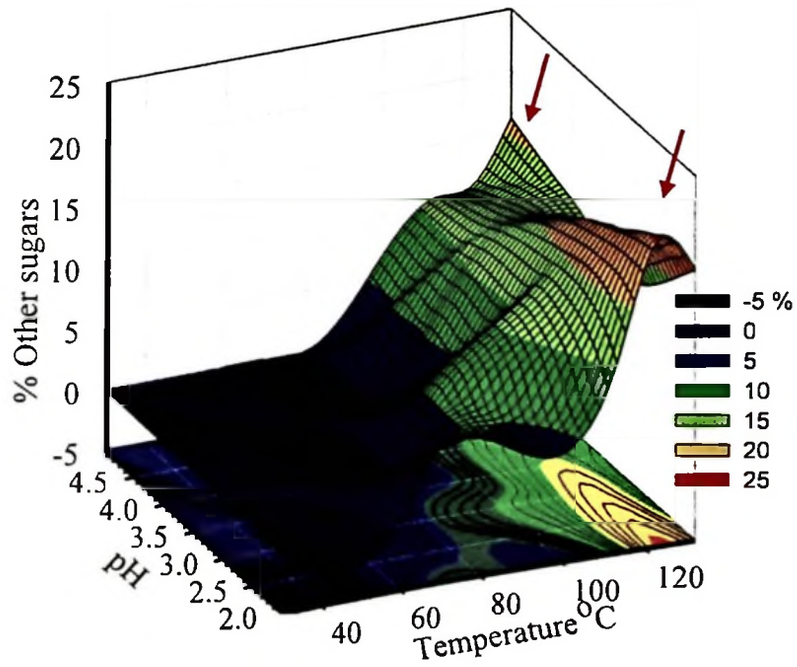

Figure 4.50: Effect of pH and temperature on other sugars

Values for the other sugars were highest at temperatures region with values 80-132°C and pH 2-3.5. The shoulder seen at pH 5 and temperature range of 100-132°C is probably due to un-accounted for sugars, which may either been enriched from breaking down of cellulose (Figure 4.50).

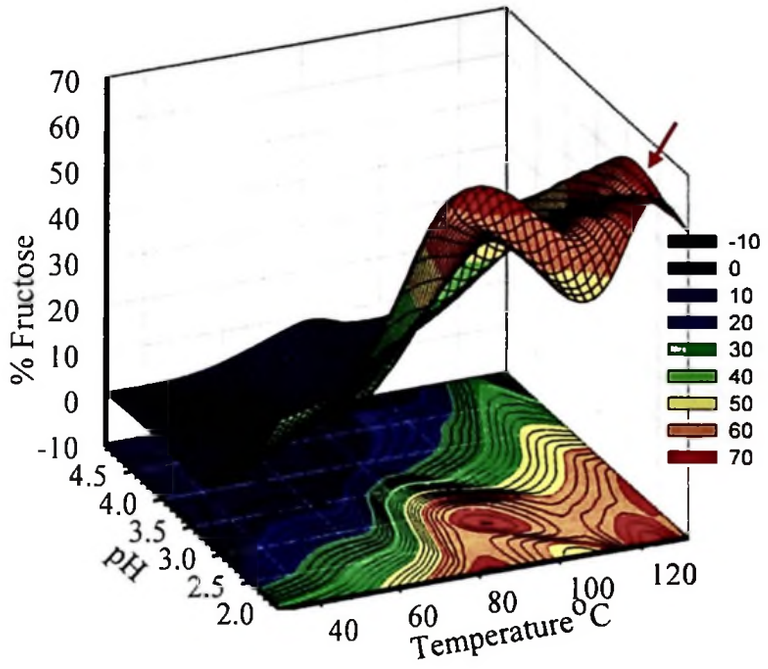

Figure 4.51: Effect of pH and temperature on fructose

Figure 4.51 Shows the highest fructose yield occurrence at temperatures 80-132°C, and pH 2-3.5, which corresponded with the highest total hydrolysates yield observed in Figure 4.52. It was therefore, deduced that the main constituent of sisal inulin hydrolysates sugar was fructose (Abasaeed and Lee, 1995; Abasaeed and Lee, 1996; De Leenheer, 1996; Praznik, *et al.*, 2002; Ngonyani-Mhaiki *et al.*, 2007).

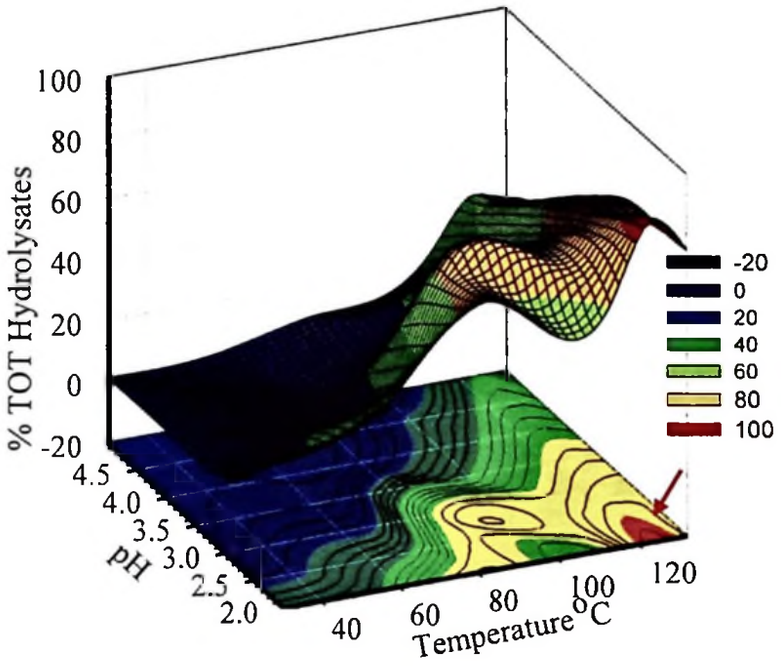

Figure 4.52: Effect of pH and temperature on total hydrolysates

The surface plot also showed quadratic effects in terms of both pH and temperature by exhibiting curvature as you change either factor while holding the other constant (equation [22]). Sisal inulin hydrolysis was most effective at further region with pH 2 and temperature 132°C otherwise; yield decreased rapidly with decrease pH at constant temperature. Yield also decreases at constant pH, as the temperature decreases.

4.5.10 Statistical modelling of the effects of initial hydrolysis conditions

Table 4.7 to 4.10 present statistical modelling of the effects of initial conditions (temperature and pH) on fructose yield.

Table 4.7: Responses for a 2^2 hydrolysis design experimental and predicted yields

		factors		Responses at 120 minutes					
S/Ord	R/Ord	pH	t. (°C)	Res. Inu (g/l)		Fructose (g/l)		TOT Hydroly (g/l)	
		X_1	X_2	Y_1	Y_1'	Y_2	Y_2'	Y_3	Y_3'
				actual	fit	actual	fit	actual	fit
10	1	3	110	5.3	5.1	145.7	144.3	175.6	175.1
3	2	2	132	5.6	5.5	152.8	155.5	175.1	178.7
7	3	2	132	5.3	5.5	158.1	155.5	182.3	178.7
1	4	2	80	18.9	16.5	132.9	134.2	151.7	151.8
4	5	4	132	35.1	35.6	101.6	100.5	143.4	142.6
6	6	4	80	156.6	154.8	22.5	21.7	22.5	21.7
5	7	2	80	14.1	16.5	135.4	134.2	151.9	151.8
8	8	4	132	36.1	35.6	99.4	100.5	141.8	142.6
9	9	3	110	4.8	5.1	142.9	144.3	174.5	175.1
2	10	4	80	152.9	154.8	20.9	21.7	20.9	21.7

Residual inulin S = 1.95115, R-Sq = 99.94%, R-Sq(adj) = 99.89%;
Fructose yield S = 2.22666, R-Sq = 99.89%, R-Sq(adj) = 99,81%;
Total Hydrolysates S = 2.41267, R-Sq = 99.91%, R-Sq(adj) = 99.84%

Table 4.8: Estimated coefficients for fructose using data in uncoded units

Term	Coefficient
Constant	305.773
pH	-103.220
Temperature	-0.696154
pH *Temperature	0.552885
CtPt	41.3500

Model equation for fructose production is shown bellow;

$$Y_1 = [305.773 + (-103.220)X_1 + (-0.696154)X_2 + (0.552885)X_1X_2 + 41.3500] \quad [31]$$

Table 4.9: Factorial Fit: Fructose yield vs. pH, Temperature, Estimated Effects and Coefficients for Fructose (coded units)

Term	Effect	Coef	SECoef	T	P	
Constant		102.95	0.7872	130.77	0.000	***sig**
pH	-83.70	-41.85	0.7872	-53.16	0.000	***sig**
Temperature	50.05	25.03	0.7872	31.79	0.000	***sig**
pH *Temperature	28.75	14.38	0.7872	18.26	0.000	***sig**
CtPt		41.35	1.7603	23.49	0.000	***sig**

S = 2.22666 R-Sq = 99.89% R-Sq(adj) = 99.81%
***sig** = significant at alpha = 0.05; ***N-sig** = Not-significant at alpha = 0.05

The p-values for each individual general linear model parameters shown in (Table 4.9), indicated that both main interactions named initial pH and initial temperature had a significant effect on hydrolysates (fructose) yield, both had values (p = 0.000) at confidence interval (CI) of 95%. Two-way interaction effect between initial pH and initial temperature, was also significant with values (p = 0.000).

Table 4.10: ANOVA-of variance for fructose yield (coded units)

Source	DF	SeqSS	AdjSS	AdjMS	F	P	
Main Effects	2	19021.40	19021.40	9510.69	1918.25	0.000	***sig**
2-Way Interactions	1	1653.10	1653.10	1653.12	333.43	0.000	***sig**
Curvature	1	2735.70	2735.70	2735.72	551.78	0.000	***sig**
Residual Error	5	24.80	24.80	4.96			
Pure Error	5	24.80	24.80	4.96			
Total	9	23435.00					

* **Sig** = significant at alpha = 0.05; * **N-sig** = Not-significant at alpha = 0.05

The sequential sums of squares (Seq SS) and adjusted sums of squares (Adj SS) shown in the ANOVA (Table 4.10) were the same therefore the model was orthogonal and did not contain covariates (Tukey, 1993; Jones and Tukey, 2000; Lehmann and Romano, 2005; McCloskey, 2008). The analysis of variance presented

block effect for both the main and two way interactions and also Figure 4.53 to 4.55, all of them supported the analysis of variance; which implied the fructose yields was significantly affected by both the initial pH and temperature.

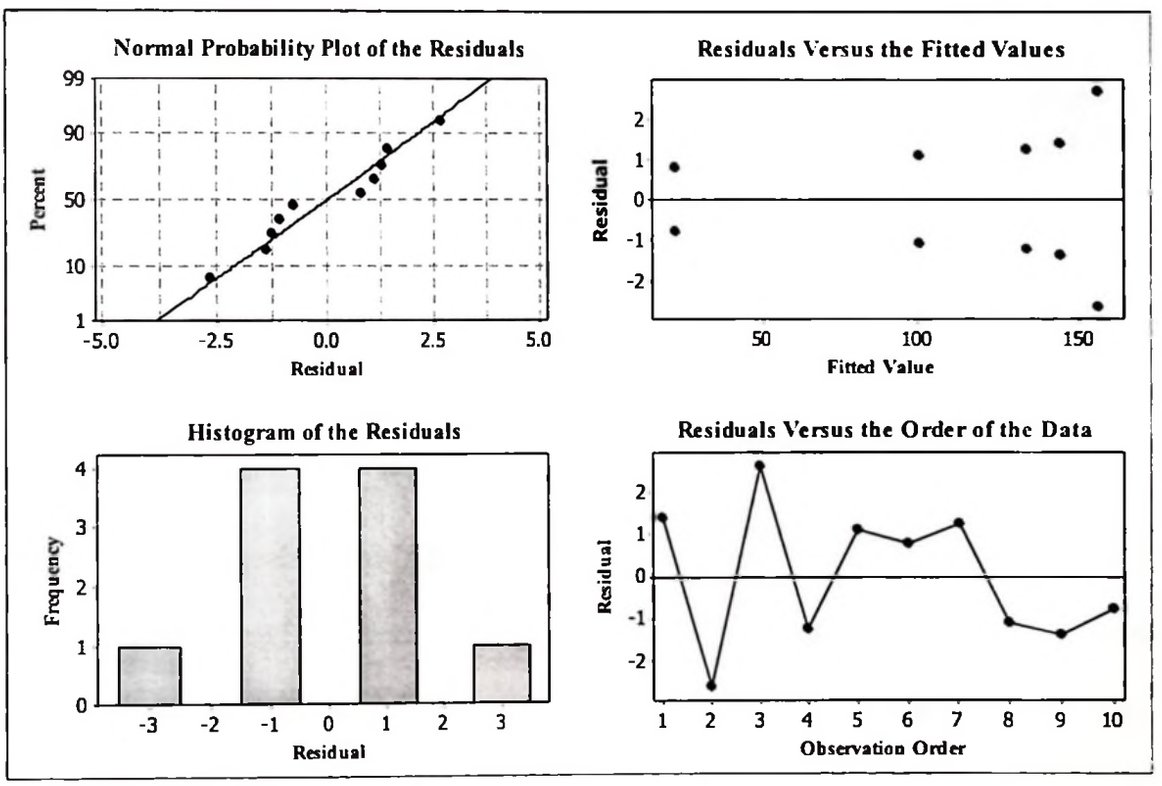

Figure 4.53: The four-in-one residual plot for fructose yield

The four-in-one residual plot, displayed four different residual plots together in one graph window. These determined whether the model meets the assumptions of the analysis as follows:

(a) The bell-shaped residual histogram shown in Figure 4.53 justified that fructose data was not skewed thus there was no outliers (Neter *et al.*, 1993; Miller, 1997; NIST/SEMATECH, 2006).

(b) Normal probability plot of the residuals indicated that the residuals followed a straight line; therefore the residuals from the analysis were normally distributed without skewness, outliers, or unidentified variables. In practice, for balanced or nearly balanced designs or for data with a large number of observations, moderate departures from normality do not seriously affect the results (Neter *et al.*, 1993; Miller, 1997; NIST/SEMATECH, 2006).

(c) Residuals versus fitted values for fructose yield appeared to be randomly scattered about zero consequently portrayed a constant-variance, without missing terms or outliers. This justified that the variance was linearly distributed (Neter *et al.*, 1993; Miller, 1997).

(d) Residuals versus order graph appeared to be randomly scattered about zero. This showed that there was no evidence that the error terms are correlated with one another. The ascending or descending trend and rapid changes in signs of adjacent residuals showed correlation among residuals (Neter *et al.*, 1993; Miller, 1997; NIST/SEMATECH, 2006).

Therefore the four-in-one residual plot Figure 4.53 indicated that both effects are important, also justified by the nature and source of residual error and pure errors; which are not due to the lack of fit of yield data (Neter *et al.*, 1993; Miller, 1997).

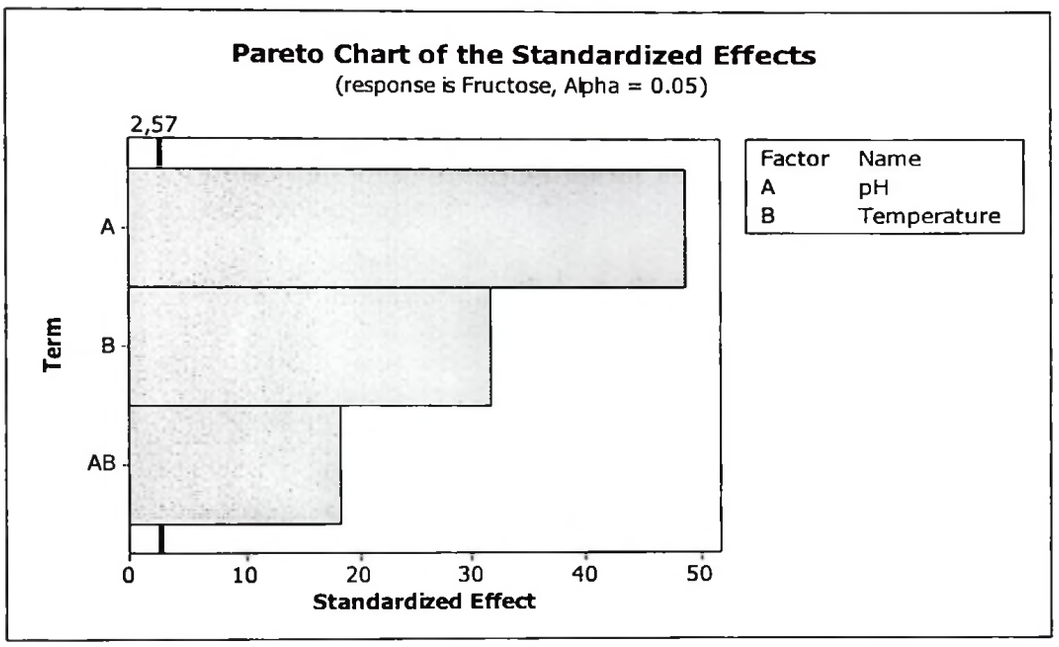

Figure 4.54: Pareto effects of standardized effects for hydrolysates-fructose

Pareto chart of the effects is shown by Figure 4.54, whereas the effects of initial pH, temperature and combined initial pH and Temperature extended past the reference line at significance ($\alpha = 0.05$). Results showed that the largest effect was coming from initial pH, followed by initial reaction temperature and then the combined effect of initial pH and initial reaction temperature. Both results were very significant at α - level of 0.05 (Neter *et al.*, 1993; Miller, 1997).

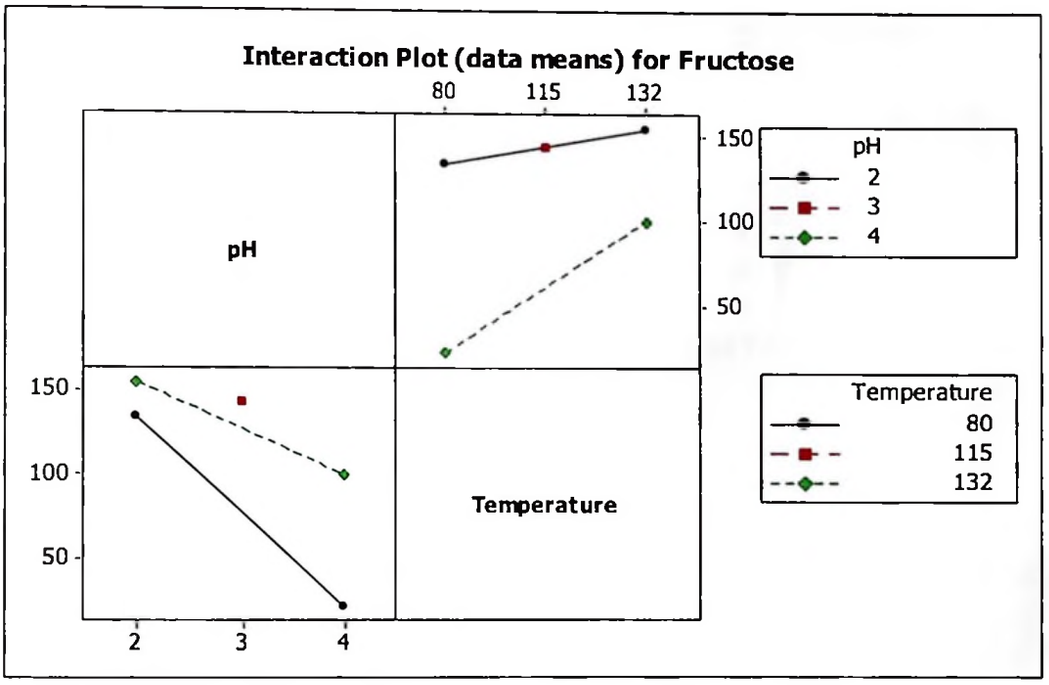

Figure 4.55: Interaction Plot for fructose

The interaction plot indicated the great degree of interaction, which is justified by looking at the interaction lines that departed from being parallel. Big departures from parallel occurred when combining both pH and reaction temperature (Figure 4.55).

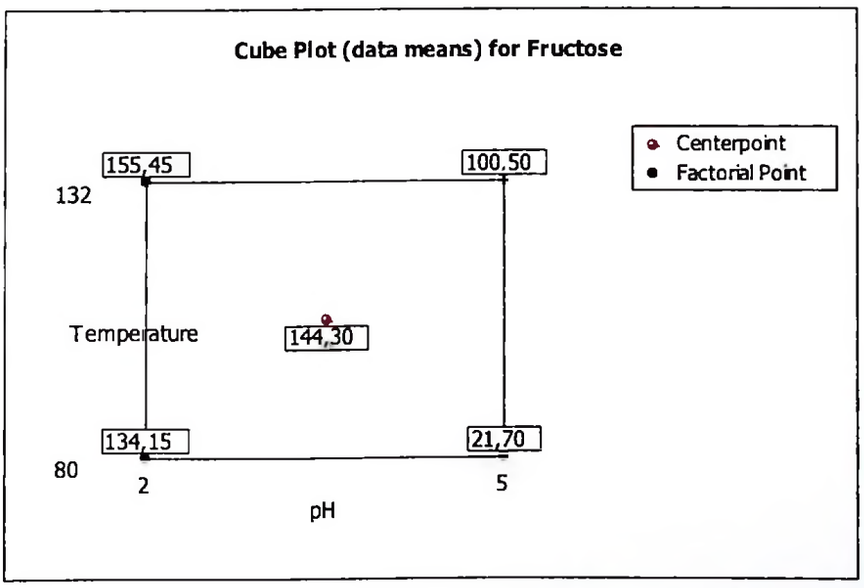

Figure 4.56: Cube plot (data means for hydrolysates-fructose yield)

The cube plot showed that increased yield is greater when reaction temperature is high (132°C) than when reaction temperature is low (80 °C). While the increase in yield is greater when reaction pH is low (2), contrary to when reaction pH is high (5). Supported by Table 4.9 and 4.10, the corners of the cube plot (Figure 4.56) show that high yields are obtained at 132°C and pH 2, for example as you move towards the pH 5 and temperature 80°C the lowest hydrolysates-fructose yield is achieved.

To sum up the ANOVA, interaction effects and by using General Linear Model (GLM), Multiple Comparisons-Tukey Method, for hydrolysis supported the null hypothesis. This justified that the initial claim is true as showed by levels of p-values at the levels of significance $\alpha = 0.05$ (Tukey, 1993; Jones and Tukey, 2000; Lehmann and Romano, 2005; McCloskey, 2008). The null hypothesis was not rejected, as the analysis supported the fact that initial hydrolysis conditions of sisal bole inulin (pH and temperature), significantly affects total hydrolysates-fructose yield and consequently citric acid yield.

4.5.11 Prediction of fructose yield optimization at 120 minutes

Table 4.11 and 4.12 present constraints and optimum levels of initial conditions and predicted yields for residual inulin and fructose by Minitab V15 numerical optimization FFD after 120 hours.

Table 4.11: Constraints for optimization of fructose yield from (SBJ-F)

variables	Goal	Lower limit (g/l SBJ-I)	Upper limit (g/l SBJ-I)
pH	-	2	5
Temperature (°C)	-	80±5	132±5
Fructose yield 120 (min)	maximize	16.70	84.96

Table 4.12: Optimum levels of initial hydrolysis conditions and predicted yields Minitab V15 numerical optimization FFD after 120 hours

	Initial		Response(g/l SB-I)			
Solution	pH	Temperature	Res. inulin	Fructose		TOT Hydrolysates
			(g/l SB-I)	(g/l SB-I)	%	(g/l SB-I)
	X_1	X_2	Y_1	Y_2		Y_3
1	2	80	16.50	134.15	88.4	151.80
2	**3**	**110**	**5.05**	**144.30**	**82.4**	**175.05**
3	4	132	5.45	155.45	86.9	178.70
4	5	132	35.60	100.50	70.5	142.60

Solution (1) predicted optimization of fructose yield at a lower temperature of 80±5°C ~ (80°C) and pH 2 which resulted to 88.4% of total hydrolysates. Solution (2) predicted at moderate temperature of 110±5°C ~ (115°C) and pH 3. At this range fructose yield was 144.3 g/l of SBJ-Inulin, which was 82.4% of total hydrolysates. Solution (3) predicted optimization at highest temperature of 132°C±5°C ~ (132°C) and pH 4 which was 86.9% of total hydrolysates. Solution (4) predicted optimization at low temperature and high pH 5, which was 70.5% of total hydrolysates. However, hydrolysing at temperatures below 80°C require prolonged reaction time beyond 120 hours. When considering the energy serving, solution (1) and (2) are more feasible and recommended. Nevertheless, solution (2) which requires pH 3 was best as reaction occurs within the sterilisation temperatures therefore save energy cost.

4.8 Oxygen Transfer and Rheology during Fermentation

The *A. niger* was cultured in pellet morphologies, which resulted in a pseudo Newtonian rheological behaviour. Because of this, the consistence of the inulin hydrolysates, which had a comparative higher viscosity 2.56 mPaS, changed with time during the fermentation course, therefore a careful monitoring of oxygen transfer and heat distribution was required.

Dissolved oxygen (DO) in the broth was routinely estimated by equations [16], [26] and [27]. The calculated average, *OUR* values were between 38 and 49±2 mmol O_2/l-hour during the production phase i.e. at the 24 to 144 fermentation hours. These values were within the range values for *OUR* in large-scale cultures, which is 40 to 200 mmol O_2/l-hour (Shuler and Kargi, 2004).

Maintenance of equal distribution of heat and oxygen transfers, by increasing agitation especially during nights of May to July was very tricky. During night times the Dar es Salaam ambient temperatures fell to the range of 20±2°C (recalling that viscosity tends to increase or, alternatively, its *fluidity* tends to decrease as its temperature decreased). To assure that there was no deprivation of dissolved oxygen tension and hence maximum respiration in growing *A. niger* more air was needed.

Unfortunately, aquarium pumps used had a maximum flow rate of 1.5±0.3 vvl, thus another option was to adjust the reactor internal pressure, which was not feasible, as

the reactor used was made of borosilicate glassware, which would withstand pressures at the range of 1-3 psi. In such circumstances, a low-pressure gauged air supply with a sterile filter was used instead of the aquarium pump. The use of gauged air compromised maintenance of the aseptic conditions.

On the course of counter affecting the broth apparent viscosity (μ_{ap}); and the compensations by increasing impeller speed (N) = the agitation and (Q) = aeration rate) to maintain adequate k_La values, inflicted stress on fungi cells (Olsvik and Kristiansen, 1994; Gavrilescu *et al.*, 1993; Kusai *et al.*, 2002) the other hand, in.

This was evident as growth rates and consequently substrates consumption rates were retarded just after 144 fermentation hours. It was hard to continue fermentation beyond 168hrs, and this could be another reason why the residual sugars remained high at the end of fermentation processing (Olsvik and Kristiansen, 1994; Gavrilescu *et al.*, 1993; Kusai *et al.*, 2002).

4.7 Pilot Scale Fermentation Production of Citric Acid

Shown in (Photo 4.6), are various *A. niger* pellets morphologies during fungal culture preparations. Inoculation was done aseptically from *A. niger* BYF KT labeled (BF KT), which had pellets of desired morphology (Photo 4.6 C).

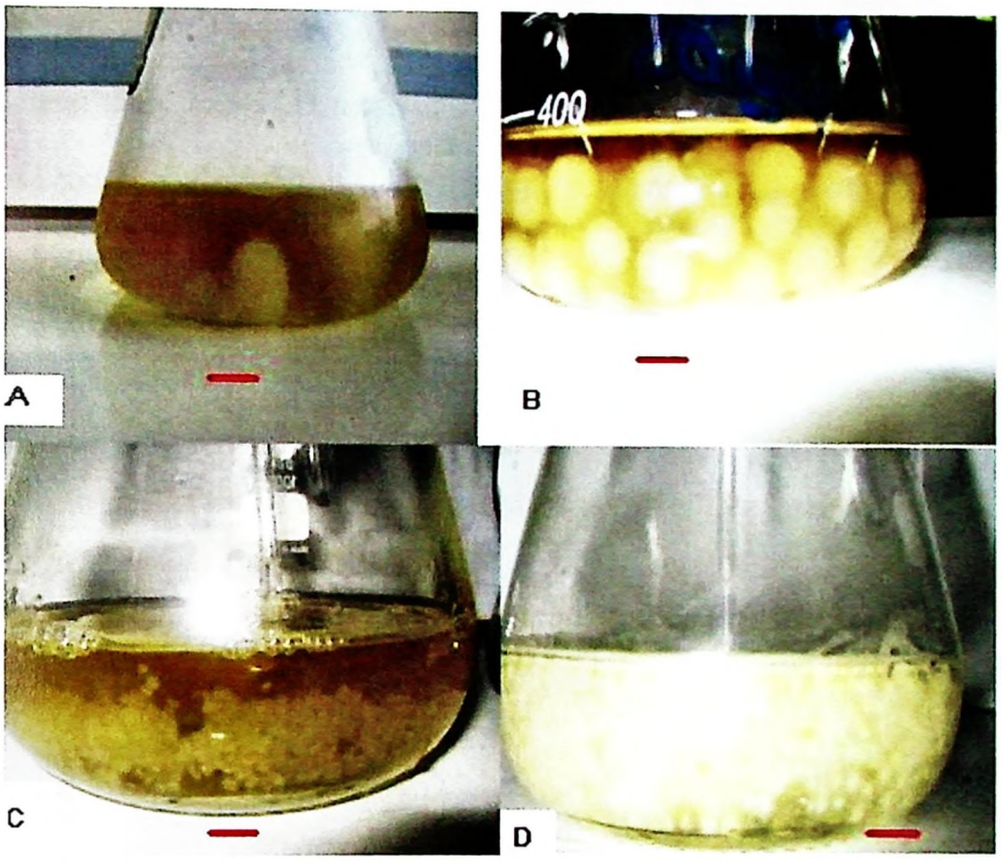

Photo 4.6: Diameters of different fungal pellets against 10mm red bar

Shown in Photo 4.6 (A) are the over grown 72 hours old pellets in low sugar concentration (diameters 20 &15±0.5 mm); (B) = 72-hours old pellets in high sugar concentration (diameter 11±0.5 mm); (D) = typical 24 hours old pellets (diameter 2±0.5 mm) before cleaning, (E) = 24 hours old, pellets cleaned.

Results obtained after 168 hours of fermentation, indicated that highest citric acid yield 46.66% (103.15±13.08 g/l) was obtained at an initial fructose concentration value of (203g/l) and pH 5, without nutrients addition. Concurrently when the fermentation was conducted with nutrients additives incorporated, yield value obtained was (100.13±1.31 g/l); this value corresponded to 44.92% and was slightly low but was within the expected ranges from substrates other than glucose (Drysdale

and McKay, 1995; Hossain *et al.*, 1984; Grewal and Kalra, 1995; Othmer, 2001). The lowest citric acid yield value of 18.32±2.40g/l (15.69%) was obtained, when fructose concentration value of (102g/l) without nutrients additives was used.

4.7.1 Effects of initial fructose concentration (102g/l) and pH on citric acid

The results presented in Figure 4.57 to 4.61, show citric acid yield at initial fructose concentration 102g/l (40%) and pH 2-5, while nutrients additives were either included or omitted.

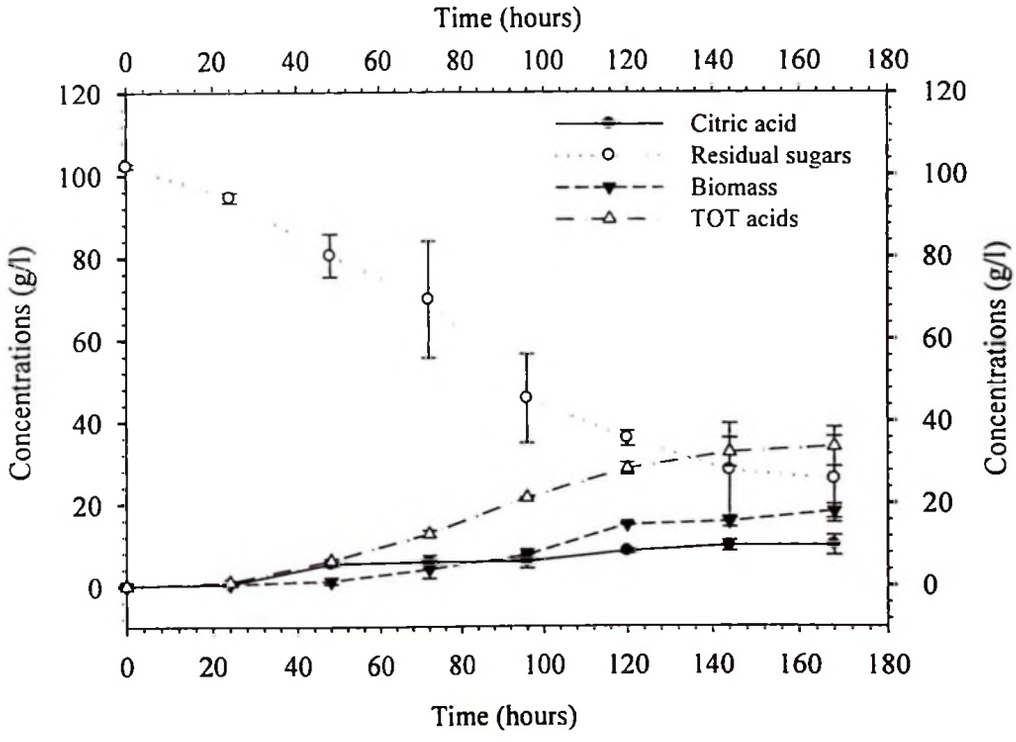

Figure 4.57: Initial hydrolysates 40% (Fructose 102g/l) pH 2 with nutrients

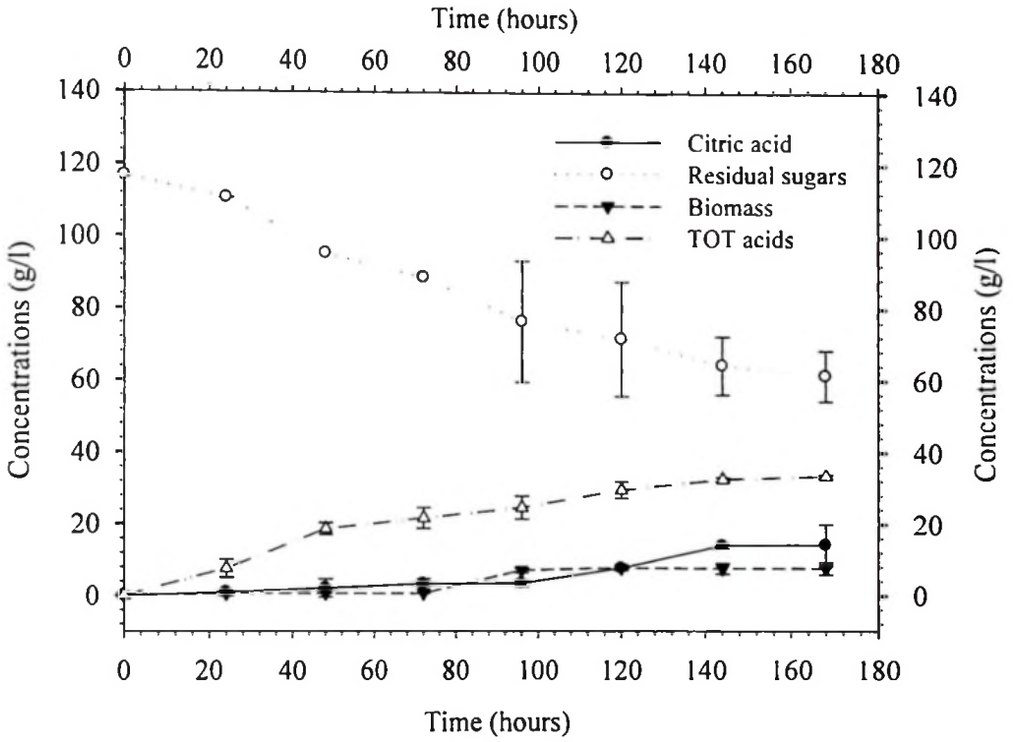

Figure 4.58: Initial hydrolysates 40% (Fructose 102g/l) pH 2 no nutrients

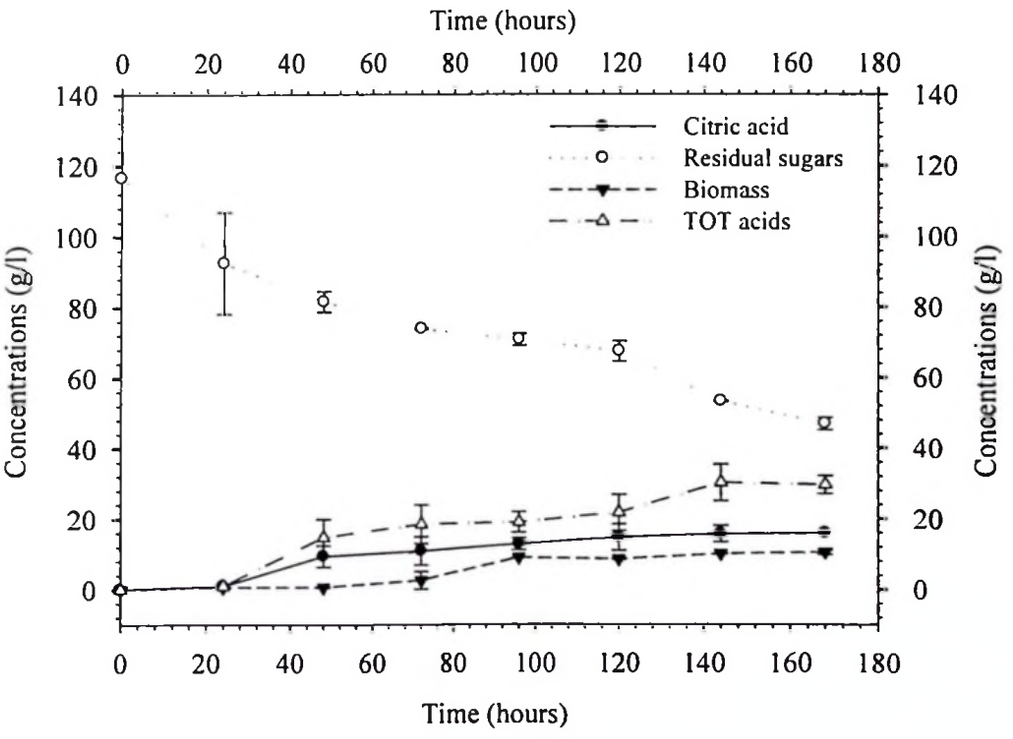

Figure 4.59: Initial hydrolysates 40% (Fructose 102g/l) pH 3.5 with nutrients

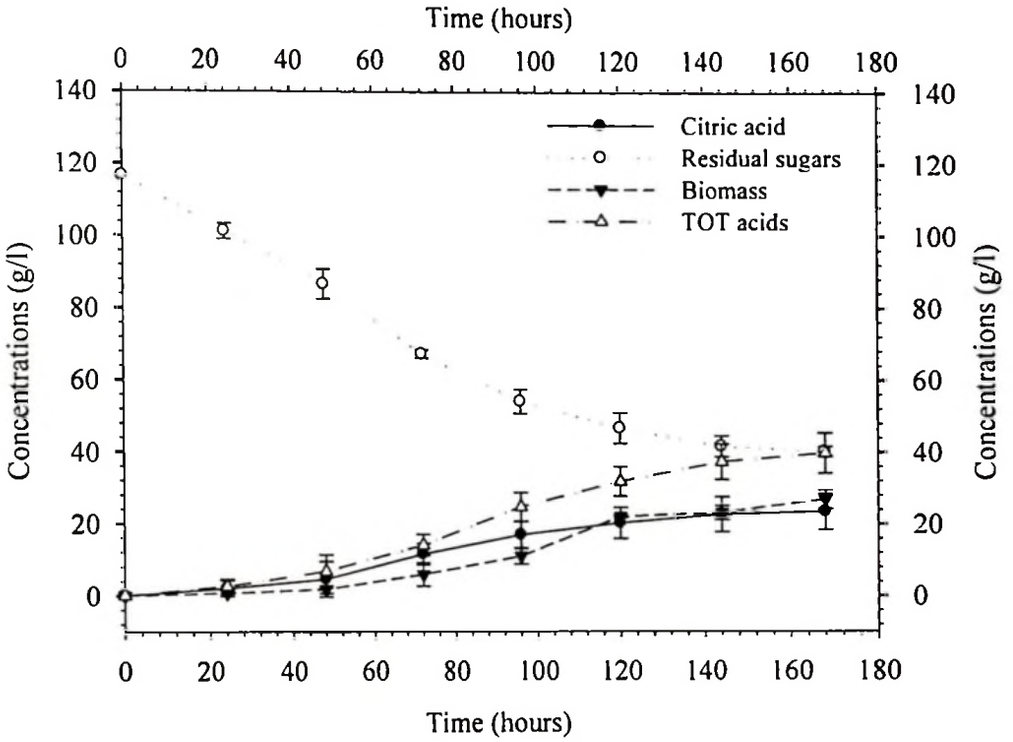

Figure 4.60: Initial hydrolysates 40% (Fructose 102g/l) pH 3.5 no nutrients

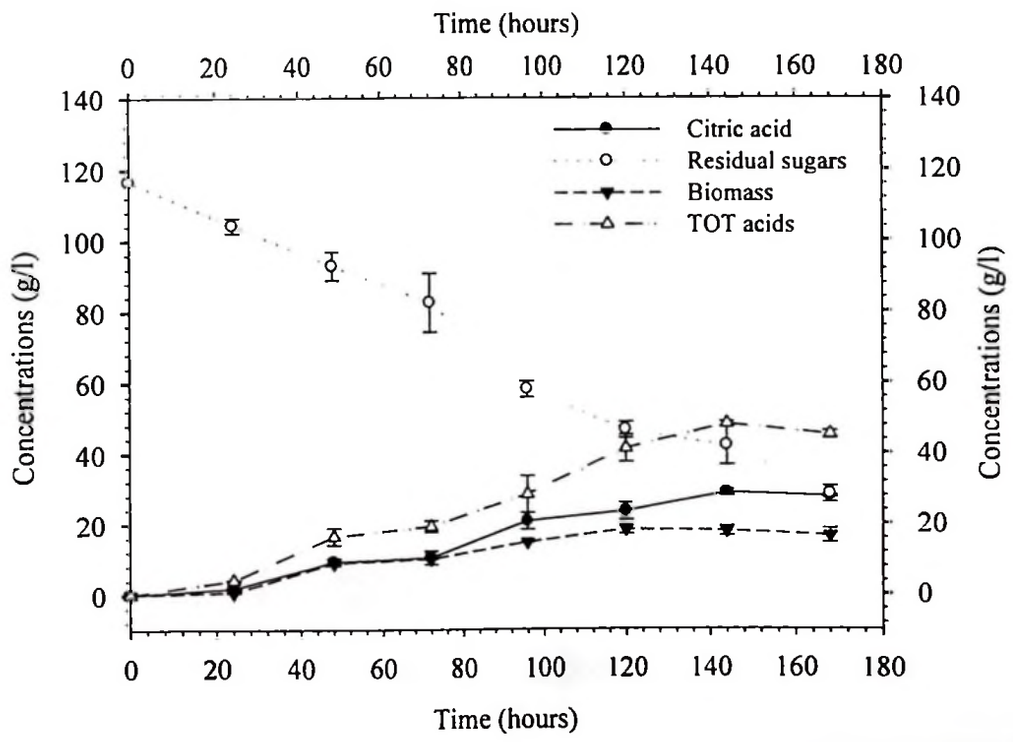

Figure 4.61: Initial hydrolysates 40% (Fructose 102g/l) pH 5 with nutrients

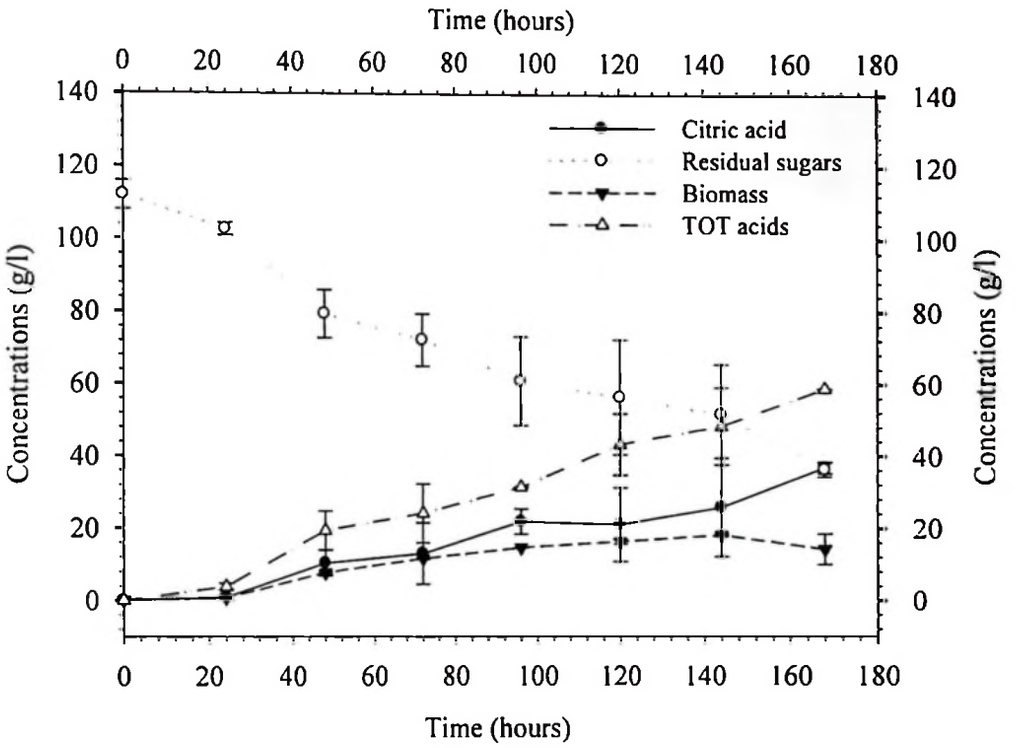

Figure 4.62: Initial hydrolysates 40% (Fructose 102g/l) pH 5 no nutrients

Results shown in Figure 4.60 indicated that after 168 hours of fermentation at pH 5, the amount of citric acid yield obtained without nutrients additives was 36.85±1.63g/l. This value corresponded with the lowest residual sugars value of 28.20±2.26g/l. Conversely lower citric acid yield 9.70±2.40g/l occurred at pH 2 with nutrients additives (Figure 4.57), and corresponded with the highest residual sugars value of 61.35±7.04g/l.

Higher citric acid yield occurred without nutrients additives and pH 5, probably because both pH and nutrients additives are growth-limiting factors (Figure 4.62). Since limiting factor concept, is based upon the Law of the minimum (states that yield is proportional to the amount of the most limiting nutrient, whichever nutrient it may be).

The functioning of *A. niger* metabolism seems to be controlled or essentially limited by fermenter environmental factors (pH) and growth-limiting factors (sugar concentrations and nutrients additives); in as much as the least favorable amounts for the later (Taylor, 1934; Arts *et al.*, 1987; Soule, 1986; Chen and Christensen, 1985).

4.7.2 Effects of fructose concentration of (152g/l) and pH on citric acid

At initial fructose concentration (152g/l) and pH values of 2, 3.5 and 5 the effect of increasing fructose concentration in citric acid yield is shown in Figure 4.63 to 4.67,

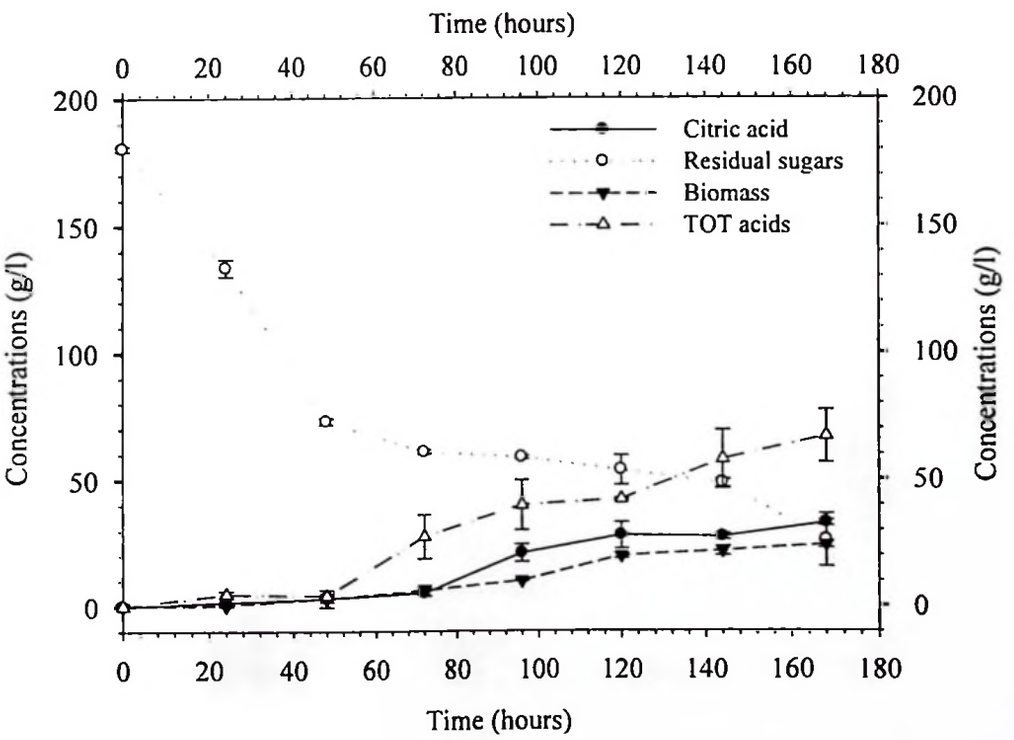

Figure 4.63: Initial hydrolysates 60% (Fructose 152 g/l) pH 2 with nutrients

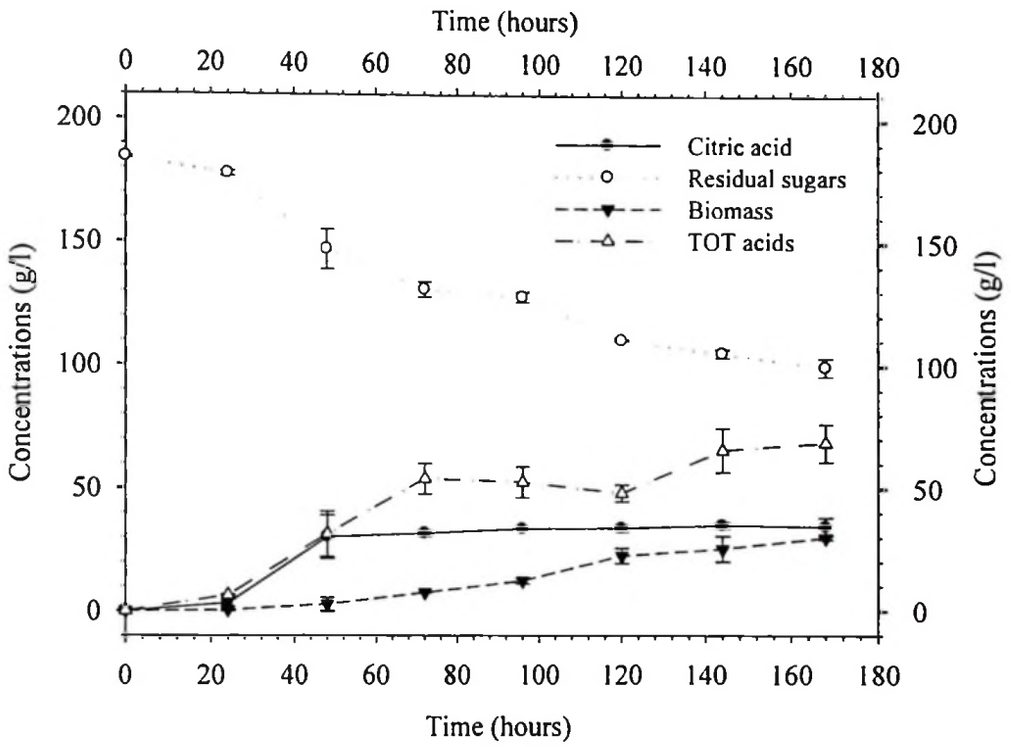

Figure 4.64: Initial hydrolysates 60% (Fructose 152 g/l) pH 2 no nutrients

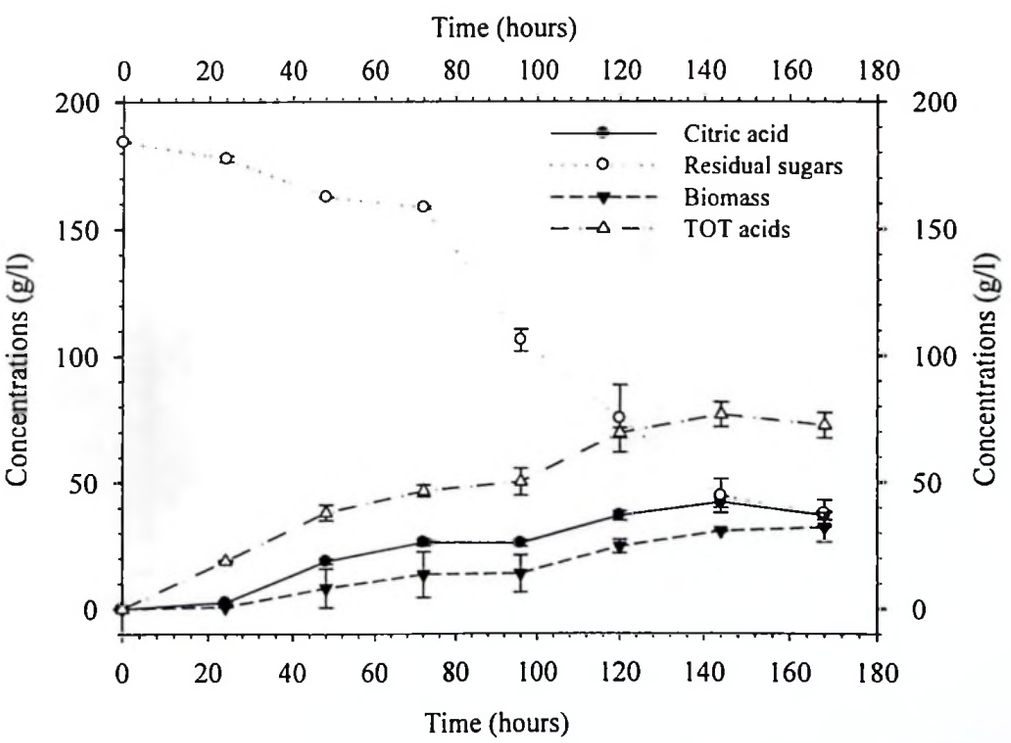

Figure 4.65: Initial hydrolysates 60% (Fructose 152 g/l) pH 3.5 with nutrients

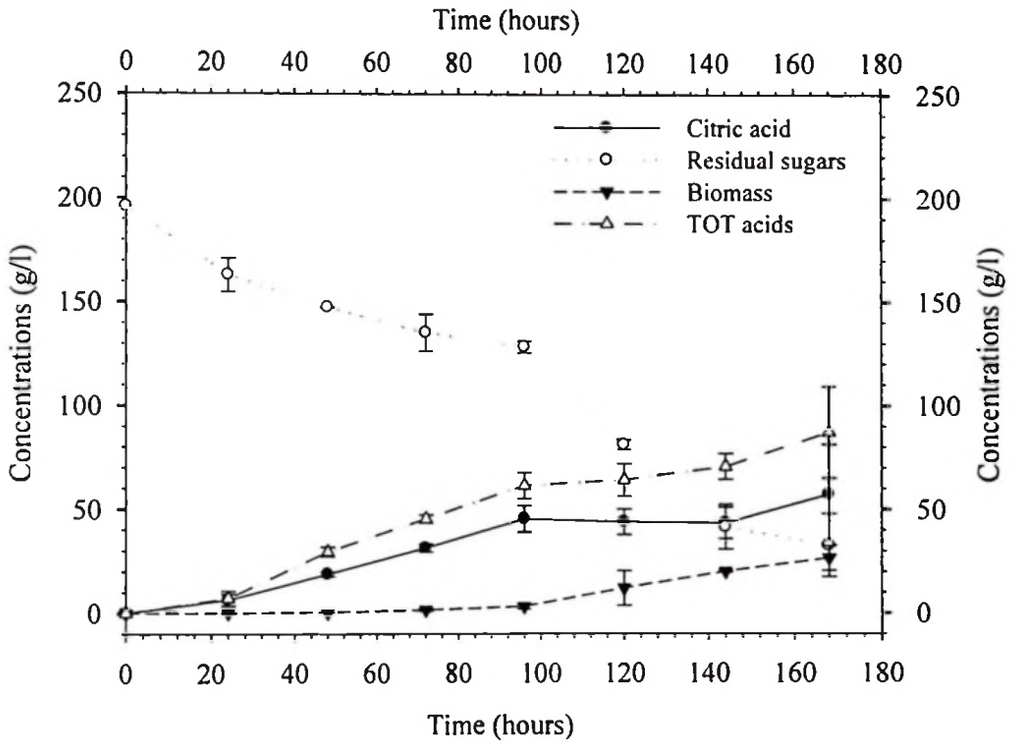

Figure 4.66; Initial hydrolysates 60% (Fructose 152 g/l) pH 3.5 no nutrients

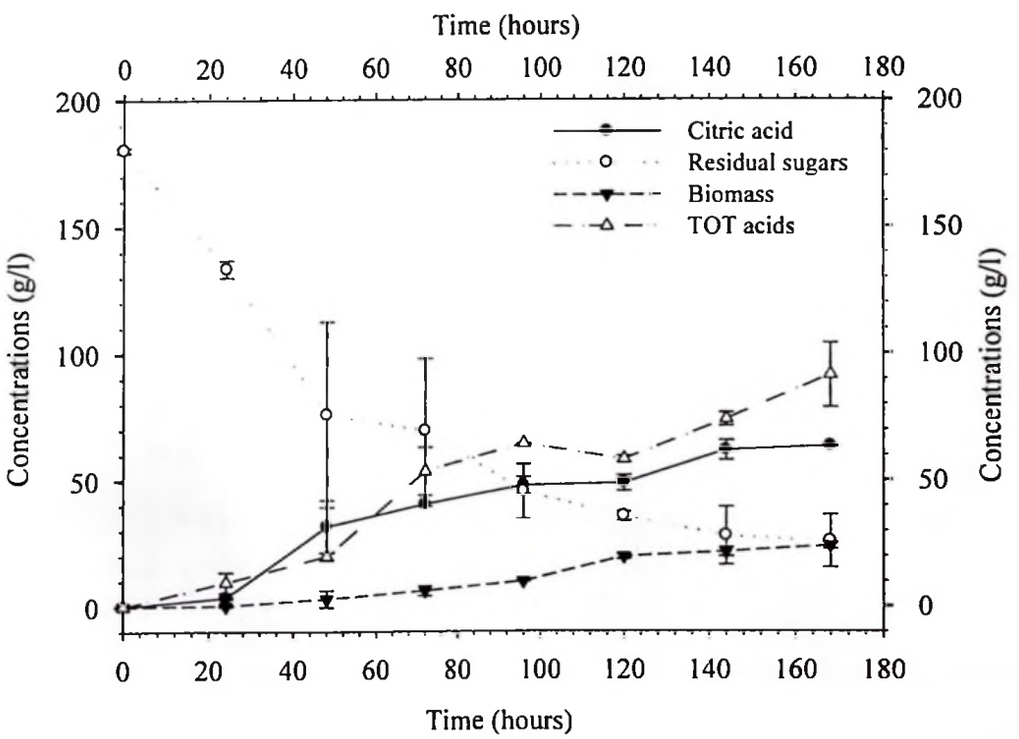

Figure 4.67: Initial hydrolysates 60% (Fructose 152 g/l) pH 5 with nutrients

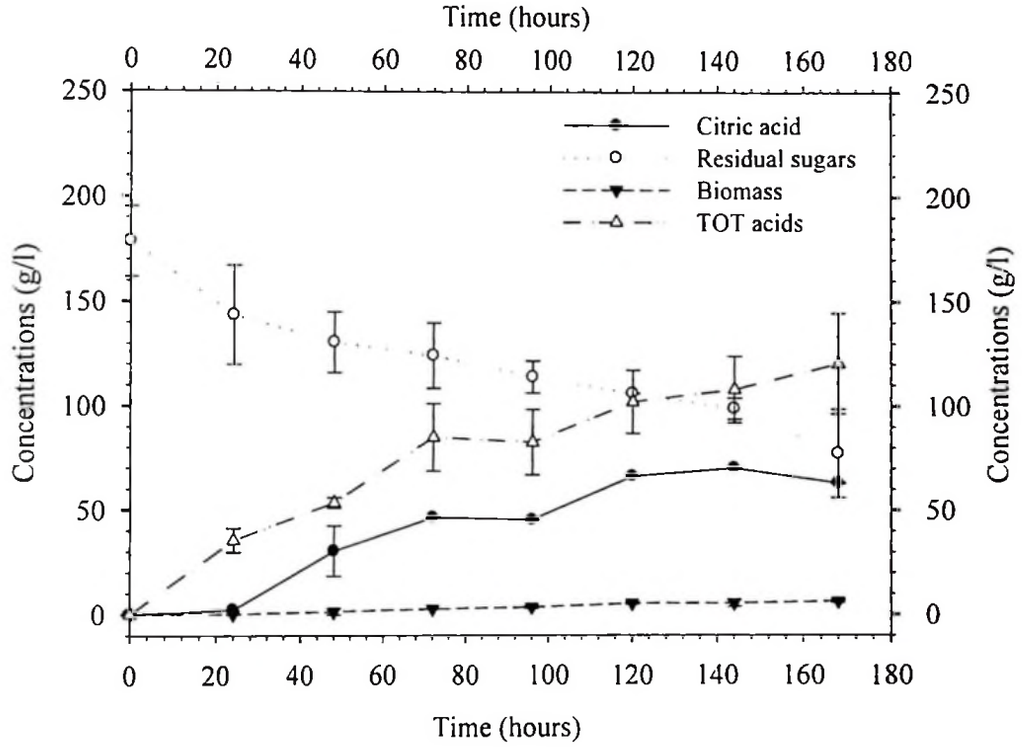

Figure 4.68: Initial hydrolysates 60% (Fructose 152 g/l) pH 5 no nutrients

Results showed that, the high citric acid yield 68.85±7.57g/l was recovered after 168 hours of the fermentation of hydrolysates-fructose with concentration value of 152 g/l, at pH 5 and without nutrients (Figure 4.68). This value corresponded to values with the low residual sugars 25.72±10.47 g/l recorded at pH 5 (Figure 4.67).

On the other hand lower citric acid yield (32.60±1.13g/l) occurred at pH 2 with nutrients added. This value corresponded with the highest residual sugars (99.49±3.76g/l). Reason for increased citric acid yields in absence of nutrients additives could be that pH and nutrients additives are both growth-limiting factors.

The metabolism of *A. niger* organism seems to be limited by either pH of the growth environment or by the combination of pH, initial sugar concentration and nutrients

additives, with the later being in the least favorable amounts (Taylor, 1934; Soule, 1986; Chen and Christensen, 1985).

4.7.3 Effects of initial fructose concentration (203g/l) and pH on citric acid

Figure 4.69 to 4.73 show time results for effect of time on citric acid production at initial fructose concentration value of 203.70g/l and initial pH values of 2, 3.5 and 5.

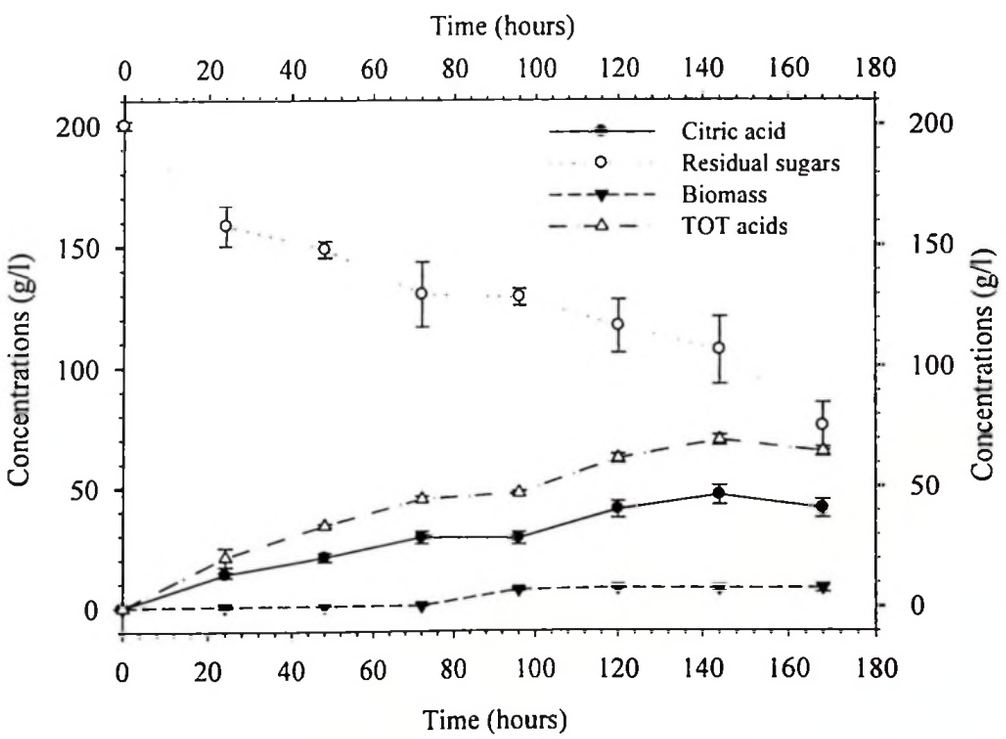

Figure 4.69: Initial hydrolysates 80% (Fructose 203 g/l) pH 2 with nutrients

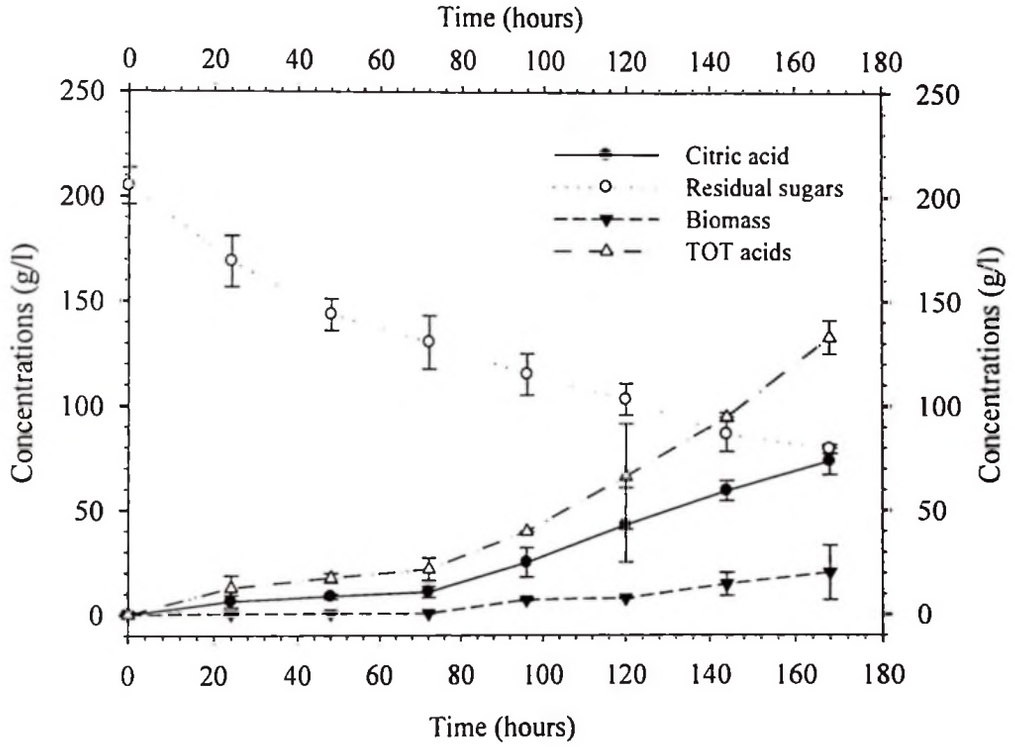

Figure 4.70: Initial hydrolysates 80% (Fructose 203 g/l) pH 2 no nutrients

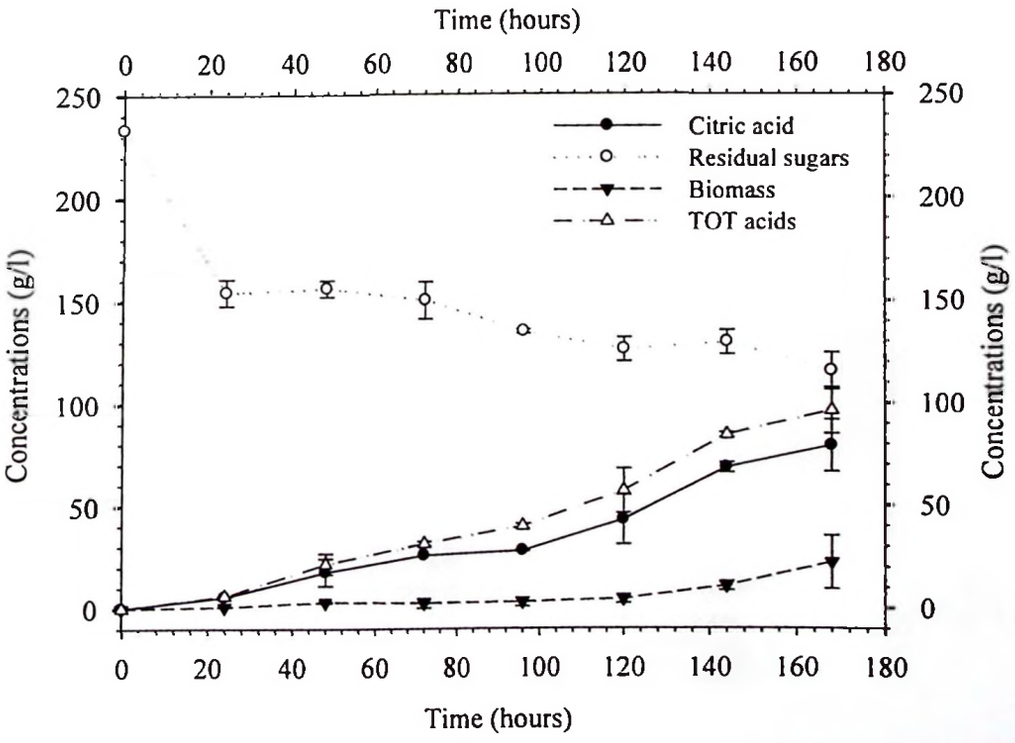

Figure 4.71: Initial hydrolysates 80% (Fructose 203 g/l) pH 3.5 with nutrients

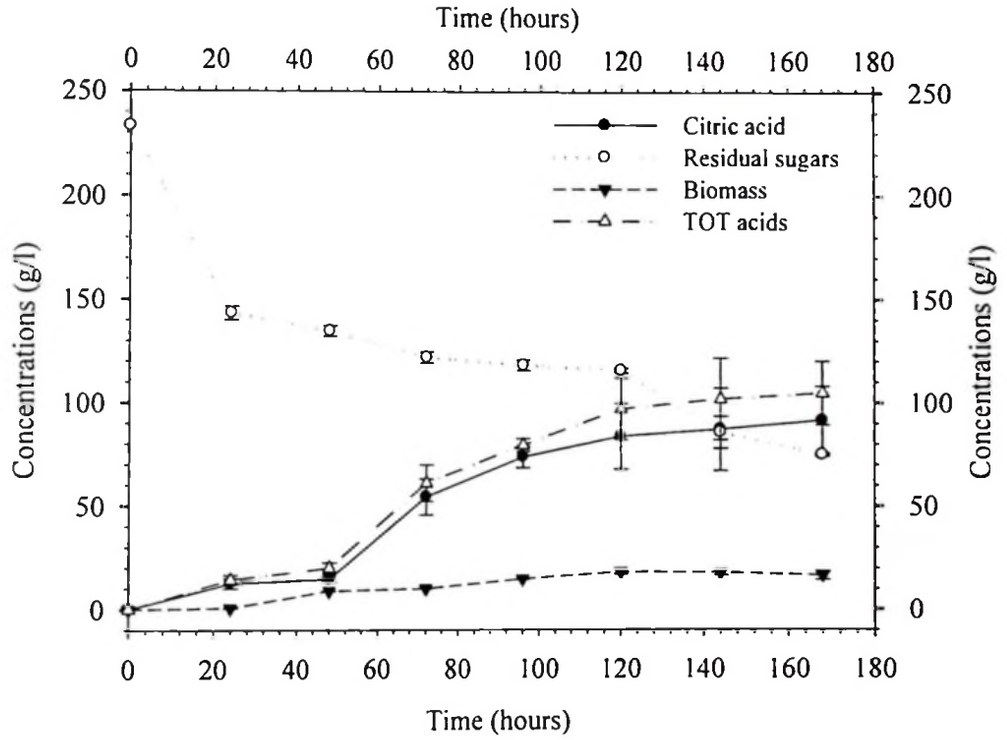

Figure 4.72: Initial hydrolysates 80% (Fructose 203 g/l) pH 3.5 no nutrients

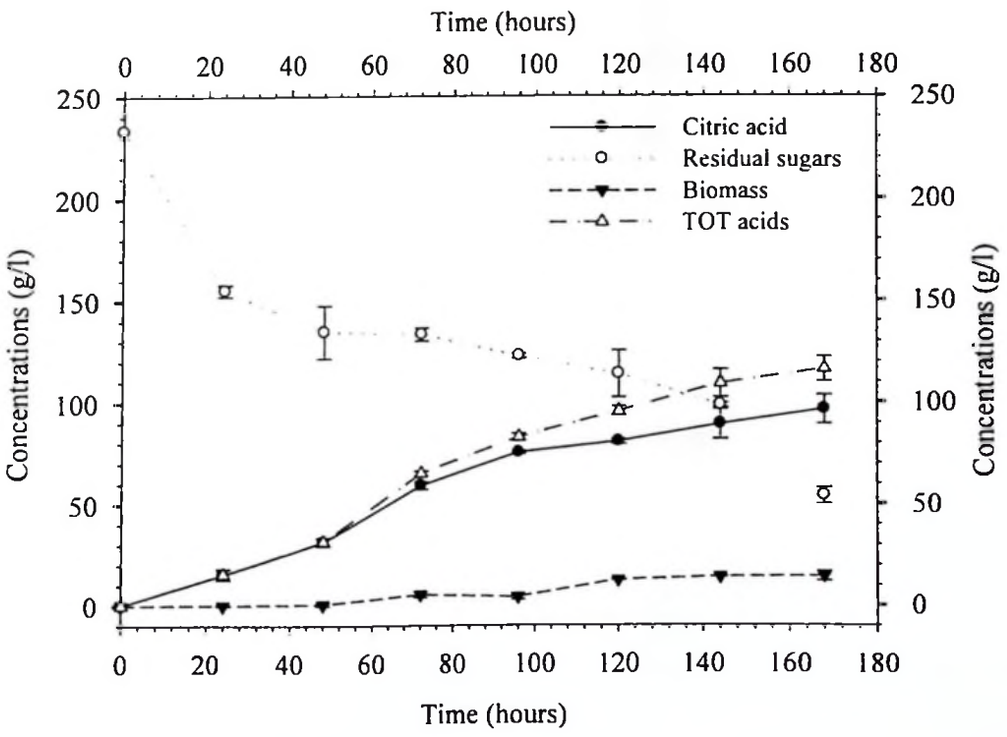

Figure 4.73: Initial hydrolysates 80% (Fructose 203 g/l) pH 5 with nutrients

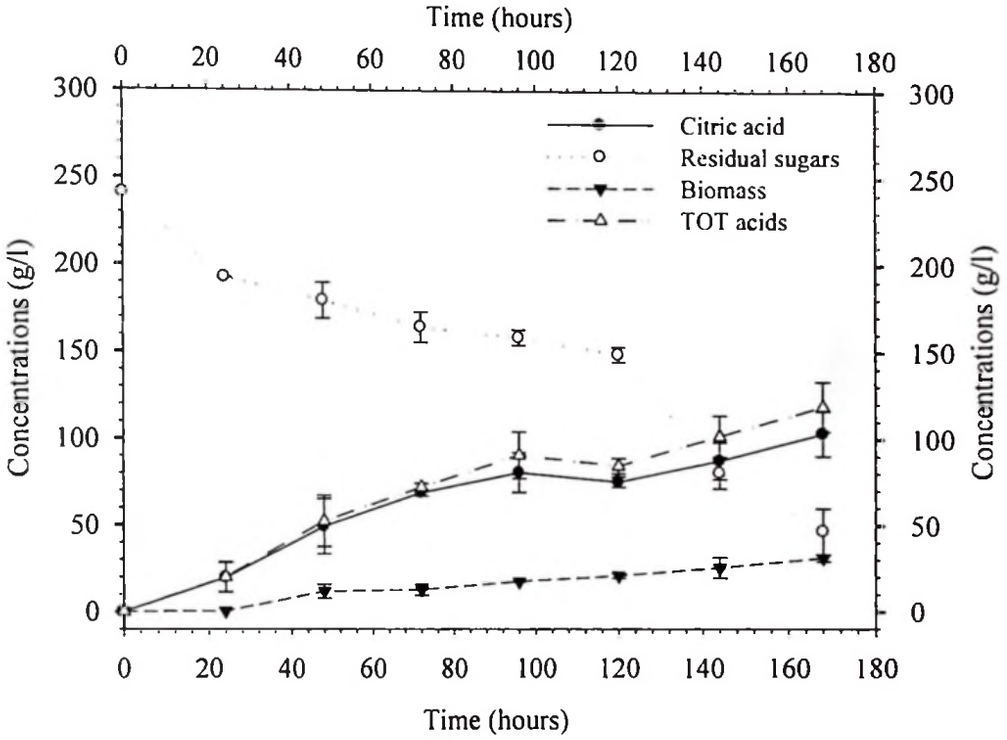

Figure 4.74: Initial hydrolysates 80% (Fructose 203 g/l) pH 5 no nutrients

Results indicated that after 168 hours of fermentation of initial fructose concentration (203.70g/l), the highest citric acid yield (103.15±13.08 g/l) occurred at pH 5 without nutrient additives. This value coincided with the lowest residual sugars (46.66±13.22 g/l) with no nutrients added (Figure 4.74). On the contrary lowest citric acid yield (40.75±3.89g/l) occurred at pH 2 with nutrients additives the value which corresponded with the high residual sugars (82.01±8.39g/l) as shown in Figure 4.67.

4.7.4 Effect of nutrient additives and pH on citric acid yield

Figure 4.75 shows effect of initial pH and nutrient addition on citric acid yield during the pilot scale fermentation processing.

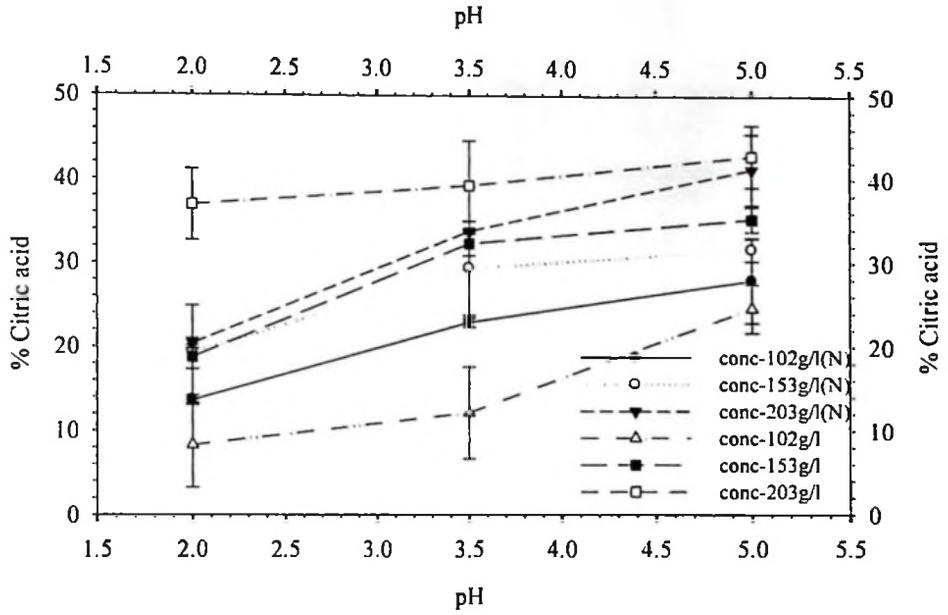

Figure 4.75: Effect of initial pH and nutrient addition on citric acid yield

The general trend in Figure 4.75 showed that higher citric acid yield was at pH 5, fructose concentration of 203g/l and without nutrient additives. Lower citric acid yield was associated with low pH 2 and nutrients additives.

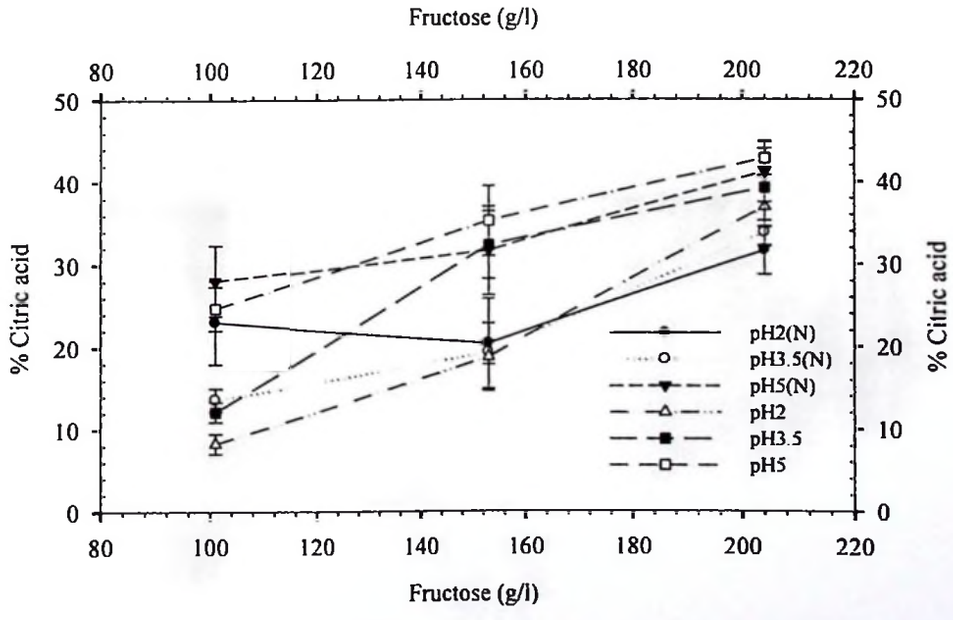

Figure 4.76: Effect of initial fructose conc. and nutrients on citric acid yield

Fermentation results in Figure 4.76 showed that, highest recovery of citric acid was obtained after employing the feedstock with hydrolysates-fructose concentration value of 203g/l without nutrient additives. The lowest citric acid yield was associated with low initial fructose concentrations value of 102 g/l and with nutrients added.

The reason for this trend could be the generic nature of sisal hydrolysates-fructose which is very reducing; when subjected to the rapid lowering of the pH at the beginning of the growth phase, the culture metabolism might have been disrupted; especially when fermentation process resulted into secretion of organic acids, which further lowers the pH.

As discussed by several for example (Torres, 1998; Pandey *et al.*, 2001), the lowering of initial pH at the beginning of fermentation production decreased the yield and the effect was dependent on type of microorganism. For that reason *A. niger* isolates used in this study, seems to be acclimatized to pH values of 5±0.2, therefore abrupt lowering of pH was suspected to disrupt its general physiology and consequently the fungal productivity (Torres, 1998; Pandey *et al.*, 2001).

4.7.5 Effect of reaction time on citric acid yield at fixed pH and nutrient additives

Figure 4.77 to 4.78 show effect of reaction time on citric acid yield at a fixed pH.

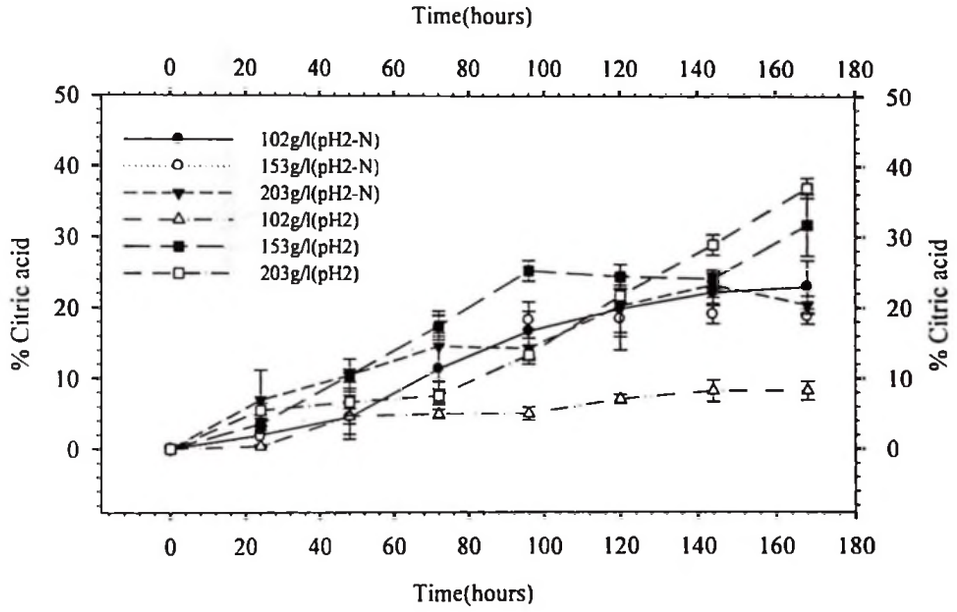

Figure 4.77: Effect of time on citric acid yield at initial pH 2

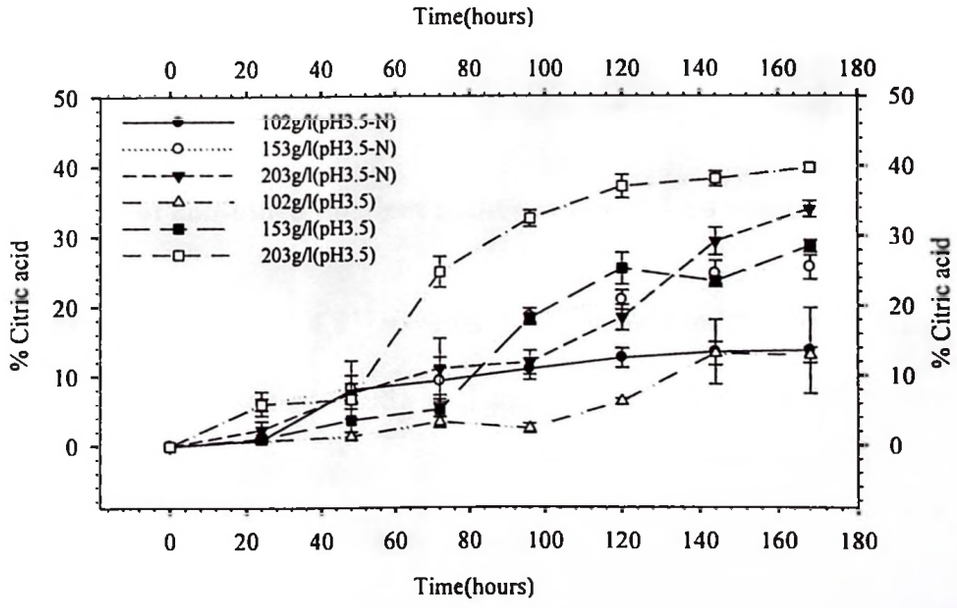

Figure 4.78: Effect of time on citric acid yield at initial pH 3.5

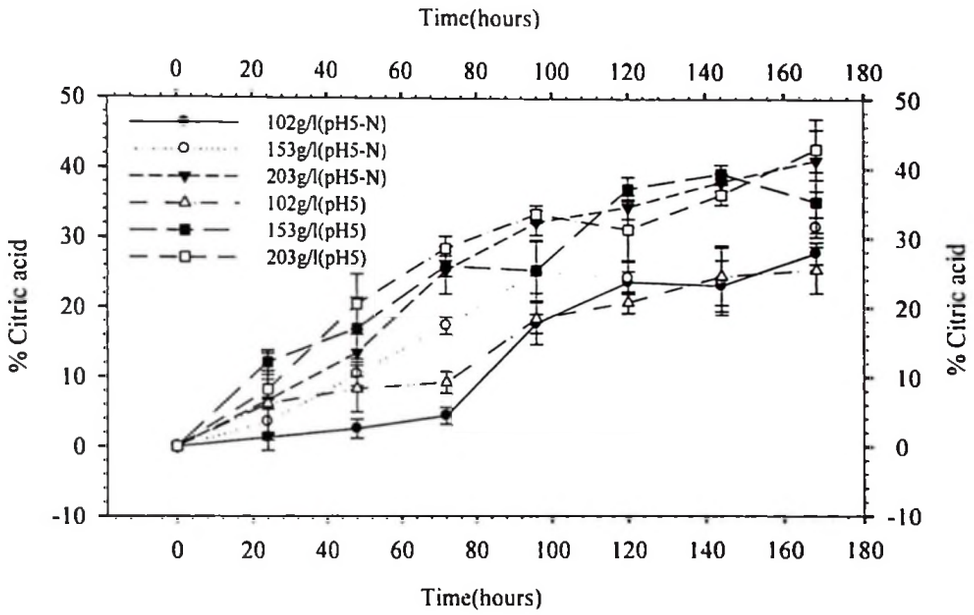

Figure 4.79: Effect of time on citric acid yield at initial pH 5

The results show that increase in reaction time resulted in higher yields, specifically when reaction pH was set at value of 5, and higher sugar concentration of 203g/l without nutrient additives.

4.7.6 Effect of combined nutrient additives and pH on yields

Surface plots (Figure 4.80 to 4.83) were used to visualize the combined effects of pH and nutrients additives on citric acid yield, and this established the desirable response values and operating conditions.

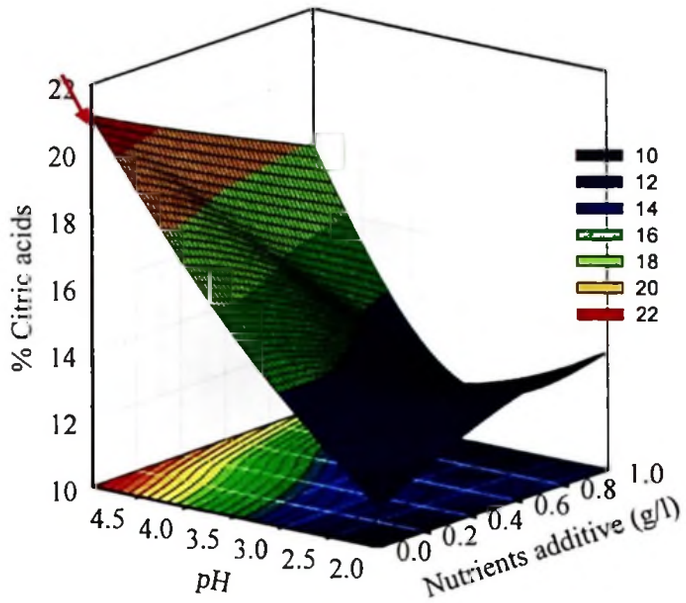

Figure 4.80: Effect of pH and nutrients addition on citric acid yield

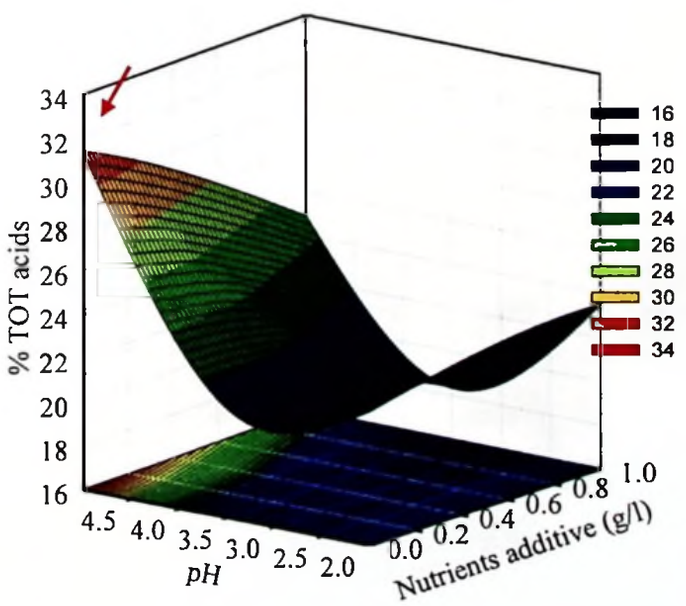

Figure 4.81: Effect of pH and nutrients addition on total acids

Evidently, citric acid yield and therefore total acid were maximized by setting pH near the value of 5 and nutrients additives near the minimum setting at a concoctions value of 0 g/l (0% nutrients additives). The yields of citric acid yield decreased as the nutrients additives increased while initial fructose concentration is held constant. Yield also decreased as the pH is held constant and the initial fructose concentration is decreased (Figure 4.80 and 4.80).

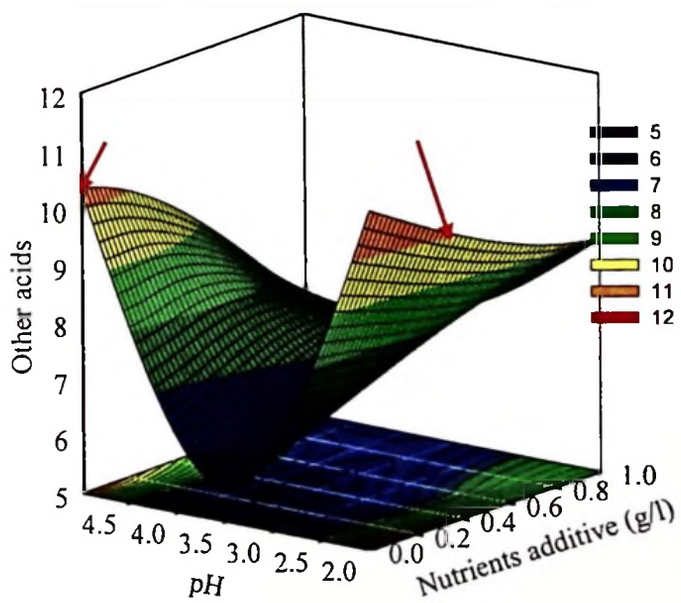

Figure 4.82: Effect of pH and nutrient addition on other acids

The plot for effect of pH and nutrient addition on other acids, assumed the hemi ellipsoid shape such that yields were maximized at edges at pH 5 and also without nutrients additives. With the same analysis, yield decreased towards pH 3 (Figure 4.82).

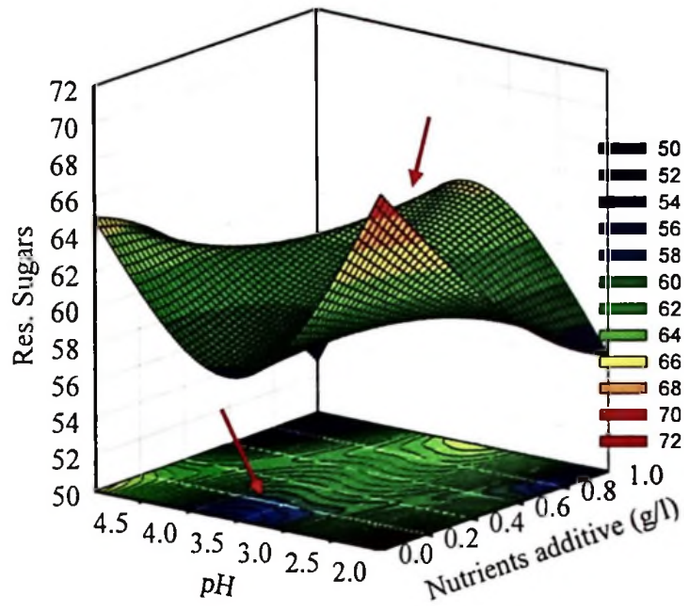

Figure 4.83: Effect of pH and nutrients addition on total residual sugars

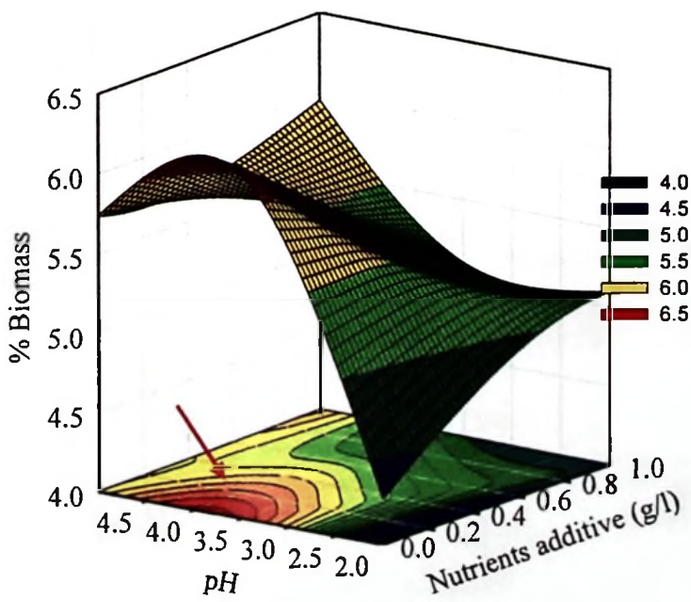

Figure 4.84: Effect of pH and nutrients addition on biomass

In addition to that

Figure 4.84 revealed that both residual sugars and biomass were minimized while citric acid yield was optimized. Further, biomass yields increased at pH 3 and without nutrients additives as residual sugars followed a similar trend.

4.7.7 Effects of reaction time on citric acid yield at fixed fructose concentrations

The results (Figure 4.85 to 4.86) show effects of reaction time on citric acid yield at fixed initial fructose concentrations. General trend showed that; combined effect of higher pH (pH 5) and initial fructose concentrations resulted in higher yield (203g/l).

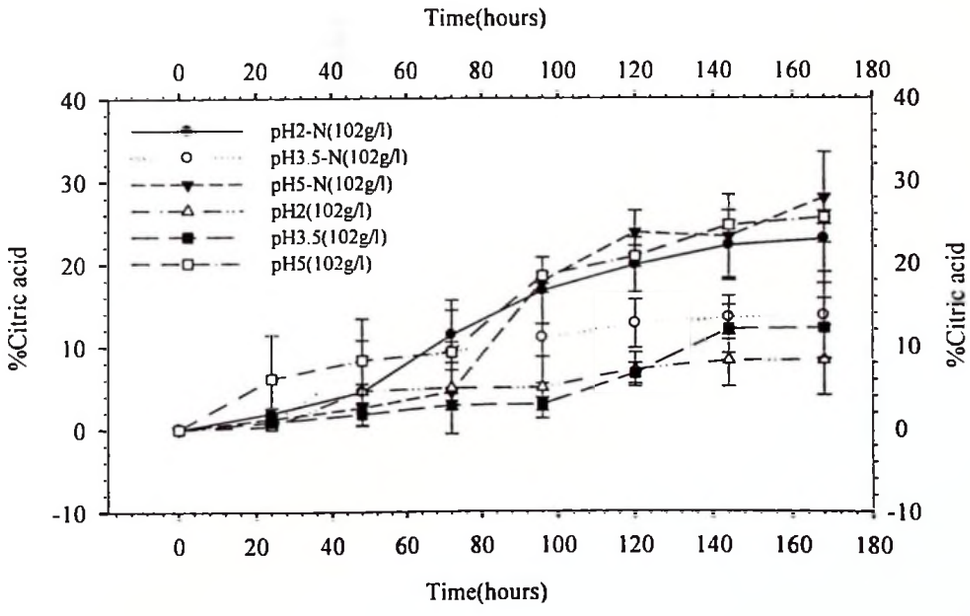

Figure 4.85: Effect of time on citric acid yield at fructose concentration 102g/l

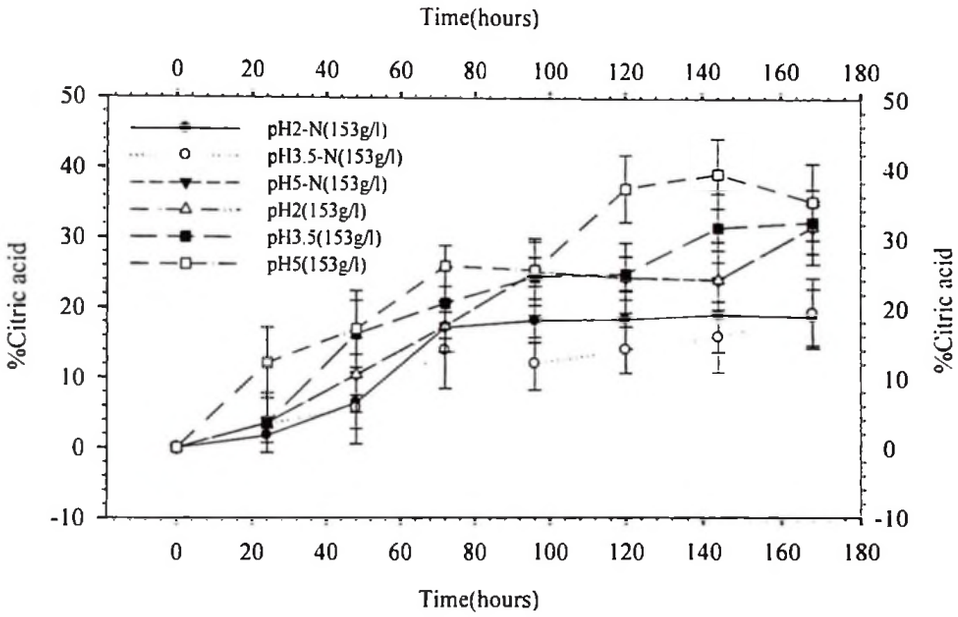

Figure 4.86: Effect of time on citric acid yield at fructose concentration 153g/l

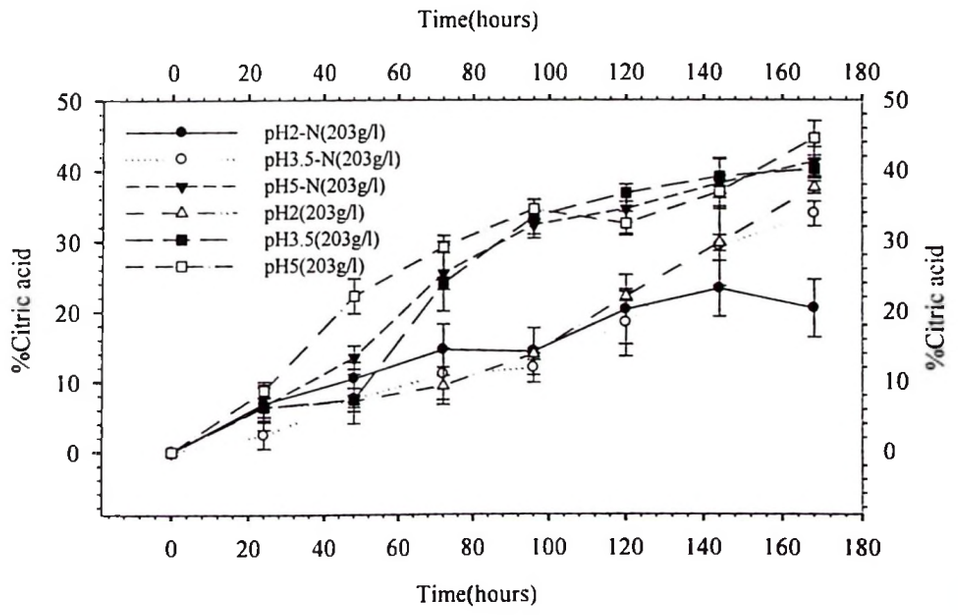

Figure 4.87: Effect of time on citric acid yield at fructose concentration 203g/l

Because of lower initial sugar concentration (102g/l) the microorganism entered the production phase after the prematurely exhaustion of food supply. Therefore, the metabolic starvation was likely causing low citric acid yields (Kubicek and Röhr, 1987; Žnidaršić and Pavko, 2001; Papagianni and Mattey 2006).

4.7.8 Effect of combined initial sugar concentration and pH on yields

Surface plots presented by (Figure 4.89 to 4.91), were used for visualizing combined effects of pH and initial hydrolysates-fructose concentration on citric acid yield, with an intention for establishing desirable response values and operating conditions at pH range of 2 to 5 and initial fructose set at concentrations range of 102 to 203g/l.

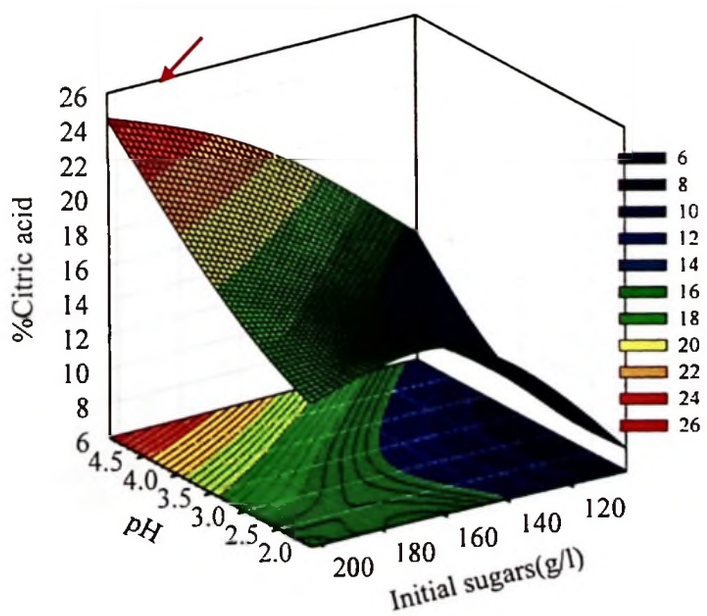

Figure 4.88: Effects of pH and initial sugar concentration on citric acid yield

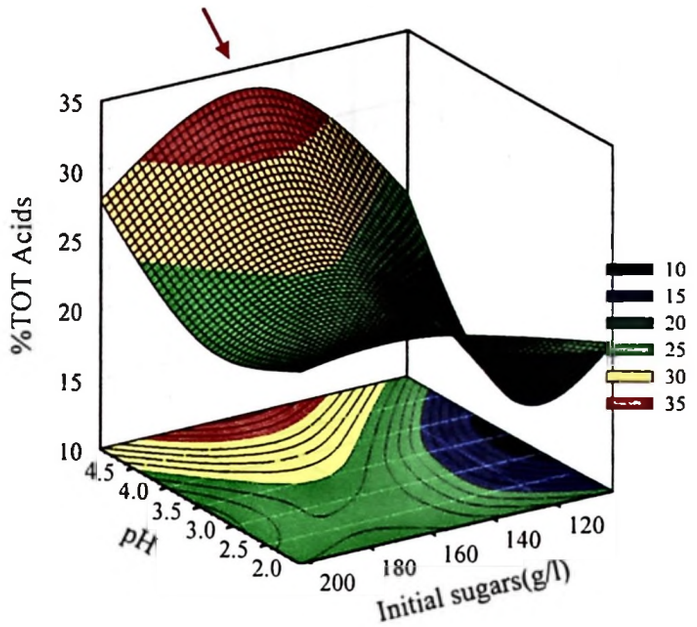

Figure 4.89: Effect of pH and initial sugar concentration on total acids

Higher citric acid yield was recovered at higher initial fructose concentrations (203g/l). The higher sugar concentrations seemed to have a positive effect on citric acid yield probably due to the fungi physiological responses to cell metabolism under excess sugars. The α-ketoglutarate dehydrogenase tends to be suppressed by presence of high concentrations of the carbon source (Hossain *et al.*, 1984; Papagianni *et al.*, 1999a, b).

Conversely, the lower initial hydrolysates-fructose concentrations resulted in lower citric acid yield. This could be as fungi experiences food scarcity; they tend to utilize almost all food resources for the cell maintenance. On the other hand, at low glucose levels, the mycelium size is reduced, and shape is affected thus interrupting citric acid metabolism at the expenses of sporullation (Röhr, 1998; Kubicek and Röhr, 1998; Röhr *et al.*, 1987; Žnidaršić and Pavko, 2001; Papagianni and Mattey 2006).

Citric acid yield and therefore total acids presented by Figure 4.88 and 4.88 were maximized by setting pH value near the maximum setting of (5) and initial sugar concentration near the maximum setting value of 203 g/l. From these settings, yield decreases with pH while the initial fructose concentration is held constant. Yield also decreases at constant pH as the initial fructose concentrations are decreased.

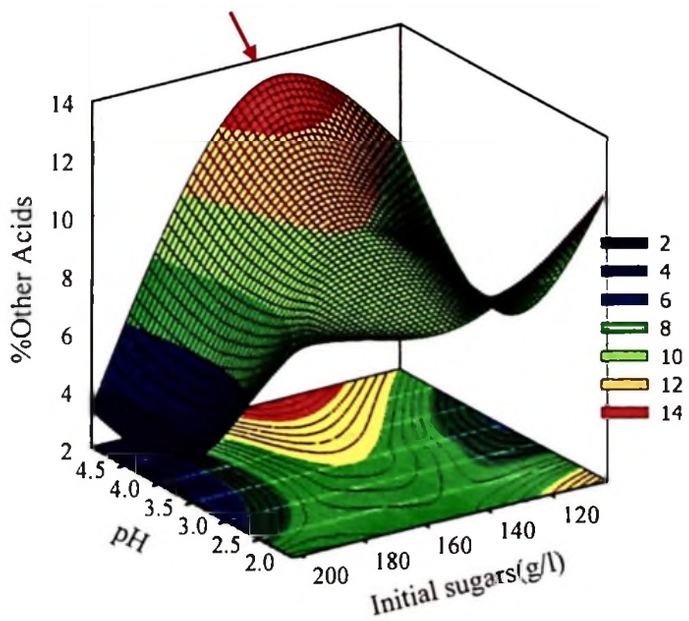

Figure 4.90: Effect of pH and initial sugar concentration on other acids

Yield for other acids maximized at edges specifically at pH 5 and initial sugars concentration (153 g/l), while yield decreased towards the pH 3 (Figure 4.90).

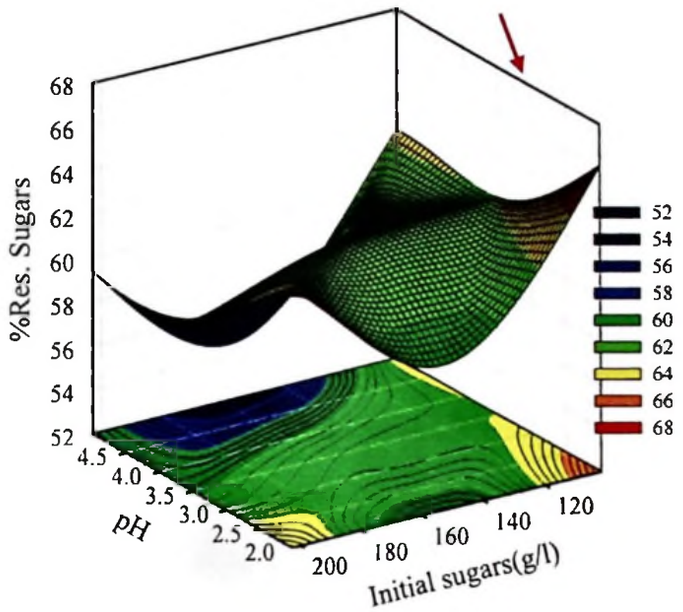

Figure 4.91: Effect of pH and initial sugar concentration on total residual sugars

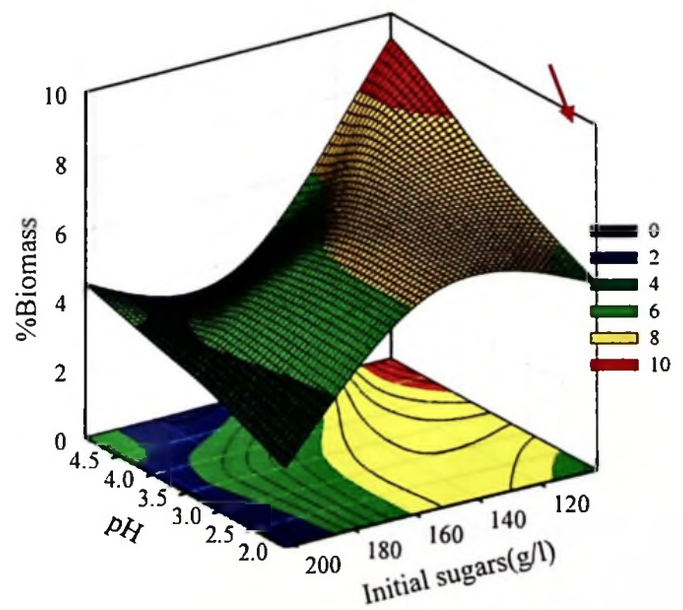

Figure 4.92: Effect of pH and initial sugar concentration on biomass

Results presented in (Figure 4.91 and 4.91) indicated that while residual sugars seemed to not to have well defined decreasing trend, an increase in biomass yield corresponded to minimum citric acid yield. This was very consistent to the findings

by Papagianni and Mattey (2006), who demonstrated that the fungi vegetation increase at the expense of lowering citric acid yield. This could be due to the fact that as fungi experiences food scarcity, they tend to utilize almost all food resources for the cell maintenance (Papagianni *et al.,* 1999b; Žnidaršić and Pavko, 2001; Papagianni and Mattey 2006).

Trend for the effect of pH, showed that, citric acid yield increased as pH 5 was approached, conversely yield decreased as pH values decreased. The reason could be the combination of the generic nature of sisal hydrolysates-fructose. The great reducing power of fructose molecules, existence of carbonyl groups under acidic environment and rapid lowering of the culture pH at the beginning of the growth phase could have limited the fungal metabolism.

A. niger used in this study seems to have been adapted to pH 5±0.2 of sisal bole juice therefore needed extended time for adaptation. From the overall pattern of surface curves, one can deduce that combined effect of higher pH, initial fructose concentration at (203g/l) without nutrients additives resulted in higher citric acid yield. While, the combination of low pH 2 and nutrients addition worsened yields.

4.7.9 Statistical modelling of fermentation conditions

Statistical modelling of effects of initial fermentation conditions after 144 hours of citric acid production are presented in Table 4.13 to 4.14.

Table 4.13: Responses for 2^3 design for citric acid fermentation (experimental and predicted yields)

		factors			Responses at 144 hours					
S/Ord	R/Ord	pH	IF	Nut	Citric acid		Res. Fructose		Biomass	
		X_1	X_2	X_3	Y_1	Y_1'	Y_2	Y_2'	Y_3	Y_3'
					actual	Fit	actual	Fit	actual	Fit
8	1	5	203	1	76.24	78.08	106.43	96.61	10.86	16.06
3	2	2	203	-1	39.99	44.40	141.52	135.16	1.97	6.01
16	3	5	203	1	79.92	78.08	86.78	96.61	21.26	16.06
11	4	2	203	-1	48.81	44.40	128.80	135.16	10.04	6.01
10	5	5	102	-1	29.17	28.60	61.43	51.61	17.90	18.15
18	6	3.5	153	0	50.02	51.13	88.98	88.33	10.37	10.76
9	7	2	102	-1	26.08	22.64	19.70	27.85	16.34	15.43
2	8	5	102	-1	28.03	28.60	41.79	51.00	18.40	18.15
4	9	5	203	-1	84.96	84.34	73.99	86.51	29.72	29.31
6	10	5	102	1	27.90	27.09	45.80	41.95	18.70	17.75
1	11	2	102	-1	19.20	22.64	36.00	27.85	14.51	15.43
12	12	5	203	-1	83.72	84.34	99.02	86.51	28.90	29.31
14	13	5	102	1	26.28	27.09	38.10	41.95	16.80	17.75
15	14	2	203	1	49.24	48.80	116.43	106.56	8.62	7.57
17	15	3.5	153	0	52.24	51.13	87.68	88.33	11.15	10.76
13	16	2	102	1	17.50	17.10	69.90	69.20	8.60	7.55
7	17	2	203	1	48.35	48.80	96.69	106.56	6.51	7.57
5	18	2	102	1	16.70	17.10	68.50	69.20	6.50	7.55

S/Ord = Std Order; R/Ord = Run Order; IF = Initial fructose; Res = Residual; Nut = Nutrients additives

Citric acid S = 2.89215, R-Sq = 99.18%, R-Sq(adj) = 98.45%;

Residual sugars S = 11.2508, R-Sq = 94.17%, R-Sq(adj) = 88,98%;

Biomass S = 3.25437, R-Sq = 89.99%, R-Sq(adj) = 81. 10%

Table 4.14: Estimated coefficients for citric acid using data in uncoded units

Parameter	Coef
Constant	5.49368
pH	-6.30841
Init.Fructose	0.0888201
Nutrients	-14.0718
pH*Init.Fructose	0.0879092
pH*Nutrients	3.14306
Init.Fructose*Nutrients	0.0976320
pH*Init.Fructose*Nutrients	-0.0242294
CtPt	7.24913

Equation for citric acid production Model

$$Y_1 = \begin{bmatrix} 5.49368 + (-6.30841)X_1 + (0.0888201)X_2 + (-14.0718)X_3 + \\ (0.0879092)X_1X_2 + (3.14306)X_1X_3 + (0.0976320)X_2X_3 \\ + (-0.0242294)X_1X_2X_3 + 7.2491 \end{bmatrix} \quad [32]$$

Table 4.15: Factorial fit for citric acid yield versus parameters "estimated effects and coefficients for citric acid (coded units)"

Parameter	Effect	Coef	SECoef	T	P	
Constant		43.88	0.72	60.69	0.00	***sig**
pH	21.29	10.65	0.72	14.72	0.00	***sig**
Init.Fructose	40.05	20.02	0.72	27.69	0.00	***sig**
Nutrients	-2.23	-1.12	0.72	-1.54	0.16	***N-sig**
pH*Init.Fructose	13.32	6.66	0.72	9.21	0.00	***sig**
pH*Nutrients	-1.66	-0.83	0.72	-1.14	0.28	***N-sig**
Init.Fructose*Nutrients	1.30	0.65	0.72	0.90	0.39	***N-sig**
pH*Init.Fructose*Nutrients	-3.67	-1.84	0.72	-2.54	0.03	***sig**
CtPt		7.249	2.1691	3.34	0.009	***sig**

*** sig** = significant at alpha = 0.05; *** N-sig** = Not-significant at alpha = 0.05

S = 2.89215 R-Sq = 99.18% R-Sq(adj) = 98.45%

The estimated effects and coefficients presented by Table 4.15, indicated that p-values associated with each individual general linear model parameters i.e. two main interactions initial sugar concentration and initial pH; had a significant effect on citric acid yield both with values (p = 0.000). The other main interaction named nutrients additives had no significant effect (p = 0.16).

Only one two-way interaction initial pH* initial sugar concentration had a significant effect with values (p = 0.000), while the other two-way interactions namely pH*Nutrients and Init.Fructose*Nutrients had no significant effects with (p) values 0.28 and 0.39 respectively. The three main effects initial pH*initial fructose*initial nutrients value was significant with (p = 0.03).

Table 4.16: ANOVA -Analysis of Variance for citric acid (coded units)

Source	DF	SeqSS	AdjSS	AdjMS	F	P	
Main Effects	3	8248.460	8248.460	2749.490	328.710	0.000	***sig**
2-WayInteractions	3	727.190	727.190	242.400	28.980	0.000	***sig**
3-WayInteractions	1	53.900	53.900	53.900	6.440	0.032	***sig**
Curvature	1	93.420	93.420	93.420	11.170	0.009	***sig**
Residual Error	9	75.280	75.280	8.360			
Pure Error	9	75.280	75.280	8.360			
Total	17	9198.240					

* sig = significant at alpha = 0.05; * N-sig = Not-significant at alpha = 0.05

The sequential sums of squares (Seq SS) and adjusted sums of squares (Adj SS) shown in the ANOVA Table 4.16 were the same therefore the model was orthogonal and did not contain covariates. The analysis of variance table presented block effect

for both the main and two way interactions. Also as was justified by Figure 4.93 to 4.95; that effect for the main, two way and three way interactions suggested that citric acid yield is affected by initial sisal bole hydrolysates-fructose and pH while Nutrient additives have less effect.

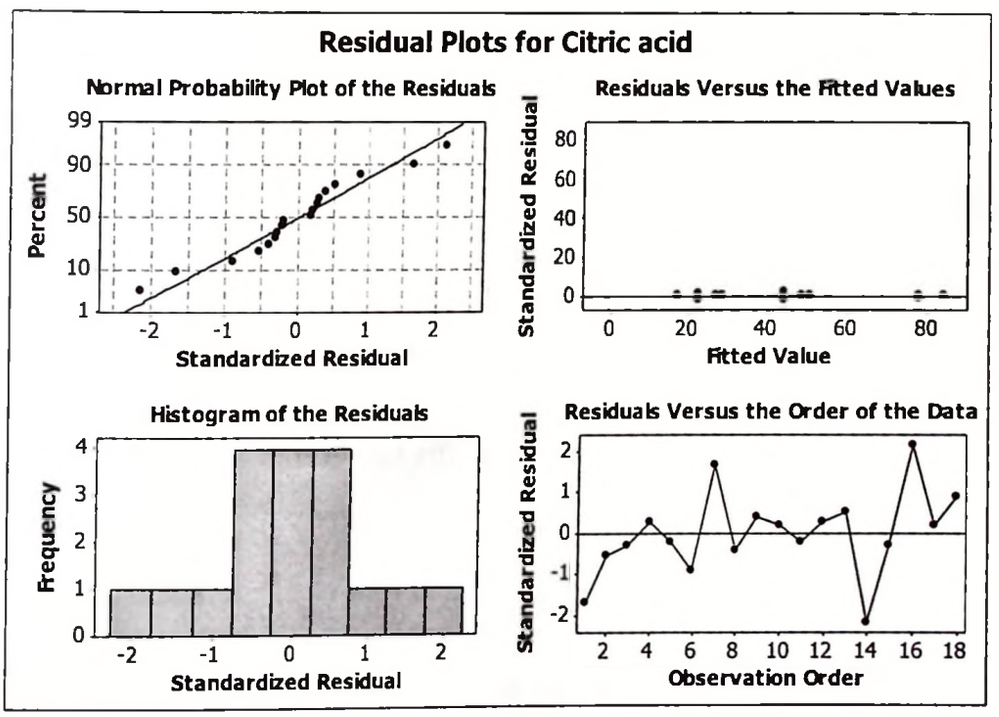

Figure 4.93: four-in-one residual plot for citric acid

The four-in-one residual plot in Figure 4.93 indicated that both effects are important, thus justified that the nature and source of residual error and pure errors; are not due to the lack of fit.

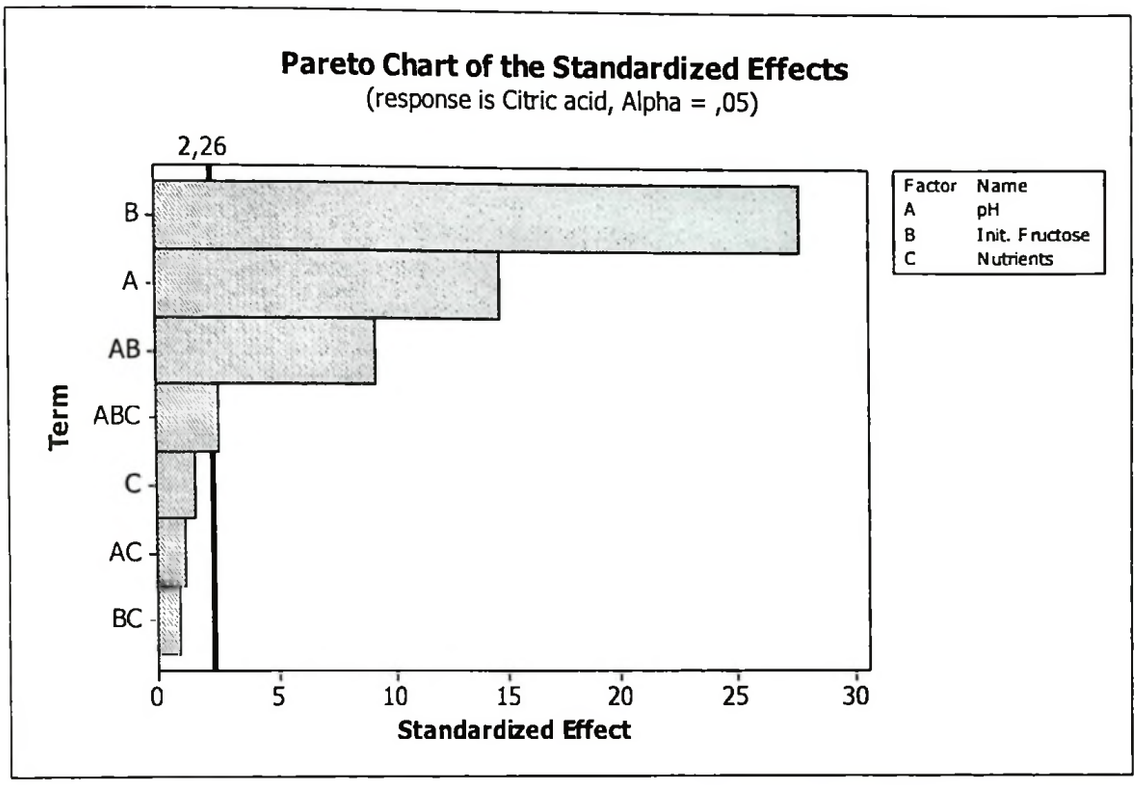

Figure 4.94: Pareto effects plot effects interaction paretro plot for citric acid

Pareto chart of the effects on Citric acid yield is presented by Figure 4.94, where as the effects of, initial fructose concentration, initial pH, nutrients; and combined pH and fructose concentration extended past the reference line at significance α -level of 0.05. These results showed that the largest effect was coming from initial fructose concentration, followed by initial pH. The combination of initial fructose concentration and pH also had significant effect at α value of 0.05. There was no significant effect to citric acid yield from effects of neither nutrients additives nor the combinations of nutrients additives with either initial fructose concentration or initial pH at α -level of 0.05 (Neter *et al.*, 1993; Miller, 1997).

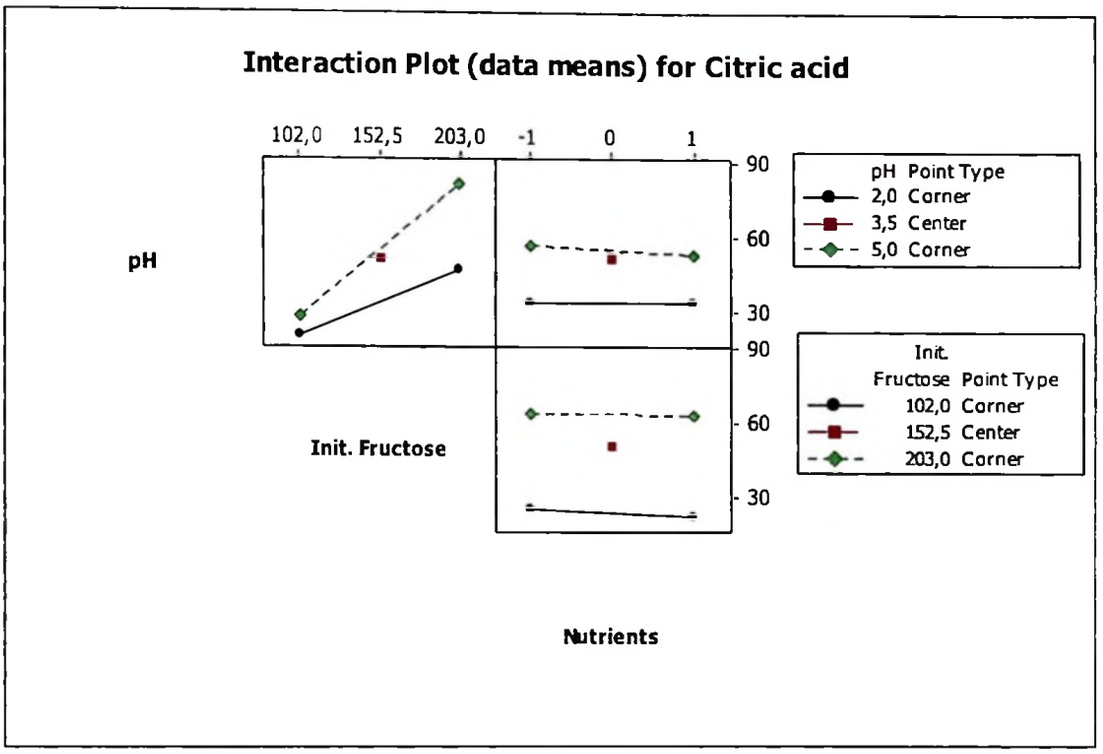

Figure 4.95: Interaction Plot for citric acid

The interaction plot (Figure 4.95) indicated the enormous degree of interaction, is justified by interaction lines that were not parallel. As great departures from parallel occurred when combining the interactions from combining pH and initial fructose concentrations, the increase in yield occurred when initial sugar concentration was high (203g/l). Less effect was observed when combining either pH or initial fructose concentration with nutrients additives as shown by parallel lines.

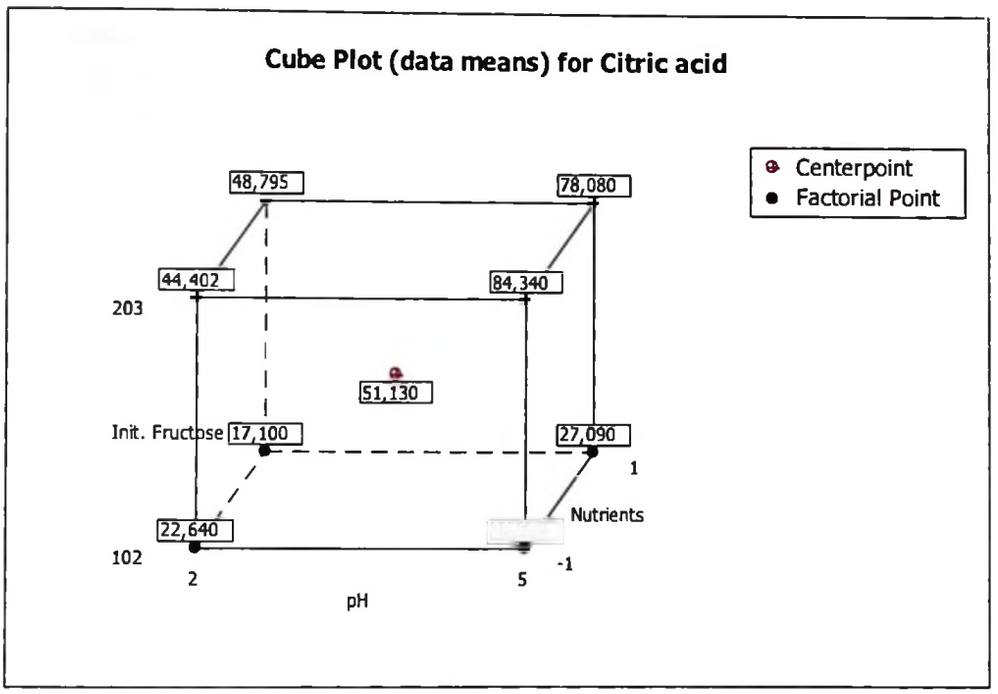

Figure 4.96: Cube plot (data means) for citric acid yield

Supported by p-values in **Table 4.15** and 4.16, corners of the cube plot Figure 4.96; showed that high yields are obtained at initial fructose (203 g/l), without nutrients additives and at pH 5. Therefore the cube plot, ANOVA, interaction effects and by using General Linear Model (GLM), Multiple Comparisons-Tukey Method indicated that the null hypothesis (initial claim) is true as justified by levels of p-values at alpha (α) the levels of significance of 0.05 (Tukey, 1993; Jones and Tukey, 2000; Lehmann and Romano, 2005; McCloskey, 2008). As the null hypothesis was not rejected then it was justified that the decrease in the initial concentration of sisal bole hydrolysates-fructose, pH and nutrients additives lowered citric acid yield, for example as you move towards the corners specifically towards sugar concentration of 102g/l, pH 2 and with nutrients additives the lowest yield is achieved.

4.7.10 Predicting citric acid yield at the pilot scale

Shown by Table 4.17 are the constraints for optimization of initial medium components; initial pH conditions, initial fructose concentration values (g/l sisal juice hydrolysates) Nutrient solution (g/l sisal juice hydrolysates) for a Pilot scale citric acid fermentation processing after 144 hours fermentation.

Table 4.17: Constraints for optimization of citric acid yield from sisal bole juice-fructose (SBJ-F) medium composition up to 144 hours

variables	Goal	Lower limit	Upper limit
pH	-	2	5
Initial fructose (g/l SBJ-F)	-	102	203
Nutrient solution (g/l SBJ-F)	-	-1	1
Citric acid yield (g/l SBJ-F) at 144 hours	maximize	16.70	84.96

Table 4.18: Optimum levels of initial conditions and predicted yields by Minitab V15 numerical optimization FFD after 144 hours of fermentation

	Initial			Response(g/lSBJ-F)		
Solution number	pH	SBJ-Fructose	Nutrients	Citric acid	Res. Fructose	Biomass
	X_1	X_1	X_1	Y_1	Y_1	Y_1
1	**5**	**203**	**-1**	**84.340 (41.55%)**	**86.505**	**29.310**
2	5	203	1	78.080 (38.46%)	96.605	16.060
3	3.5	152.5	0	51.130 (33.53%)	88.330	10.760
4	2	203	1	48.784 (24.03%)	106.560	7.565

As was seen in Table 4.17 and 4.18, universal solution indicated that optimization occurred at initial pH 5, initial fructose concentration 203g/l SBJ-fructose without nutrients addition.

Analysis of solutions 1 to 4 was done and predicted value for citric acid was 84.34g/l SBJ-fructose, which is closer to the actual value by 0.73%. When expressed as a percentage this value correspond to 41.55%, which is far less by 26.45% from the theoretical yield value of 68% as was indicated in Table 2.1. Predicted value for residual fructose at 144 hours was 86.50 g/l SBJ-fructose, which is lower by 38.875% meaning that fructose, is used for metabolic processes without affecting yield. However, this value reflects that values of sugars left un-assimilated probably because of fungal cells exhaustion or premature deaths.

Biomass formation also slightly increases by 1.38% meaning that more growth without compromising yields. However, an increase in biomass values suggested the successions (contaminations) by invasive microorganisms metabolising the remaining sugars. This tendency has been justified by emergence of other acids e.g. lactic acid specifically beyond 144 hours of fermentation and sharp decreasing in residual sugars. Concurrently values of total acids increased while citric acid profiles remains stable seen in Figure 4.88 to 4.91.

4.7.11 Initial fermentation conditions on citric acid yield

Based on ANOVA and the overall pattern of curves; one can deduce that, the null hypothesis is valid, that is the decrease in initial sisal inulin concentration, pH and nutrients additives decreases citric acid yield. The combined effect of higher pH and initial fructose concentrations (203g/l) resulted in higher citric acid yield. This is

because exposing *A. niger* to high concentrations of sugar, the intracellular concentration of fructose-6-phosphate. Fructose-6-phosphateis an important enzyme in carboxylic acid cycle (Arts *et al.*, 1987; Kubicek-Pranz *et al.*, 1990; Legiša and Bencina, 1994; Habison, *et al.*, 2006; Legiša and Mattey, 2007).

Simultaneously, the excess of sugar concentrations shifts the fructose-6-phosphateformation step downward to glycerinaldehyde-3-phosphatedehydrogenase (Peksel *et al.*, 2002) thereby enabling increased rate of carbon uptake and channeling more of it into glycolysis. Further crucially important cytosolic pyruvate carboxylase, is also induced by high carbohydrate concentrations, thus the rate of citric acid accumulation increases (Torres *et al.*, 1996, Hossain *et al.*, 1984; Papagianni *et al.*, 1999a, b; Peksel *et al.*, 2002).

Yields from sisal bole inulin substrates were fairly good and concurred with results from study by Tran *et al.*, (1998), which indicated that yields from inulin are usually 20-30% (63-72% yield) lowers than from sucrose the traditional substrate (90% yield). Slightly lower values are typical as from findings by Hossain *et al.*, (1984), on the effect of the sugar source in citric acid fermentation.

4.8 Important Findings

1. Sisal boles are rich in microbe biodiversity, most of which have a fermentative potential. In this research, *A. niger* was isolated and its performance compared with the commercial strain. When compared with commercial strains the local isolate performed equally well as the industrial DMSZ 8 strain.

2. Sisal boles consisted of inulin rich carbohydrates, which were successfully hydrolysed prior fermentation processing. The current study investigated effects of combined acid and cooking hydrolysis. The ideal hydrolysis conditions suggested were; the pH 3 and temperature of 115±5°C for 120 minutes. This temperature was ideal as it corresponds to the sterilization temperature and scanty maillard compounds are likely at this range. Results obtained were used for the optimization of production of fructose yield for citric acid processing.

3. Citric acid production via fermentation process using glucose or fructose is well documented (Shuler and Kargi, 2001; Ruijter *et al.*, 2002). Production of the acid using sisal juice inulin *Agave* hybrid H 11648, which is composed of high levels of fructose, has been sparingly studied. Masalla, (2003), did citric acid process-development studies in small volumes of fermentation media not exceeding 250ml; his findings needed supporting data from larger scale (Seletzky *et al.*, 2007. Scaling up is feasible as conditions studied at the current pilot scale (10 liters) generated enough data for the scaling up towards the industrial scale using indigenous *A. niger*.

CHAPTER FIVE

CONCLUSSION AND RECOMMENDATION

5.1 Conclusions

Based on the results obtained in this study on the feasibility of citric acid production by fermentation of sisal bole juice, the following are the conclusions:

(a) Sisal bole rots are very rich in microorganisms (fungi, yeasts, moulds and bacteria) the later, which comprised of some motile endo spore forming bacteria (coccid and rods);

(b) The microorganisms identified were *Brevundimonas diminuta sp, Shewanella putrefaciens sp, Brevundimonas vesicularis sp* and *Pasteurella sp*; however, these were not intensively studied in this work. Four major groups of fungi and moulds isolates were established as *Aspergilla sp* = (36±2) %, *Penicillin* = (28±2) %, *Yeast* = (15±2) % and *Fusarium* = (10±2) %. While the dominant *Aspergilli* species observed were *A. nidulans*, *A. tamari* and *A. niger* in ratios of (3:2:2) respectively;

(c) *A. niger* species generic to sisal boles which was inferred as fungi BYF CPE-KT, was effective in processing citric acid and the performance was comparable to commercial strain DMSZ 8. As fructose was being utilized in citric acid production, isolated fungi species produced better yield which

disadvantage of introducing strong mineral acids that would tamper with the fungi general physiology. However, prolonged hydrolysis time beyond 120 minutes while maintaining lower temperatures 70-80°C and higher pH 4-5; increased total hydrolysates yield. This seemed to be the best solution whenever issues of sterility and time are not crucial. Results indicated that prolonged heat treatment at this temperature range did not only break down complex carbohydrate molecules, but also enhanced hydrolysis with minimum mallard reactions.

(g) Based on the results of the hydrolysis experiments, two independent variables for the full factorial 2^2 design (initial pH and temperature) was subjected to the multiple regression analysis using Minitab (Version 15). A polynomial model presented by equation [31], developed from statistical results as, presented in Table 4.7 to 4.10). Regression-Square (adjusted) = 99.81% indicated that the model could explain up to 99.81% of the variations observed in fructose production;

(h) The factorial fit for fructose yield vs. pH, temperature, estimated effects (coded units) indicated that both the main and two-way interactions effects were significant (p=0.000) at confidence interval (CI) of 95%. The sequential sums of squares (Seq SS) and adjusted sums of squares (Adj SS) shown in the ANOVA (Table 4.10) were the same which justified that the model was orthogonal. These outstanding statistical results proved the feasibility of production of fructose rich feedstock's from sisal bole inulin by fermentation;

(i) In the absence of nutrients additives, the combined effect of higher initial hydrolysates-fructose concentration (203g/l) and higher initial pH 5 resulted in the highest citric acid yield 46.66% (103.15±13.08 g/l). However, at the end of fermentation (after 168 hours) larger amounts of residual hydrolysates-fructose (46.66±13.22 g/l) and retarded biomass growth (31.18±2.06g/l) was evident. This suggested premature fungal cells fatigue, which could be due to inflicted pellets disintegrations and incomplete fermentation processing. Concurrently minimum citric acid yield (9.70±2.40 g /l) was obtained at pH 2, low initial fructose concentration of 102 g/l and nutrient additives; whereas residual hydrolysates-fructose (25.72±10.46g/l) and comparatively high biomass value (17.94±1.74g/l) were recovered;

(j) High biomass recovery at low initial sugar concentrations justified that the specific production rate decreased in favor of the specific growth rate so that the available carbon source was utilized solemnly for cell growth leaving scanty carbon levels for citric acid accumulations;

(k) In this set of experiment, there was no controlled supply of oxygen. A magnet stirrer was used to promote air dispersion in the broth. An "on-off" temperature control was also used. Thus, the temperature varied around the set point, which was set ambient temperatures (30±2 ^{o}C) during the months of April to August. The results showed that Oxygen uptake rate was between 38 and 49±2 mmol O_2/l-hour at the 36 to 144 fermentation hours respectively. The temperature setting was not altered to compensate for

possible temperature drop during the night for fear of stressing out the fungi. As a result, night temperatures seemed to drop to about ±5 °C, which could have some effect to fungal metabolism.

(l) In this study, fructose was the main carbon source; although glucose was also present in the substrate, still glucose and fructose are not equivalent substrates in citric acid accumulation by *A. niger*. In fact, the uptake of fructose in the *A. niger* strains turned out to be the yield-limiting factor. Despite to the fact that mechanism of fructose utilization and metabolism by *A. niger* is reported to be a complex phenomenon, in this work the uptake phenomenon for maximization of fructose metabolism was not thoroughly investigated. This observation might particularly be important to consider in future when complex cultivation media e.g. inulin hydrolysates are used;

(m) The utilization of fructose by *A. niger* which rapidly undergoes mallard's reactions at lower pH was somewhat slowed, consequently at the end of fermentation large amounts of total residual hydrolysates were observed. As the main carbon source (unlike glucose), inhibition of the uptake of fructose, competitions by alien microorganism and consumptions by mallards/reactions especially at elevated concentration of citric acid were likely the cause of this inhibition;

(n) Based on the results of the Fermentation experiments; three independent variables for the full factorial 2^3 design (initial fructose-hydrolysates concentration, initial pH and nutrients additives); were subjected to multiple

regression analysis using Minitab (Version 15). Initial fructose concentration and initial pH were found to be significant (Table 4.13 to 4.16) and using these observations, a polynomial model equation [32] was developed. Regression-Square (adjusted) = 98.45% indicated that the model could explain up to 98.45% of the variations observed in citric acid production;

(o) From the factorial fit for citric acid yield vs. initial fructose-hydrolysates concentration, initial pH and nutrients additives, The estimated effects and coefficients presented by Table 4.14, indicated that p-values associated with two main interactions from initial sugar concentration and initial pH; had a significant effect on citric acid yield both with values (p=0.000). Also, the main interaction from nutrients additives had no significant effect (p=0.16). A two-way interactions effects between pH and Initial fructose concentration (pH*Initial fructose concentration) was significant (p=0.000), whereas three way interaction of pH, Initial Fructose concentration and Nutrients additive (pH*Initial Fructose concentration *Nutrients additive) was also significant (p=0.009), at confidence interval (CI) of 95%. The sequential sums of squares (Seq SS) and adjusted sums of squares (Adj SS) shown in the ANOVA (Table 4.16) were the same therefore the model was orthogonal. Therefore, it is possible to conclude that the current results established the viability of utilization of sisal bole juice–Inulin as an alternative chemical feedstock in citric acid production.

5.2 Recommendations

The successful utilization of sisal bole juice–Inulin as an alternative chemical feedstock in citric acid production, and further that the Inulin rich feedstock from sisal boles could be used to produce other valuable products thus adding value to sisal industry. However, additional study is required to clarify limitations, such as:

(a) To further, explore the potential of bio-diversified microorganism from sisal boles for use in fermentation processing. Since *A. niger* strains utilized fructose as carbon source in citric acid fermentation it is possible that alternative fructose presents an alternative transport system for metabolism, further studies regarding productivity of indigenous strains is important;

(b) Minimize the effects of micronutrients (such as Fe, Mn and Zn) during fermentation process. A use of inert processing equipment in juice production is a possibility, as sisal juice is acidic in nature of juice there will always be a possibility that some metallic species would end up being dissolved (especially unwanted manganese). Effect of other nutrients such as salt addition would help identify the main limiting nutrients;

(c) To establish the effect of genotype;

(d) The acid catalyzed hydrolysis process needs to be optimized (yield basis);

(e) To improve citric acid fermentation by introducing both temperature control and air supply (dissolved oxygen levels);

(f) Unravel the biochemical mechanisms controlling fructose transport under complex cultivation media is required. However, as indigenous strains showed high competence, there is a need for further studies regarding either sequencing of *A. niger* strains generic to sisal boles or by mutagenic the parent stock that has already been proven competent in fructose utilization for future industrial productions. This might particularly be of interest to indentify strains that would give similar yields at a relatively shorter fermentation time;

(g) Determine the effect of fructose uptake by sisal bole rot *A. niger* in conjunction with citric acid formation. Non-completely utilized residual fructose, suggest an integration of production of other carboxylic acids for example lactic acid. Alternatively, biogas or/and bio fertiliser could be processed from the residual substrate and the polyols formed. However the strategy for controlling premature fungal cells fatigue which lead to the recovery of larger amounts of residual hydrolysates-fructose at the end of fermentation would be to introduce a fresh *A. niger* cultures midway (semi continuous fed batch).

(h) Scale up fermentation experiments to volumes as large as 100L (larger than 10 litres) as pre-cursor for moving into industrial scale production. The set up must use industrial type of controls such as agitation speed, temperature, pH and DO, with real time sampling to follow up the reaction.

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APPENDIX I: Historia ya Uzalishaji Katani Na Bodi ya Mkonge Tanzania

MWAKA	TANI	MWAKA	TANI	MWAKA	TANI	MWAKA	TANI
1898	0,6	1930	49962	1962	214000	1994	28902
1899	0,6	1931	55939	1963	214000	1995	25022
1900	7,5	1932	60554	1964	230000	1996	22180
1901	1,5	1933	69600	1965	214000	1997	21400
1902	225	1934	54246	1966	222000	1998	23228
1903	350	1935	62048	1967	216000	1999	20584
1904	800	1936	67450	1968	193783	2000	20489
1905	1400	1937	83171	1969	209180	2001	23542
1906	1600	1938	103192	1970	202180	2002	23641
1907	3000	1939	103376	1971	181108	2003	23858
1908	4000	1940	101810	1972	156850	2004	26958
1909	4800	1941	81041	1973	155407	2005	27794
1910	7000	1942	99061	1974	143442	2006	30934
1911	10000	1943	102436	1975	127840	2007	
1912	17080	1944	111228	1976	119077	2008	
1913	20835	1945	112218	1977	105018	2009	
1914	11175	1946	106806	1978	91873	2010	
1915	Vita Kuu	1947	105548	1979	81384	2011	
1916	Vita Kuu	1948	120662	1980	85978	2012	
1917	3344	199	123296	1981	73753	2013	
1918	7954	1950	121598	1982	60635	2014	
1919	16744	1951	145223	1983	46187	2015	
1920	16663	1952	162185	1984	38255	2016	
1921	7923	1953	165725	1985	32270	2017	
1922	10224	1954	178250	1986	30151	2018	
1923	12845	1955	176499	1987	33170	2019	
1924	18428	1956	185762	1988	33268	2020	
1925	18276	1957	184877	1989	32265	2021	
1926	25022	1958	196567	1990	33743	2022	
1927	33012	1959	205273	1991	36001	2023	
1928	36186	1960	204868	1992	24303	2024	
1929	45728	1961	198000	1993	29601	2025	

APPENDIX II: Table 3a: Tanzania Sisal Board Sisal Projections Revised Projected Land Development and Fibre Production 2006-2015

CUTTING CYCLE					1	2	3	4	5
METRES/HECTARE					36.00	46.00	46.00	45.00	45.00
METRES/TON					50.00	45.00	36.00	30.00	25.00
TONS/HECTARE					0.73	1.03	1.29	1.50	1.80
YEAR					2006	2007	2008	2009	2010
	PLANTING[HA]								
		MATURE	IMMATURE	TOTAL					
PRODUCTION IN METRES									
1989- 1999	18 915	0	0	18 915	596 109	478 197	450 068	421 938	337 552
2000	2 012	0	18 915	20 927	90 357	90 357	85 601	76 090	66 579
2001	3 531	0	20 927	24 458	163 582	158 574	158 474	150 228	133 536
2002	3 610	18 915	5 542	28 067	167 241	167 241	162 122	162 122	153 589
2003	3 373	20 927	7 141	31 441	122 655	156 262	156 262	151 478	151 478
2004	3 129	24 458	6 983	34 570		113 782	144 958	144 958	140 521
2005	3 671	28 068	6 502	38 241			133 491	170 067	170 068
2006	10 000	31 441	13 671	55 112				363 636	463 273
2007	15 000	34 570	25 000	74 570					545 455
2008	20 000	38 241	35 000	93 241					
2009	20 000	48 241	40 000	108 241					
2010	20 000	63 241	40 000	123 241					
2011	20 000	83 241	40 000	143 241					
2012	20 000	84 326	40 000	144 326					
2013	20 000	102 314	40 000	162 314					
2014	20 000	118 783	40 000	178 783					
2015	20 000	135 173	40 000	195 173					
TOTAL METRE CUT					1 139 944	1 164 413	1 291 076	1 640 518	2 162 049

Tanzania Sisal Board Sisal Projections Revised Projected Land Development and Fibre Production 2006-2015(Continues)

YEAR					2006	2007	2008	2009	2010
	PLANTING[HA]								
		MATURE	IMMATURE	TOTAL					
PRODUCTION IN TONS									
1989 -1999	18 915	0	0	18 915	16 559	13 406	12 617	11 828	9 463
2000	2 012	0	18 915	20 927	3 012	3 614	4 280	3 043	2 466
2001	3 531	0	20 927	24 458	4 544	4 286	6 343	7 511	5 341
2002	3 610	18 915	5 542	28 067	3 716	4 646	5 404	6 485	7 679
2003	3 373	20 927	7 141	31 441	2 453	3 472	4 341	5 049	6 059
2004	3 129	24 458	6 983	34 570		2 276	3 221	4 027	4 684
2005	3 671	28 068	6 502	38 241			2 670	3 779	4 724
2006	10 000	31 441	13 671	55 112				7 273	10 295
2007	15 000	34 570	25 000	74 570					10 909
2008	20 000	38 241	35 000	93 241					
2009	20 000	48 241	40 000	108 241					
2010	20 000	63 241	40 000	123 241					
2011	20 000	83 241	40 000	143 241					
2012	20 000	84 326	40 000	144 326					
2013	20 000	102 314	40 000	162 314					
2014	20 000	118 783	40 000	178 783					
2015	20 000	135 173	40 000	195 173					

Tanzania Sisal Board Sisal Projections Revised Projected Land Development and Fibre Production 2006-2015 (Continues)

Year	2006	2007	2008	2009	2010
Fibre potential[tons]	30 284	32 700	36 876	48 996	61 621
Recovery line fibre	1	1	1	1	1
Recovery flume tow	0	0	0	0	0
Line fibre [tons]	27 256	29 430	34 988	44 096	55 459
Flume tow[tons]	2 726	2 943	3 499	4 410	5 546
Projected waste production(tons)	726 816	784 791	933 015	1 175 894	1 478 911
Projected electricity production(mws)	8	9	10	13	16
Matured plants	31 441	34 570	38 241	48 241	63 241
Projected sisal bole production (pcs)x10^6	251.5	276.6	305.9	385.9	505.9
Projected sisal bole production (kgs)x10^6	6 288.2	6 914.0	7 648.2	9 648.2	12 648.2
Projected sisal bole sugars inulin production potential (mts)x10^6	1 886.5	2 074.2	2 294.5	2 894.5	3 794.5
Projected citric acid production potential(mts)x10^6	1 622.4	1 783.8	1 973.2	2 489.2	3 263.2

Tanzania Sisal Board Sisal Projections Revised Projected Land Development and Fibre Production 2006-2015 (Continues)

Cutting cycle					6	7	8	9	10
Metres/hectare					43.00	38.00	33.00	28.00	32.00
Metres/ton					20.00	25.00	27.00	30.00	36.00
Tons/hectare					2.13	1.51	1.23	0.95	0.88
Year					2011	2012	2013	2014	2015
	Planting[HA]								
		Mature	Immature	Total					
Production in metres									
1989- 1999	18 915	0	0	18 915					
2000	2 012	0	18 915	20 927	57 068	63 408			
2001	3 531	0	20 927	24 458	116 844	100 152	111 280		
2002	3 610	18 915	5 542	28 067	136 524	119 458	102 393	113 740	
2003	3 373	20 927	7 141	31 441	143 506	127 561	111 616	95 671	106 301
2004	3 129	24 458	6 983	34 570	140 521	133 125	118 333	103 511	88 750
2005	3 671	28 068	6 502	38 241	164 861	164 861	156 184	138 871	121 477
2006	10 000	31 441	13 671	55 112	463 273	449 091	449 091	425 455	378 182
2007	15 000	34 570	25 000	74 570	694 909	694 909	673 636	673 626	638 182
2008	20 000	38 241	35 000	93 241	727 273	926 545	926 545	898 182	898 182
2009	20 000	48 241	40 000	108 241		727 273	926 545	926 545	898 182
2010	20 000	63 241	40 000	123 241			727 273	926 545	926 545
2011	20 000	83 241	40 000	143 241				727 273	926 545
2012	20 000	84 326	40 000	144 326					727 273
2013	20 000	102 314	40 000	162 314					
2014	20 000	118 783	40 000	178 783					
2015	20 000	135 173	40 000	195 173					
Total metre cut					2 644 777	3 506 384	4 302 897	5 029 449	5 709 618

Tanzania Sisal Board Sisal Projections Revised Projected Land Development and Fibre Production 2006-2015 (Continues)

Year					2011	2012	2013	2014	2015
	Planting[HA]								
		Mature	Immature	Total					
Production in tons									
1989 -1999	18 915	0	0	18 915					
2000	2 012	0	18 915	20 927	1 902	1 761			
2001	3 531	0	20 927	24 458	4 328	3 338	3 091		
2002	3 610	18 915	5 542	28 067	5 461	4 424	3 413	3 160	
2003	3 373	20 927	7 141	31 441	7 175	5 102	4 134	3 189	2 953
2004	3 129	24 458	6 983	34 570	5 621	6 656	4 733	3 135	2 958
2005	3 671	28 068	6 502	38 241	5 495	6 594	7 809	5 563	4 499
2006	10 000	31 441	13 671	55 112	12 869	14 970	17 964	21 273	15 127
2007	15 000	34 570	25 000	74 570	15 442	19 303	22 455	26 945	31 909
2008	20 000	38 241	35 000	93 241	14 545	20 590	25 737	29 939	35 927
2009	20 000	48 241	40 000	108 241		14 545	20 590	25 737	29 939
2010	20 000	63 241	40 000	123 241			14 545	20 540	25 737
2011	20 000	83 241	40 000	143 241				14 545	20 590
2012	20 000	84 326	40 000	144 326					14 545
2013	20 000	102 314	40 000	162 314					
2014	20 000	118 783	40 000	178 783					
2015	20 000	135 173	40 000	195 173					

Tanzania Sisal Board Sisal Projections Revised Projected Land Development and Fibre Production 2006-2015 (Continues)

Year	2011	2012	2013	2014	2015
Fibre potential[tons]	72 839	97 285	124 472	154 758	184 186
Recovery line fibre	1	1	1	1	1
Recovery flume tow	0	0	0	0	0
Line fibre [tons]	65 555	87 557	112 024	139 291	165 767
Flume tow[tons]	6 555	8 756	11 202	13 929	16 577
Projected waste production(tons)	1 748 131	2 334 848	2 987 318	3 714 424	4 420 464
Projected electricity production(MWs)	19	26	33	41	49
Matured plants	83 241	84 326	102 314	118 783	135 173
Projected sisal bole production (PCs)x10^6	665.9	674.6	818.5	950.3	1 081.4
Projected sisal bole production (KGs)x10^6	16 648.2	16 865.2	20 462.8	23 756.6	27 034.6
Projected sisal bole sugars inulin production potential (MTs)x10^6	4 994.5	5 059.6	6 138.8	7 127.0	8 110.4
Projected citric acid production potential(MTs)x10^6	4 295.2	4 351.2	5 279.4	6 129.2	6 974.9

APPENDIX III: Data-Hydrolysis at Different pH and Oven Temperature 30-80°C

Time	Temp	pH	Inulin	Sucrose	Glucose	Fructose	TOT-hydrolysates	TOT sugars
(hr)	(°C)		(g/100mL)	(g/100mL)	(g/100mL)	(g/100mL)	(g/100mL)	(g/100mL)
0	30	2	18.17	0.00	0.00	0.00	0.00	18.17
30	30	2	16.13	0.00	0.00	1.17	1.17	17.30
60	30	2	15.26	0.00	0.00	2.00	2.00	17.27
90	30	2	8.52	0.00	0.00	9.45	9.45	17.97
120	30	2	8.13	0.00	0.00	9.49	9.49	17.62
150	30	2	7.79	0.00	0.00	9.10	9.10	16.89
330	30	2	7.63	0.00	0.00	9.42	9.42	17.05
0	30	3	18.17	0.00	0.00	0.00	0.00	18.17
30	30	3	16.59	0.00	0.00	0.00	0.00	16.59
60	30	3	17.19	0.00	0.00	0.30	0.30	17.49
90	30	3	17.09	0.00	0.00	0.84	0.84	17.93
120	30	3	16.46	0.00	0.00	1.51	1.51	17.97
150	30	3	16.20	0.00	0.00	1.95	1.95	18.15
330	30	3	15.50	0.00	0.00	2.01	2.01	17.51
0	30	4	18.17	0.00	0.00	0.00	0.00	18.17
30	30	4	18.17	0.00	0.00	0.00	0.00	18.17
60	30	4	17.78	0.00	0.00	0.00	0.00	17.78
90	30	4	16.90	0.00	0.00	0.73	0.73	17.62
120	30	4	17.03	0.00	0.00	0.53	0.53	17.57
150	30	4	16.98	0.00	0.00	0.75	0.75	17.72
330	30	4	16.59	0.00	0.00	0.70	0.70	17.29
0	30	5	18.17	0.00	0.00	0.00	0.00	18.17
30	30	5	18.18	0.00	0.00	0.00	0.00	18.18
60	30	5	18.13	0.00	0.00	0.00	0.00	18.13
90	30	5	17.38	0.00	0.00	0.00	0.00	17.38
120	30	5	17.69	0.00	0.00	0.00	0.00	17.69
150	30	5	16.30	0.00	0.00	0.77	0.77	17.07

Data-Hydrolysis at Different pH and Oven Temperature 30-80°C (continues)

330	30	5	16.29	0.00	0.00	0.81	0.81	17.10
0	60	2	18.17	0.00	0.00	0.00	0.00	18.17
30	60	2	15.65	0.00	0.00	0.77	0.77	16.43
60	60	2	12.23	0.00	0.00	4.84	4.84	17.07
90	60	2	6.95	0.00	0.00	10.31	10.31	17.26
120	60	2	5.85	0.00	0.00	12.76	12.76	18.61
150	60	2	2.50	0.00	0.66	13.26	13.92	16.42
330	60	2	1.65	0.00	0.75	14.52	15.28	16.93
0	60	3	18.17	0.00	0.00	0.00	0.00	18.17
30	60	3	17.21	0.00	0.00	0.00	0.00	17.21
60	60	3	15.74	0.00	0.00	0.00	0.00	15.74
90	60	3	15.91	0.00	0.00	2.49	2.49	18.40
120	60	3	10.70	0.00	0.00	4.48	4.48	15.18
150	60	3	10.18	0.00	0.00	6.79	6.79	16.96
330	60	3	9.95	0.00	0.00	8.21	8.21	18.16
0	60	4	18.17	0.00	0.00	0.00	0.00	18.17
30	60	4	17.88	0.00	0.00	0.00	0.00	17.88
60	60	4	16.81	0.00	0.00	0.60	0.60	17.42
90	60	4	16.53	0.00	0.00	0.68	0.68	17.21
120	60	4	15.76	0.00	0.00	0.87	0.87	16.63
150	60	4	16.37	0.00	0.00	0.96	0.96	17.33
330	60	4	16.05	0.00	0.00	1.04	1.04	17.09
0	60	5	18.17	0.00	0.00	0.00	0.00	18.17
30	60	5	18.18	0.00	0.00	0.00	0.00	18.18
60	60	5	17.36	0.00	0.00	0.00	0.00	17.36
90	60	5	16.60	0.00	0.00	0.72	0.72	17.32
120	60	5	17.14	0.00	0.00	0.81	0.81	17.95
150	60	5	16.37	0.00	0.00	0.71	0.71	17.08
330	60	5	16.04	0.00	0.00	1.83	1.83	17.87
0	70	2	18.17	0.00	0.00	0.00	0.00	18.17
30	70	2	15.26	0.00	0.00	3.48	3.48	18.74

Data-Hydrolysis at Different pH and Oven Temperature 30-80°C (continues)

60	70	2	7.59	0.00	0.00	10.70	10.70	18.29
90	70	2	6.00	0.00	0.00	11.54	11.54	17.54
120	70	2	4.09	3.30	0.00	12.21	15.52	19.60
150	70	2	1.65	2.60	0.00	13.99	16.59	18.24
330	70	2	0.60	2.15	0.00	16.28	18.42	19.03
0	70	3	18.17	0.00	0.00	0.00	0.00	18.17
30	70	3	15.72	0.00	0.00	0.00	0.00	15.72
60	70	3	12.48	0.00	0.00	3.13	3.13	15.61
90	70	3	12.01	0.00	0.00	3.15	3.15	15.16
120	70	3	10.06	0.00	0.00	5.47	5.47	15.53
150	70	3	8.46	0.00	0.00	7.57	7.57	16.03
330	70	3	6.64	0.00	0.00	8.66	8.66	15.30
0	70	4	18.17	0.00	0.00	0.00	0.00	18.17
30	70	4	17.98	0.00	0.00	0.00	0.00	17.98
60	70	4	17.87	0.00	0.00	0.00	0.00	17.87
90	70	4	17.00	0.00	0.00	0.00	0.00	17.00
120	70	4	16.22	0.00	0.00	0.70	0.70	16.92
150	70	4	16.07	0.00	0.00	0.88	0.88	16.96
330	70	4	15.17	0.00	0.00	1.96	1.96	17.13
0	70	5	18.17	0.00	0.00	0.00	0.00	18.17
30	70	5	18.15	0.00	0.00	0.00	0.00	18.15
60	70	5	17.57	0.00	0.00	0.00	0.00	17.57
90	70	5	16.34	0.00	0.00	1.33	1.33	17.67
120	70	5	14.67	0.00	0.00	1.99	1.99	16.66
150	70	5	14.72	0.00	0.00	2.19	2.19	16.90
330	70	5	14.37	0.00	0.00	2.19	2.19	16.57
0	80	2	18.17	0.00	0.00	0.00	0.00	18.17
30	80	2	3.15	0.42	0.00	13.41	13.82	16.98
60	80	2	3.03	0.82	0.00	13.29	14.11	17.14
90	80	2	2.19	0.93	0.00	13.28	14.21	16.40
120	80	2	1.89	1.88	0.00	13.29	15.17	17.07

Data-Hydrolysis at Different pH and Oven Temperature 30-80°C (continues)

150	80	2	1.78	2.15	0.00	13.15	15.31	17.09
330	80	2	1.38	2.17	0.00	12.67	14.84	16.22
0	80	3	18.17	0.00	0.00	0.00	0.00	18.17
30	80	3	10.10	0.00	0.00	6.46	6.46	16.56
60	80	3	8.94	0.38	0.00	7.48	7.85	16.79
90	80	3	7.53	0.62	0.00	8.46	9.07	16.61
120	80	3	2.93	0.62	0.00	12.59	13.21	16.14
150	80	3	2.60	1.69	0.00	12.43	14.12	16.72
330	80	3	2.11	2.08	0.00	12.18	14.26	16.37
0	80	4	18.17	0.00	0.00	0.00	0.00	18.17
30	80	4	18.57	0.00	0.00	0.00	0.00	18.57
60	80	4	17.83	0.00	0.00	0.00	0.00	17.83
90	80	4	15.98	0.00	0.00	2.06	2.06	18.04
120	80	4	15.66	0.00	0.00	2.25	2.25	17.92
150	80	4	15.05	0.00	0.00	3.21	3.21	18.27
330	80	4	14.22	0.00	0.00	4.13	4.13	18.35
0	80	5	18.17	0.00	0.00	0.00	0.00	18.17
30	80	5	15.98	0.00	0.00	1.28	1.28	17.26
60	80	5	15.23	0.00	0.00	1.39	1.39	16.62
90	80	5	15.02	0.00	0.00	1.43	1.43	16.44
120	80	5	14.93	0.00	0.00	1.93	1.93	16.85
150	80	5	14.80	0.00	0.00	2.15	2.15	16.94
330	80	5	14.26	0.00	0.00	3.17	3.17	17.43

APPENDIX IV: Data-Hydrolysis at Different pH and Steam Temperatures 115-132°C (Pressure 1.6-2.9 bar)

Time	Temp	pH	Inulin	Sucrose	Glucose	Fructose	TOT-hydrolysates	TOT sugars
(hr)	(°C)		(g/100mL)	(g/100mL)	(g/100mL)	(g/100mL)	(g/100mL)	(g/100mL)
0	115	2	18.17	0.00	0.00	0.00	0.00	18.17
15	115	2	18.05	0.25	0.00	0.00	0.25	18.29
30	115	2	2.67	1.58	1.32	12.12	15.01	17.68
60	115	2	1.76	1.56	1.85	12.62	16.03	17.79
90	115	2	0.98	1.62	1.92	13.26	16.80	17.78
120	115	2	0.53	1.68	1.30	14.57	17.56	18.08
150	115	2	0.01	1.11	1.93	15.27	18.32	18.32
0	115	3	18.17	0.00	0.00	0.00	0.00	18.17
15	115	3	15.82	1.37	0.00	0.00	1.37	17.19
30	115	3	7.99	1.52	0.29	8.27	10.08	18.07
60	115	3	0.57	2.12	1.13	13.98	17.23	17.80
90	115	3	0.25	1.55	1.40	14.88	17.83	18.08
120	115	3	0.25	1.67	1.48	15.03	18.19	18.43
150	115	3	0.01	4.86	1.80	11.99	18.65	18.66
0	115	4	18.17	0.00	0.00	0.00	0.00	18.17
15	115	4	16.81	0.79	0.09	0.00	0.88	17.69
30	115	4	12.31	0.92	0.25	4.61	5.78	18.09
60	115	4	7.57	1.24	0.68	8.52	10.44	18.00
90	115	4	6.92	1.01	0.82	9.34	11.16	18.08
120	115	4	5.69	1.63	0.88	10.11	12.62	18.30
150	115	4	0.15	4.45	3.66	10.17	18.28	18.43
0	115	5	18.17	0.00	0.00	0.00	0.00	18.17
15	115	5	17.80	0.22	0.18	0.00	0.39	18.20
30	115	5	16.62	0.32	0.20	0.96	1.48	18.10
60	115	5	14.56	0.36	0.24	3.05	3.65	18.21
90	115	5	13.58	0.47	0.24	3.79	4.50	18.08
120	115	5	11.12	1.58	0.28	5.19	7.05	18.17

Data-Hydrolysis at Different pH and Steam Temperatures 115-132°C (Pressure 1.6-2.9 bar) (continues)

150	115	5	0.29	4.03	5.52	8.36	17.91	18.20
0	121	2	17.69	0.00	0.00	0.00	0.00	17.69
15	121	2	1.05	1.05	2.10	13.85	17.00	18.04
30	121	2	0.73	0.93	1.28	14.20	16.41	17.14
60	121	2	0.55	1.16	2.33	13.15	16.65	17.20
90	121	2	0.48	1.86	2.91	13.27	18.04	18.53
120	121	2	0.00	1.28	3.84	12.34	17.46	17.46
150	121	2	0.00	1.86	3.18	12.81	17.85	17.85
0	121	3	17.45	0.00	0.00	0.00	0.00	17.45
15	121	3	15.41	0.63	0.00	1.35	1.98	17.38
30	121	3	1.16	1.55	1.40	13.18	16.14	17.30
60	121	3	0.93	1.82	1.20	13.00	16.02	16.96
90	121	3	0.35	2.57	1.91	11.91	16.39	16.74
120	121	3	0.12	2.56	2.42	12.99	17.97	18.08
150	121	3	0.12	2.54	2.57	12.64	17.75	17.87
0	121	4	18.12	0.00	0.00	0.00	0.00	18.12
15	121	4	14.59	0.61	0.53	2.29	3.43	18.02
30	121	4	7.43	1.31	0.99	8.04	10.34	17.77
60	121	4	5.87	1.64	1.07	9.05	11.75	17.62
90	121	4	5.27	1.83	1.28	9.43	12.54	17.81
120	121	4	4.11	2.00	1.13	10.83	13.96	18.07
150	121	4	1.15	2.35	2.55	11.92	16.81	17.96
0	121	5	18.08	0.00	0.00	0.00	0.00	18.08
15	121	5	16.40	0.34	0.00	0.80	1.14	17.54
30	121	5	14.19	0.92	0.00	2.60	3.52	17.71
60	121	5	9.74	3.11	0.00	4.61	7.72	17.46
90	121	5	9.00	3.20	0.00	5.49	8.70	17.69
120	121	5	6.61	2.86	0.35	8.00	11.21	17.82
150	121	5	2.07	2.90	0.03	12.96	15.88	17.95
0	132	2	18.57	0.00	0.00	0.00	0.00	18.57
15	132	2	3.55	1.73	2.99	10.30	15.02	18.57

Data-Hydrolysis at Different pH and Steam Temperatures 115-132°C (Pressure 1.6-2.9 bar) (continues)

30	132	2	1.76	2.30	2.30	11.54	16.13	17.89
60	132	2	1.61	0.77	2.53	12.39	15.68	17.29
90	132	2	1.52	0.43	2.66	13.94	17.03	18.55
120	132	2	1.06	0.67	1.06	15.28	17.01	18.07
150	132	2	0.82	1.16	3.92	11.30	16.39	17.21
0	132	3	18.60	0.00	0.00	0.00	0.00	18.60
15	132	3	3.14	0.33	0.60	14.12	15.04	18.19
30	132	3	3.33	0.37	1.27	14.06	15.70	19.02
60	132	3	2.83	0.64	1.44	14.03	16.10	18.93
90	132	3	0.73	0.53	5.38	10.66	16.57	17.30
120	132	3	0.61	0.45	2.65	13.48	16.58	17.19
150	132	3	0.70	0.47	1.12	14.02	15.61	16.31
0	132	4	18.07	0.00	0.00	0.00	0.00	18.07
15	132	4	12.57	0.38	0.29	4.33	5.01	17.57
30	132	4	8.34	1.04	0.84	7.86	9.75	18.09
60	132	4	6.41	1.77	0.92	8.43	11.11	17.52
90	132	4	4.75	2.10	2.46	8.07	12.63	17.38
120	132	4	3.51	2.11	2.08	10.16	14.34	17.86
150	132	4	2.23	1.99	1.66	11.70	15.36	17.59
0	132	5	18.17	0.00	0.00	0.00	0.00	18.17
15	132	5	15.31	0.23	0.58	1.05	1.86	17.17
30	132	5	14.67	1.33	0.70	1.61	3.64	18.31
60	132	5	12.15	1.50	1.03	2.07	4.60	16.75
90	132	5	8.93	2.10	2.55	4.22	8.87	17.80
120	132	5	6.71	2.56	2.89	6.17	11.62	18.33
150	132	5	6.04	2.06	2.93	7.20	12.19	18.23

APPENDIX V: Minitab V15 Statistical Analysis of Hydrolysis Data

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Welcome to Minitab. press F1 for help.

Results for: Worksheet 3

Full Factorial Design

Factors: 2 Base Design: 2; 4
Runs: 10 Replicates: 2
Blocks: 1 Center pts (total): 2

All terms are free from aliasing.

Design Table (randomized)

Run	A	B
1	0	0
2	-	+
3	-	+
4	-	-
5	+	+
6	+	-
7	-	-
8	+	+
9	0	0
10	+	-

Factorial Fit: Inulin; Fructose; TOT-hydrolysates

Factorial Fit: Inulin versus pH; Temperature

Estimated Effects and Coefficients for Inulin (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		53.08	0.6898	76.94	0.000
pH	84.20	42.10	0.6898	61.03	0.000
Temperature	-65.10	-32.55	0.6898	-47.19	0.000
pH*Temperature	-54.05	-27.03	0.6898	-39.18	0.000
Ct Pt		-48.03	1.5425	-31.13	0.000

S = 1.95115 R-Sq = 99.94% R-Sq(adj) = 99.89%

Analysis of Variance for Inulin (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	2	22655.3	22655.3	11327.7	2975.48	0.000
2-Way Interactions	1	5842.8	5842.8	5842.8	1534.75	0.000
Curvature	1	3690.2	3690.2	3690.2	969.33	0.000
Residual Error	5	19.0	19.0	3.8		
Pure Error	5	19.0	19.0	3.8		
Total	9	32207.4				

Obs	StdOrder	Inulin	Fit	SE Fit	Residual	St Resid
1	10	5.300	5.050	1.380	0.250	0.18
2	3	5.600	5.450	1.380	0.150	0.11
3	7	5.300	5.450	1.380	-0.150	-0.11
4	1	18.900	16.500	1.380	2.400	1.74
5	4	35.100	35.600	1.380	-0.500	-0.36

6	6	156.600	154.750	1.380	1.850	1.34
7	5	14.100	16.500	1.380	-2.400	-1.74
8	8	36.100	35.600	1.380	0.500	0.36
9	9	4.800	5.050	1.380	-0.250	-0.18
10	2	152.900	154.750	1.380	-1.850	-1.34

Estimated Coefficients for Inulin using data in uncoded units

Term	Coef
Constant	-280.389
pH	157.476
Temperature	1.86635
pH*Temperature	-1.03942
Ct Pt	-48.0250

Least Squares Means for Inulin

	Mean	SE Mean
pH		
2	10.975	0.9756
4	95.175	0.9756
Temperature		
85	85.625	0.9756
137	20.525	0.9756
pH*Temperature		
2 85	16.500	1.3797
4 85	154.750	1.3797
2 137	5.450	1.3797
4 137	35.600	1.3797

Mean for Center Point = 5.050

Effects Plot for Inulin

Factorial Fit: Fructose versus pH; Temperature

Estimated Effects and Coefficients for Fructose (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		102.95	0.7872	130.77	0.000
pH	-83.70	-41.85	0.7872	-53.16	0.000
Temperature	50.05	25.03	0.7872	31.79	0.000
pH*Temperature	28.75	14.38	0.7872	18.26	0.000
Ct Pt		41.35	1.7603	23.49	0.000

S = 2.22666 R-Sq = 99.89% R-Sq(adj) = 99.81%

Analysis of Variance for Fructose (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	2	19021.4	19021.4	9510.69	1918.25	0.000
2-Way Interactions	1	1653.1	1653.1	1653.12	333.43	0.000
Curvature	1	2735.7	2735.7	2735.72	551.78	0.000
Residual Error	5	24.8	24.8	4.96		
Pure Error	5	24.8	24.8	4.96		
Total	9	23435.0				

Obs	StdOrder	Fructose	Fit	SE Fit	Residual	St Resid
1	10	145.700	144.300	1.574	1.400	0.89
2	3	152.800	155.450	1.574	-2.650	-1.68
3	7	158.100	155.450	1.574	2.650	1.68
4	1	132.900	134.150	1.574	-1.250	-0.79
5	4	101.600	100.500	1.574	1.100	0.70

6	6	22.500	21.700	1.574	0.800	0.51
7	5	135.400	134.150	1.574	1.250	0.79
8	8	99.400	100.500	1.574	-1.100	-0.70
9	9	142.900	144.300	1.574	-1.400	-0.89
10	2	20.900	21.700	1.574	-0.800	-0.51

Estimated Coefficients for Fructose using data in uncoded units

Term	Coef
Constant	305.773
pH	-103.220
Temperature	-0.696154
pH*Temperature	0.552885
Ct Pt	41.3500

Least Squares Means for Fructose

	Mean	SE Mean
pH		
2	144.80	1.113
4	61.10	1.113
Temperature		
85	77.93	1.113
137	127.98	1.113
pH*Temperature		
2 85	134.15	1.574
4 85	21.70	1.574
2 137	155.45	1.574
4 137	100.50	1.574

Mean for Center Point = 144.30

Effects Plot for Fructose

Factorial Fit: TOT-hydrolysates versus pH; Temperature

Estimated Effects and Coefficients for TOT-hydrolysates (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		123.70	0.8530	145.02	0.000
pH	-83.10	-41.55	0.8530	-48.71	0.000
Temperature	73.90	36.95	0.8530	43.32	0.000
pH*Temperature	47.00	23.50	0.8530	27.55	0.000
Ct Pt		51.35	1.9074	26.92	0.000

S = 2.41267 R-Sq = 99.91% R-Sq(adj) = 99.84%

Analysis of Variance for TOT-hydrolysates (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	2	24733.6	24733.6	12366.8	2124.52	0.000
2-Way Interactions	1	4418.0	4418.0	4418.0	758.98	0.000
Curvature	1	4218.9	4218.9	4218.9	724.78	0.000
Residual Error	5	29.1	29.1	5.8		
Pure Error	5	29.1	29.1	5.8		
Total	9	33399.7				

Obs	StdOrder	TOT-hydrolysates	Fit	SE Fit	Residual	St Resid
1	10	175.600	175.050	1.706	0.550	0.32
2	3	175.100	178.700	1.706	-3.600	-2.11R
3	7	182.300	178.700	1.706	3.600	2.11R
4	1	151.700	151.800	1.706	-0.100	-0.06

5	4	143.400	142.600	1.706	0.800	0.47
6	6	22.500	21.700	1.706	0.800	0.47
7	5	151.900	151.800	1.706	0.100	0.06
8	8	141.800	142.600	1.706	-0.800	-0.47
9	9	174.500	175.050	1.706	-0.550	-0.32
10	2	20.900	21.700	1.706	-0.800	-0.47

R denotes an observation with a large standardized residual.

Estimated Coefficients for TOT-hydrolysates using data in uncoded units

Term	Coef
Constant	391.583
pH	-141.877
Temperature	-1.29038
pH*Temperature	0.903846
Ct Pt	51.3500

Least Squares Means for TOT-hydrolysates

	Mean	SE Mean
pH		
2	165.25	1.206
4	82.15	1.206
Temperature		
85	86.75	1.206
137	160.65	1.206
pH*Temperature		
2 85	151.80	1.706
4 85	21.70	1.706
2 137	178.70	1.706
4 137	142.60	1.706

Mean for Center Point = 175.05

Effects Plot for TOT-hydrolysates

Alias Structure
I
pH
Temperature
pH*Temperature

Residual Plots for Inulin

Residual Plots for Fructose

Residual Plots for TOT-hydrolysates

Response Optimization
Parameters

	Goal	Lower	Target	Upper	Weight	Import
Inulin	Minimum	70.0	70.0	156.0	1	1
Fructose	Maximum	70.0	130.0	130.0	1	1
TOT-hydrolys	Target	151.7	151.8	151.9	1	1

Global Solution

pH = 2
Temperature = 80

Predicted Responses

Inulin = 16.50; desirability = 1
Fructose = 134.15; desirability = 1
TOT-hydrolys = 151.80; desirability = 1

Composite Desirability = 1.00000

Response Optimization
Parameters

	Goal	Lower	Target	Upper	Weight	Import
Inulin	Minimum	10.0	10.0	36.1	1	1
Fructose	Maximum	99.4	150.0	150.0	1	1
TOT-hydrolys	Target	141.8	151.8	182.3	1	1

Local Solution

pH = 3
Temperature = 115

Predicted Responses

Inulin = 5.05; desirability = 1.00000
Fructose = 144.30; desirability = 0.88735
TOT-hydrolys = 175.05; desirability = 0.23770

Composite Desirability = 0.59527

Local Solution

pH = 2
Temperature = 132

Predicted Responses

Inulin = 5.45; desirability = 1.00000
Fructose = 155.45; desirability = 1.00000
TOT-hydrolys = 178.70; desirability = 0.11803

Composite Desirability = 0.49053

Local Solution

pH = 4
Temperature = 132

Predicted Responses

Inulin = 35.6; desirability = 0.01916
Fructose = 100.5; desirability = 0.02174
TOT-hydrolys = 142.6; desirability = 0.08000

Composite Desirability = 0.03218

Local Solution

pH = 2
Temperature = 80

Predicted Responses

Inulin = 16.50; desirability = 0.75096

Fructose = 134.15; desirability = 0.68676
TOT-hydrolys = 151.80; desirability = 1.00000

Composite Desirability = 0.80194

Global Solution

pH = 2
Temperature = 80

Predicted Responses

Inulin = 16.50; desirability = 0.75096
Fructose = 134.15; desirability = 0.68676
TOT-hydrolys = 151.80; desirability = 1.00000

Composite Desirability = 0.80194

Response Optimization
Parameters

	Goal	Lower	Target	Upper	Weight	Import
Inulin	Target	4.8	5	5.3	1	1
Fructose	Target	142.9	145	145.7	1	1
TOT-hydrolys	Target	174.5	175	175.6	1	1

Local Solution

pH = 3
Temperature = 115

Predicted Responses

Inulin = 5.05; desirability = 0.83333
Fructose = 144.30; desirability = 0.66667
TOT-hydrolys = 175.05; desirability = 0.91667

Composite Desirability = 0.79857

Global Solution

pH = 3
Temperature = 115

Predicted Responses

Inulin = 5.05; desirability = 0.83333
Fructose = 144.30; desirability = 0.66667
TOT-hydrolys = 175.05; desirability = 0.91667

Composite Desirability = 0.79857

Response Optimization
Parameters

	Goal	Lower	Target	Upper	Weight	Import
Inulin	Target	4.8	5	5.3	1	1
Fructose	Target	142.9	145	145.7	1	1
TOT-hydrolys	Target	174.5	175	175.6	1	1

Local Solution

pH = 3
Temperature = 115

Predicted Responses

Inulin = 5.05; desirability = 0.83333
Fructose = 144.30; desirability = 0.66667
TOT-hydrolys = 175.05; desirability = 0.91667

Composite Desirability = 0.79857

Response Optimization

Parameters

	Goal	Lower	Target	Upper	Weight	Import
Inulin	Minimum	5.0	5	5.3	1	1
Fructose	Maximum	142.9	145	145.0	1	1
TOT-hydrolys	Target	174.5	175	175.6	1	1

Global Solution

pH = 3
Temperature = 115

Predicted Responses

Inulin = 5.05; desirability = 0.83333
Fructose = 144.30; desirability = 0.66667
TOT-hydrolys = 175.05; desirability = 0.91667

Composite Desirability = 0.79857

Response Optimization
Parameters

	Goal	Lower	Target	Upper	Weight	Import
Inulin	Target	5.3	30	36.1	1	1
Fructose	Target	99.4	145	158.1	1	1
TOT-hydrolys	Target	141.8	175	182.3	1	1

Local Solution

pH = 2
Temperature = 132

Predicted Responses

Inulin = 5.45; desirability = 0.00607
Fructose = 155.45; desirability = 0.20229
TOT-hydrolys = 178.70; desirability = 0.49315

Composite Desirability = 0.08462

Local Solution

pH = 2
Temperature = 80

Predicted Responses

Inulin = 16.50; desirability = 0.45344
Fructose = 134.15; desirability = 0.76206

TOT-hydrolys = 151.80; desirability = 0.30120

Composite Desirability = 0.47039

Global Solution

pH = 2
Temperature = 80

Predicted Responses

Inulin = 16.50; desirability = 0.45344
Fructose = 134.15; desirability = 0.76206
TOT-hydrolys = 151.80; desirability = 0.30120

Composite Desirability = 0.47039

Response Optimization
Parameters

	Goal	Lower	Target	Upper	Weight	Import
Inulin	Target	14.1	150.0	156.0	1	1
Fructose	Target	70.0	130.0	135.4	1	1
TOT-hydrolys	Target	151.7	151.8	151.9	1	1

Local Solution

pH = 2
Temperature = 85

Predicted Responses

Inulin = 16.50; desirability = 0.01766
Fructose = 134.15; desirability = 0.23148
TOT-hydrolys = 151.80; desirability = 1.00000

Composite Desirability = 0.15990

Global Solution

pH = 2
Temperature = 80

Predicted Responses

Inulin = 16.50; desirability = 0.01766
Fructose = 134.15; desirability = 0.23148
TOT-hydrolys = 151.80; desirability = 1.00000

Composite Desirability = 0.15990

---------- **2009-05-14 11:28:37** ----------

Welcome to Minitab. press F1 for help.
Retrieving project from file: 'C:\DOCUMENTS AND SETTINGS\CONSOLATA M\MY DOCUMENTS\PHD_AAB\HYDROLYSIS AT DIFF TEMP PH (THESIS).MPJ'

General Linear Model: Inulin; Fructose; TOT-hydrolysates versus

MANOVA for pH
s = 1 m = 0.5 n = 1.0

Criterion	Test Statistic	F	Num	Denom	P
Wilks'	0.02202	59.209	3	4	0.001
Lawley-Hotelling	44.40675	59.209	3	4	0.001
Pillai's	0.97798	59.209	3	4	0.001
Roy's	44.40675				

MANOVA for Temperature
s = 1 m = 0.5 n = 1.0

Criterion	Test Statistic	F	Num	Denom	P
Wilks'	0.02964	43.651	3	4	0.002
Lawley-Hotelling	32.73796	43.651	3	4	0.002
Pillai's	0.97036	43.651	3	4	0.002
Roy's	32.73796				

MANOVA for pH*Temperature
s = 1 m = 0.5 n = 1.0

Criterion	Test Statistic	F	Num	Denom	P
Wilks'	0.01346	97.746	3	4	0.000
Lawley-Hotelling	73.30948	97.746	3	4	0.000
Pillai's	0.98654	97.746	3	4	0.000
Roy's	73.30948				

Response Surface Regression: Inulin; Fructose; ... versus pH; Temperature

The following terms cannot be estimated. and were removed.

Temperature*Temperature

Response Surface Regression: Inulin versus pH; Temperature

The analysis was done using uncoded units.

Estimated Regression Coefficients for Inulin

Term	Coef	SE Coef	T	P
Constant	23.004	15.3835	1.495	0.195
pH	-68.267	9.8028	-6.964	0.001
Temperature	1.866	0.0839	22.244	0.000
pH*pH	36.758	1.5609	23.549	0.000
pH*Temperature	-1.039	0.0265	-39.176	0.000

S = 1.951 R-Sq = 99.9% R-Sq(adj) = 99.9%

Analysis of Variance for Inulin

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	4	32188.3	32188.35	8047.09	2113.76	0.000
Linear	2	24234.3	2460.51	1230.25	323.16	0.000
Square	1	2111.2	2111.21	2111.21	554.56	0.000
Interaction	1	5842.8	5842.81	5842.81	1534.75	0.000
Residual Error	5	19.0	19.04	3.81		
Pure Error	5	19.0	19.03	3.81		
Total	9	32207.4				

Obs	StdOrder	Inulin	Fit	SE Fit	Residual	St Resid
1	10	5.300	5.050	1.380	0.250	0.18
2	3	5.600	5.450	1.380	0.150	0.11
3	7	5.300	5.450	1.380	-0.150	-0.11

4	1	18.900	16.500	1.380	2.400	1.74
5	4	35.100	35.600	1.380	-0.500	-0.36
6	6	156.600	154.750	1.380	1.850	1.34
7	5	14.100	16.500	1.380	-2.400	-1.74
8	8	36.100	35.600	1.380	0.500	0.36
9	9	4.800	5.050	1.380	-0.250	-0.18
10	2	152.900	154.750	1.380	-1.850	-1.34

Response Surface Regression: Fructose versus pH; Temperature

The analysis was done using uncoded units.

Estimated Regression Coefficients for Fructose

Term	Coef	SE Coef	T	P
Constant	40.7923	17.5557	2.324	0.068
pH	95.6692	11.1870	8.552	0.000
Temperature	-0.6962	0.0957	-7.271	0.001
pH*pH	-32.6875	1.7813	-18.350	0.000
pH*Temperature	0.5529	0.0303	18.260	0.000

S = 2.227 R-Sq = 99.9% R-Sq(adj) = 99.8%

Analysis of Variance for Fructose

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	4	23410.2	23410.23	5852.56	1180.43	0.000
Linear	2	20087.6	805.10	402.55	81.19	0.000
Square	1	1669.5	1669.55	1669.55	336.74	0.000
Interaction	1	1653.1	1653.13	1653.13	333.43	0.000
Residual Error	5	24.8	24.79	4.96		
Pure Error	5	24.8	24.79	4.96		
Total	9	23435.0				

Obs	StdOrder	Fructose	Fit	SE Fit	Residual	St Resid
1	10	145.700	144.300	1.574	1.400	0.89
2	3	152.800	155.450	1.574	-2.650	-1.68
3	7	158.100	155.450	1.574	2.650	1.68
4	1	132.900	134.150	1.574	-1.250	-0.79
5	4	101.600	100.500	1.574	1.100	0.70
6	6	22.500	21.700	1.574	0.800	0.51
7	5	135.400	134.150	1.574	1.250	0.79
8	8	99.400	100.500	1.574	-1.100	-0.70
9	9	142.900	144.300	1.574	-1.400	-0.89
10	2	20.900	21.700	1.574	-0.800	-0.51

Response Surface Regression: TOT-hydrolysates versus pH; Temperature

The analysis was done using uncoded units.

Estimated Regression Coefficients for TOT-hydrolysates

Term	Coef	SE Coef	T	P
Constant	76.6538	19.0223	4.030	0.010
pH	94.0000	12.1216	7.755	0.001
Temperature	-1.2904	0.1037	-12.438	0.000
pH*pH	-38.5596	1.9301	-19.978	0.000
pH*Temperature	0.9038	0.0328	27.550	0.000

S = 2.413 R-Sq = 99.9% R-Sq(adj) = 99.8%

Analysis of Variance for TOT-hydrolysates

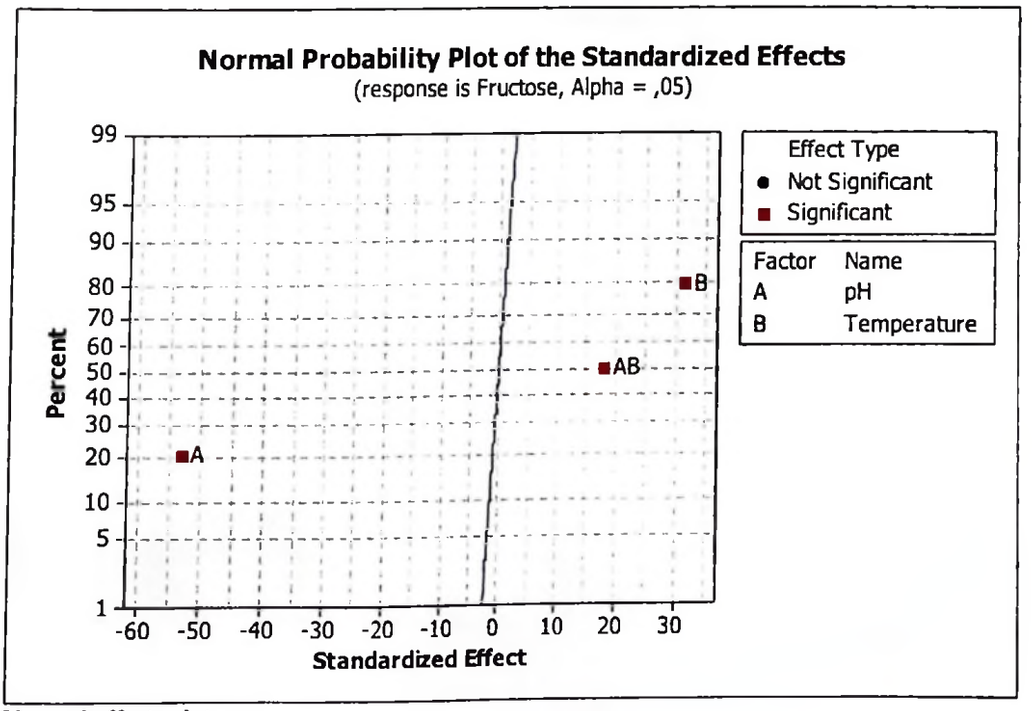
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	4	33370.6	33370.56	8342.64	1433.20	0.000
Linear	2	26629.3	1585.19	792.60	136.16	0.000
Square	1	2323.3	2323.27	2323.27	399.12	0.000
Interaction	1	4418.0	4418.00	4418.00	758.98	0.000
Residual Error	5	29.1	29.11	5.82		
Pure Error	5	29.1	29.10	5.82		
Total	9	33399.7				

Obs	StdOrder	TOT-hydrolysates	Fit	SE Fit	Residual	St Resid
1	10	175.600	175.050	1.706	0.550	0.32
2	3	175.100	178.700	1.706	-3.600	-2.11 R
3	7	182.300	178.700	1.706	3.600	2.11 R
4	1	151.700	151.800	1.706	-0.100	-0.06
5	4	143.400	142.600	1.706	0.800	0.47
6	6	22.500	21.700	1.706	0.800	0.47
7	5	151.900	151.800	1.706	0.100	0.06
8	8	141.800	142.600	1.706	-0.800	-0.47
9	9	174.500	175.050	1.706	-0.550	-0.32
10	2	20.900	21.700	1.706	-0.800	-0.47

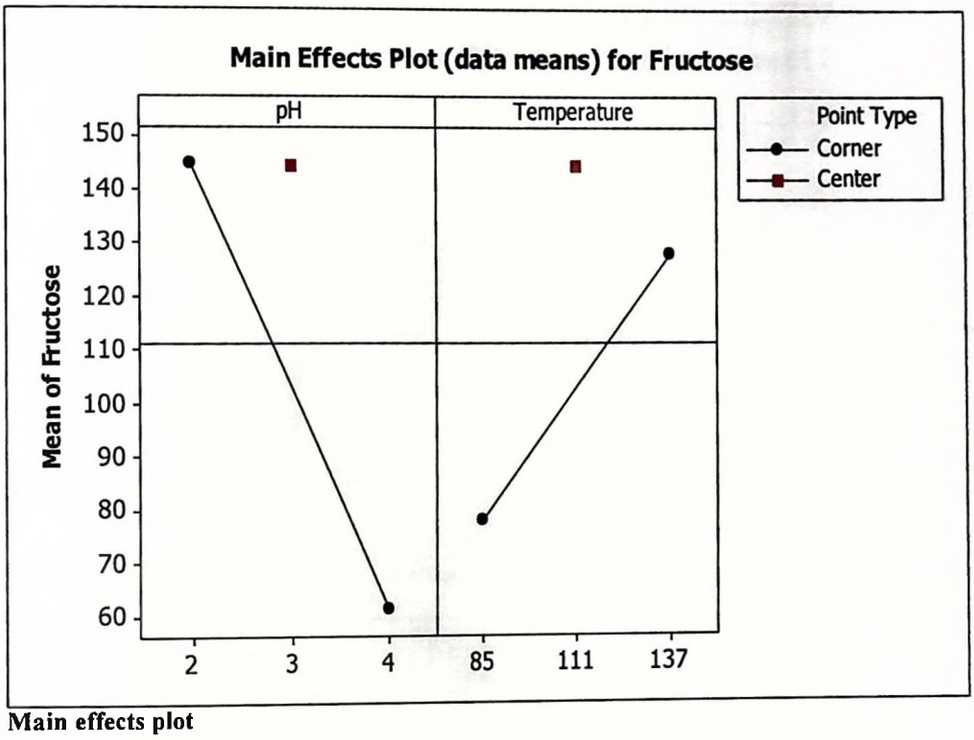
R denotes an observation with a large standardized residual.

Four-in-one residual plot

Histogram -effects plot



Normal effects plot



Main effects plot

Interaction effects plot

cube plot

APPENDIX VI: Descriptive Statistics: pH values of 2. 3. 4 and 5

2008-12-28 09:28:04

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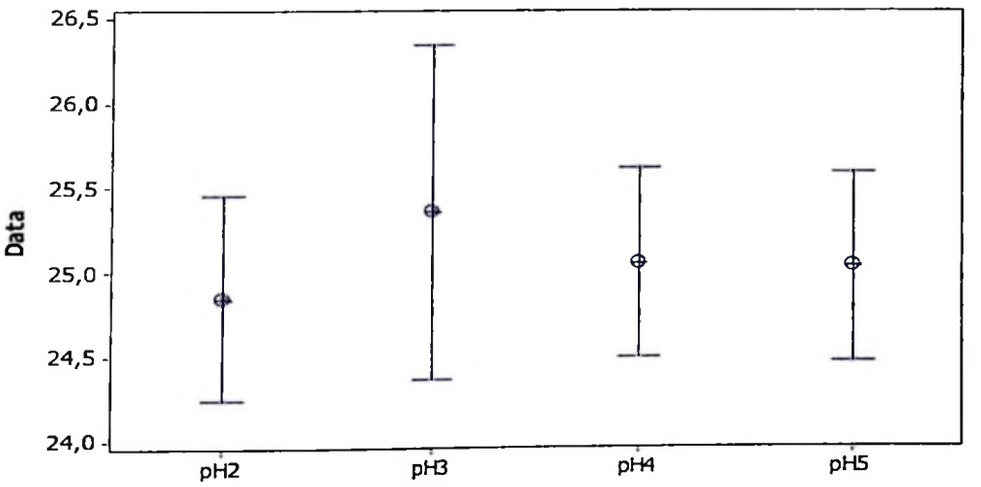
Descriptive Statistics: pH 2; pH 3; pH 4; pH 5

Variable	Total Count	N	N *	Mean	SEMean	StDev	Variance	CoefVar	Sum of Squares
pH 2	36	36	0	24.853	0.299	1.795	3.222	7.22	22349.052
pH 3	36	36	0	25.356	0.488	2.925	8.558	11.54	23445.082
pH 4	36	36	0	25.054	0.276	1.659	2.751	6.62	22692.884
pH 5	36	36	0	25.030	0.276	1.654	2.735	6.61	22650.264

Variable	Minimum	Q1	Median	Q3	Maximum	Range	MSSD	AD	P
pH 2	22.19	23.333	24.250	26.463	29.79	7.60	3.008	1.303	0.099
pH 3	21.87	22.740	24.275	27.038	31.20	9.33	8.701	0.618	<0.005
pH 4	21.81	24.305	25.095	26.568	27.89	6.08	3.812	0.658	0.079
pH 5	21.71	24.475	25.220	26.005	29.93	8.22	2.365	1.013	0.01

Interval Plot of pH2; pH3; pH4; pH5

95% CI for the Mean



Mean of means of fructose yield after hydrolysis at pH's 2-5: of 10 years old *Agave* hybrid H 11648 from Highland sisal estates

	Average total inulin (g/100g sisal juice)					Average fructose equivalent (g/100g sisal juice)			
	pH 2	pH 3	pH 4	pH 5	Avg				
avg	24.85	25. 56	25.05	25.03	25.07	100%	80%	60%	40%
std	1.79	2.92	1.65	1.65	0.21	25.07	20.06	15.04	10.03
max	29.79	31.20	27.89	29.93	25.36	±0.21	±0.21	±0.21	±0.21
min	22.19	21.87	21.81	21.71	24.85				
var	3.22	8.56	2.75	2.73	0.04				
confidence level	0.59	0.97	0.55	0.56	0.07				
Population					36				

APPENDIX VII: Descriptive Statistics properties of sisal bole juice

2008-12-28 09:28:04

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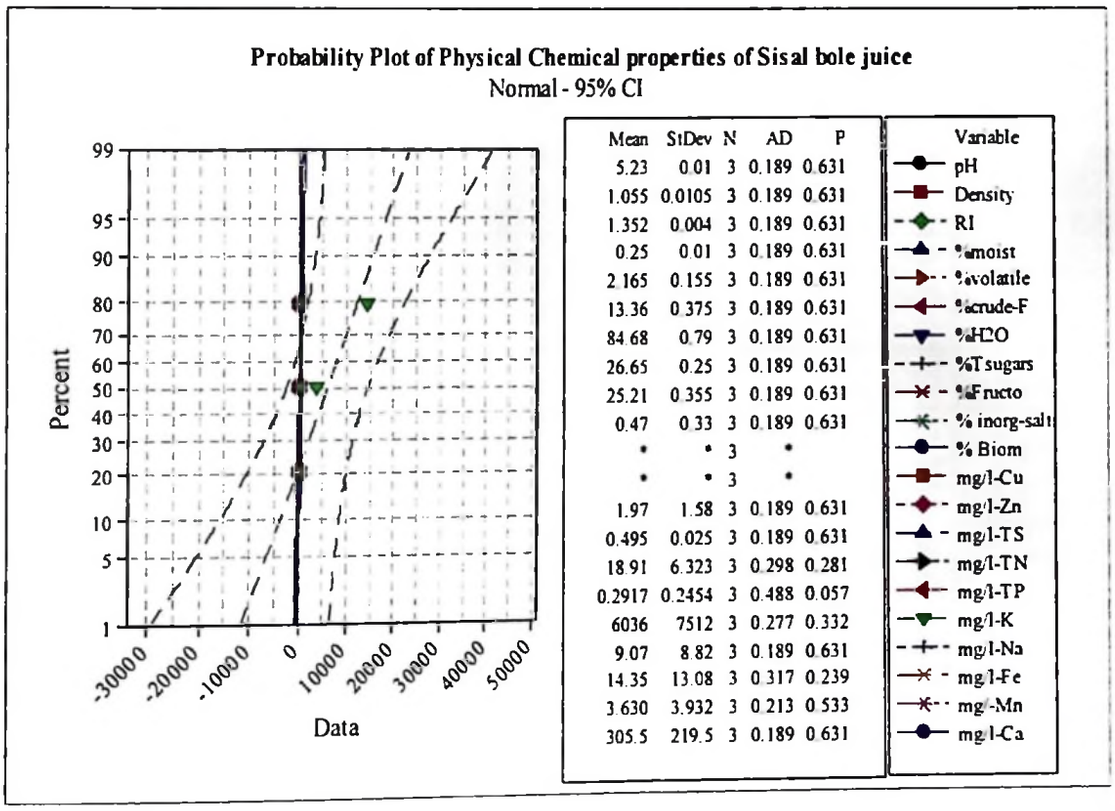

APPENDIX VIII: Citric Acid Production Potential Tests

Citric acid production trends for (W) Aspergillus niger wild1 (BYF Aspergillus niger) as compared with (D) DSMZ 8 standard strains

	40% SBJ Inulin (W)	80% SBJ Inulin(W)	80% SBJ Inulin (D)	10% sucroseSTD(D)	40% SBJ Inulin (D)
12	0.12	0.12	0.20	0.23	0.11
24	0.24	0.20	0.40	0.47	0.12
48	0.24	0.44	0.40	0.57	0.22
72	0.28	0.49	0.43	0.69	0.23
96	0.35	0.57	0.48	0.72	0.26
120	0.38	0.58	0.50	0.81	0.27
144	0.38	0.69	0.83	0.89	0.29
Time hrs	40% SBJ Inulin (W)	80% SBJ Inulin(W)	80% SBJ Inulin (D)	10% sucroseSTD(D)	40% SBJ Inulin (D)
12	0.12	0.12	0.20	0.22	0.11
24	0.22	0.18	0.38	0.45	0.12
48	0.22	0.42	0.38	0.55	0.20
72	0.27	0.47	0.41	0.67	0.21
96	0.33	0.55	0.46	0.70	0.24
120	0.36	0.56	0.48	0.79	0.25
144	0.36	0.67	0.81	0.87	0.27
Time hrs	40% SBJ Inulin (W)	80% SBJ Inulin(W)	80% SBJ Inulin (D)	10% sucroseSTD(D)	40% SBJ Inulin (D)
	0.043274	0.053675	0.041576	0.041576	0.020788
12	0.12	0.12	0.2	0.225	0.11
24	0.23	0.19	0.39	0.46	0.12
48	0.23	0.43	0.39	0.56	0.21
72	0.275	0.48	0.42	0.68	0.22
96	0.34	0.56	0.47	0.71	0.25
120	0.37	0.57	0.49	0.8	0.26
144	0.37	0.68	0.82	0.88	0.28

APPENDIX IX: Pilot Scale Citric Acid Fermentation Data

Time	pH	nutrients	initial sugar	CA	CA	total resid	total resid	Biomass	Biomass	Other Acid	Other Acid	TOT acids	TOT acids
										malic	malic		
			(% w/v)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)	(g/L)
0	2	1	40	0.0	0.0	102.60	101.82	0.00	0.00	0.00	0.00	0.0	0
24	2	1	40	0.3	0.5	93.63	95.41	0.80	0.11	0.48	0.48	4.04	0.96
48	2	1	40	5.0	5.8	84.10	76.75	1.68	0.65	0.80	0.69	8.89	1.8
72	2	1	40	4.6	6.9	49.62	89.67	5.48	2.42	7.38	6.27	16.88	19.92
96	2	1	40	4.6	7.0	37.82	53.12	8.36	6.33	16.64	14.30	31.07	33.92
120	2	1	40	7.9	8.6	36.96	34.38	14.57	14.84	21.47	18.63	38.62	41.94
144	2	1	40	10.5	8.7	35.98	19.74	14.51	16.34	24.23	21.04	43.43	47.11
168	2	1	40	11.4	8.0	33.12	18.32	16.71	19.17	25.62	22.24	45.5	49.24
0	2	0	40	0.0	0.0	116.61	116.61	0.00	0.00	0.00	0.00	0	0
24	2	0	40	1.0	1.0	110.53	110.67	0.04	0.94	9.09	5.27	9.38	5.79
48	2	0	40	3.9	0.3	95.28	95.28	0.58	0.66	12.49	14.03	17.49	19.83
72	2	0	40	4.3	2.3	88.59	88.59	0.58	0.63	15.02	16.87	19.66	23.71
96	2	0	40	4.1	2.8	88.33	64.49	7.49	6.64	17.75	19.92	22.36	26.9
120	2	0	40	7.2	8.2	82.82	60.31	7.94	7.61	20.04	22.50	27.91	31.13
144	2	0	40	13.5	14.6	69.91	58.45	8.62	6.51	21.55	24.20	32.07	32.86
168	2	0	40	18.3	10.1	66.33	56.37	8.90	6.44	22.34	25.08	33.69	33.03
0	3.5	1	40	0.0	0.0	116.61	116.61	0.00	0.00	0.00	0.00	0	0
24	3.5	1	40	1.0	1.0	102.79	82.42	0.74	0.57	0.00	0.00	1.04	1.04
48	3.5	1	40	7.3	11.6	83.71	79.59	0.74	0.66	3.90	6.85	11.22	18.44
72	3.5	1	40	8.0	13.7	74.06	73.74	0.75	4.33	6.56	8.63	14.58	22.32
96	3.5	1	40	11.6	14.0	71.96	69.57	8.94	8.91	5.29	6.99	16.89	20.96
120	3.5	1	40	12.0	17.3	65.39	69.45	8.33	8.62	6.02	7.95	18.02	25.21
144	3.5	1	40	14.0	17.3	53.34	53.16	10.05	10.00	12.50	16.52	26.47	33.84
168	3.5	1	40	15.9	15.9	45.66	48.07	10.01	10.97	15.50	11.89	31.4	27.79

Pilot Scale Citric Acid Fermentation Data (continues)

0	3.5	0	40	0.0	0.0	116.61	116.61	0.00	0.00	0.00	0.00	0	0
24	3.5	0	40	3.6	0.5	102.79	99.80	1.20	0.17	0.40	0.69	1.44	1.73
48	3.5	0	40	8.1	1.1	83.71	89.58	2.51	0.97	1.93	2.43	5.83	2.72
72	3.5	0	40	9.5	13.7	66.44	68.09	8.22	3.63	2.56	2.50	6.89	4.78
96	3.5	0	40	14.4	19.6	56.44	51.71	12.54	9.50	7.49	7.97	11.56	10.77
120	3.5	0	40	17.2	23.3	43.66	49.65	21.85	22.25	11.79	11.42	19.03	19.61
144	3.5	0	40	19.2	26.1	39.49	43.66	21.76	24.51	14.62	14.67	28.12	29.23
168	3.5	0	40	19.9	27.0	39.19	41.14	25.06	28.76	15.89	16.70	34.19	26.77
0	5	1	40	0.0	0.0	116.61	116.61	0.00	0.00	0.00	0.00	0	0
24	5	1	40	1.5	1.9	102.79	105.80	0.74	0.57	2.47	2.22	3.82	3.81
48	5	1	40	8.6	10.0	90.04	95.70	8.94	8.91	6.19	8.15	10.09	10.23
72	5	1	40	9.0	11.7	76.64	88.35	10.05	10.00	9.09	8.68	13.42	14.73
96	5	1	40	22.3	19.0	56.37	59.62	14.62	14.67	9.68	5.56	27.98	28.75
120	5	1	40	25.1	21.6	44.80	48.03	18.94	17.37	18.77	16.83	50.14	40.84
144	5	1	40	28.0	29.2	38.08	45.80	16.83	18.75	19.60	19.37	45.87	47.32
168	5	1	40	27.4	27.8	29.79	26.60	15.01	17.88	18.48	16.57	51.88	48.37
0	5	0	40	0.0	0.0	114.84	109.19	0.00	0.00	0.00	0.00	0	0
24	5	0	40	1.4	0.4	101.42	103.78	1.20	0.17	3.09	2.77	7.6	12
48	5	0	40	7.8	12.9	74.64	84.00	7.49	7.97	7.74	10.19	16.38	20.16
72	5	0	40	7.0	19.0	67.04	77.27	11.79	11.42	11.36	10.86	20.39	22.51
96	5	0	40	19.4	24.3	52.09	69.55	14.62	14.67	12.10	6.94	34.44	25.98
120	5	0	40	13.8	28.3	45.10	67.64	15.89	16.70	23.46	21.04	48.52	42.68
144	5	0	40	16.2	35.5	41.79	61.43	18.38	17.88	24.49	20.42	52.53	49.59
168	5	0	40	35.7	38.0	37.65	34.91	17.22	11.27	23.10	20.71	50.45	48.48

Pilot Scale Citric Acid Fermentation Data (continues)

0	2	1	60	0.0	0.0	181.16	179.73	0.00	0.00	0.00	0.00	0	0
24	2	1	60	1.4	1.6	136.00	131.13	0.59	0.04	2.50	4.00	6.78	12.5
48	2	1	60	3.9	2.1	72.03	73.82	5.42	0.62	0.90	1.42	18.86	21.02
72	2	1	60	4.3	6.0	60.20	61.53	4.76	7.50	16.90	27.51	46.87	60.31
96	2	1	60	18.3	23.2	59.04	58.12	9.95	9.68	14.50	23.51	64.41	64.46
120	2	1	60	31.4	24.0	57.58	49.34	20.28	18.92	10.90	17.66	59.15	57.26
144	2	1	60	26.3	28.0	49.34	47.20	20.26	22.81	23.30	37.83	72.09	75.78
168	2	1	60	33.4	31.8	33.12	18.32	23.05	24.87	26.00	42.29	100.22	82.34
0	2	0	60	0.0	0.0	184.50	184.04	0.00	0.00	0.00	0.00	0	0
24	2	0	60	4.2	2.2	178.50	176.89	0.59	0.04	2.50	4.00	6.7	6.15
48	2	0	60	24.0	36.5	141.42	152.85	4.76	0.78	0.90	1.42	24.9	37.94
72	2	0	60	32.6	30.8	133.11	128.70	7.45	7.50	16.90	27.51	49.5	58.3
96	2	0	60	33.6	33.5	129.29	126.30	11.54	13.12	14.50	23.51	48.1	56.97
120	2	0	60	34.8	32.9	110.68	110.10	20.47	24.84	10.90	17.66	45.7	50.61
144	2	0	60	36.0	34.1	103.89	106.35	29.18	21.75	23.30	37.83	59.3	71.96
168	2	0	60	37.2	31.9	96.83	102.15	30.61	29.57	26.00	42.29	63.2	74.16
0	3.5	1	60	0.0	0.0	184.50	184.04	0.00	0.00	0.00	0.00	0	0
24	3.5	1	60	2.1	3.0	178.50	176.89	0.83	0.66	16.60	16.20	17.96	16.6
48	3.5	1	60	18.2	19.6	162.50	162.90	2.85	13.75	22.14	16.40	29.97	29.25
72	3.5	1	60	25.3	27.2	158.00	158.79	7.11	19.90	22.90	17.80	49.91	42.14
96	3.5	1	60	24.9	26.7	103.00	109.15	8.54	18.88	28.88	19.60	48.25	58.63
120	3.5	1	60	35.3	37.9	84.40	65.56	26.32	22.53	32.55	32.80	46.33	70.76
144	3.5	1	60	40.5	43.5	49.16	39.70	30.76	30.40	32.63	36.60	48.79	64.85
168	3.5	1	60	35.6	38.0	41.40	33.84	27.99	36.25	33.30	38.00	68.98	73.5

Pilot Scale Citric Acid Fermentation Data (continues)

0	3.5	0	60	0.0	0.0	195.90	195.90	0.00	0.00	0.00	0.00	0	0
24	3.5	0	60	4.3	8.5	168.80	157.35	0.05	0.10	0.38	1.10	1.47	4.3
48	3.5	0	60	18.0	19.6	147.60	147.23	0.41	0.37	13.20	8.30	52.39	32.82
72	3.5	0	60	30.0	32.8	128.80	141.51	1.04	1.97	14.04	13.99	55.12	53.97
96	3.5	0	60	49.9	41.0	126.40	130.32	2.33	3.95	15.96	16.02	61.5	66.23
120	3.5	0	60	48.3	39.6	82.80	79.40	17.93	6.13	21.49	19.04	68.05	70.02
144	3.5	0	60	48.8	38.0	49.16	33.84	19.71	20.33	26.22	28.27	85.17	92.76
168	3.5	0	60	74.2	40.1	43.32	21.91	22.25	30.70	28.49	31.40	91.71	94.91
0	5	1	60	0.0	0.0	181.16	179.73	0.00	0.00	0.00	0.00	0	0
24	5	1	60	3.9	3.2	136.00	131.13	0.59	0.04	2.50	4.00	6.78	12.5
48	5	1	60	39.2	24.5	50.15	101.83	5.42	0.62	0.90	1.42	18.86	21.02
72	5	1	60	41.1	40.0	49.62	89.67	4.76	7.50	16.90	27.51	46.87	60.31
96	5	1	60	45.5	50.2	37.82	53.12	9.95	9.68	14.50	23.51	64.41	64.46
120	5	1	60	46.6	51.0	36.96	34.38	20.28	18.92	10.90	17.66	59.15	57.26
144	5	1	60	59.0	64.5	35.98	19.74	20.26	22.81	23.30	37.83	72.09	75.78
168	5	1	60	63.2	63.5	33.12	18.32	23.05	24.87	26.00	42.29	100.22	82.34
0	5	0	60	0.0	0.0	190.40	166.70	0.00	0.00	0.00	0.00	0	0
24	5	0	60	2.2	2.2	160.20	126.80	0.25	0.20	9.80	17.96	31.41	39.57
48	5	0	60	38.9	21.8	140.80	120.20	0.31	2.38	16.40	29.97	55.27	51.77
72	5	0	60	46.3	46.6	135.20	113.10	2.63	2.54	27.30	49.91	73.59	96.47
96	5	0	60	45.2	45.5	119.40	108.50	3.79	3.06	26.40	48.25	71.56	93.79
120	5	0	60	66.0	66.5	106.00	106.10	5.41	4.90	25.30	46.33	91.27	112.81
144	5	0	60	70.0	70.4	95.20	102.40	6.40	4.13	26.70	48.79	96.7	119.23
168	5	0	60	62.7	63.2	62.20	92.30	6.66	5.78	40.60	74.22	103.34	137.44

Pilot Scale Citric Acid Fermentation Data (continues)

0	2	1	80	0	0	201.16	211.07	0	0	0	0	0	0
24	2	1	80	14.7	13	152.62	157.07	0.04	0.94	9.09	5.27	23.75	18.24
48	2	1	80	22.2	19.6	151.03	151.07	0.58	0.66	12.49	14.03	34.65	33.62
72	2	1	80	30.8	27.2	139.41	136.69	0.58	0.63	15.02	16.87	45.83	44.11
96	2	1	80	30.3	26.7	126.17	130.75	7.49	6.64	17.75	19.92	47.99	46.66
120	2	1	80	42.9	37.9	124.46	119.71	7.94	7.61	20.04	22.5	62.95	60.43
144	2	1	80	49.2	43.5	116.43	114.15	8.62	6.51	21.55	24.2	70.79	67.73
168	2	1	80	43.5	38	82.01	88.39	8.9	6.44	22.34	25.08	65.84	63.08
0	2	0	80	0	0	201.16	198.81	0	0	0	0	0	0
24	2	0	80	4.3	8.5	152.62	164.4	0.04	0.94	9.09	5.27	23.75	18.24
48	2	0	80	18	19.6	151.03	146.22	0.58	0.66	12.49	14.03	34.65	33.62
72	2	0	80	30	32.8	139.41	120.44	0.58	0.63	15.02	16.87	45.83	44.11
96	2	0	80	49.9	41	126.17	131.17	7.49	6.64	17.75	19.92	47.99	46.66
120	2	0	80	48.3	39.6	124.46	108.77	7.94	7.61	20.04	22.5	62.95	60.43
144	2	0	80	48.8	38	116.43	96.69	8.62	6.51	21.55	24.2	70.79	67.73
168	2	0	80	74.2	40.1	82.01	67.97	8.9	6.44	22.34	25.08	65.84	63.08
0	3.5	1	80	0	0	233.23	233.23	0	0	0	0	0	0
24	3.5	1	80	5.5	5.5	158.75	149.62	0.81	0.72	0.45	0.6	5.99	6.14
48	3.5	1	80	22.4	13	158.75	153.17	3.22	2.84	2.93	5.13	25.28	18.1
72	3.5	1	80	25.8	25.8	156.59	143.86	4.06	0.8	4.92	6.47	30.68	32.23
96	3.5	1	80	27.6	27.9	134.17	135.64	4.38	1.35	11.26	12.65	38.9	40.5
120	3.5	1	80	51.3	34.3	130.05	121.77	5.73	2.8	13.31	14.94	64.58	49.21
144	3.5	1	80	69.9	66.4	125.2	133.48	8.96	12.26	15.03	16.88	84.94	83.31
168	3.5	1	80	87.8	69.9	121.62	108.77	12.94	31.18	16.16	18.15	103.92	88.06

Pilot Scale Citric Acid Fermentation Data (continues)

0	3.5	0	80	0	0	233.23	233.23	0	0	0	0	0	0
24	3.5	0	80	10.9	14.4	145.77	141.1	0.74	0.57	1.85	1.66	12.71	16.02
48	3.5	0	80	13.6	15.7	136.44	132.91	8.94	8.91	4.65	6.11	18.2	21.85
72	3.5	0	80	60.6	48.3	123.82	120	10.05	10	6.82	6.51	67.37	54.81
96	3.5	0	80	70.1	77.5	119.93	116.39	14.62	14.67	7.26	4.17	77.34	81.66
120	3.5	0	80	72.5	95	116.39	114.83	18.94	17.37	14.08	12.63	86.54	107.59
144	3.5	0	80	73.1	101.3	91.17	80.14	16.83	18.75	14.7	14.53	87.82	115.79
168	3.5	0	80	79.9	103.2	74.32	75.4	15.01	17.88	13.86	12.43	93.77	115.61
0	5	1	80	0	0	233.23	233.23	0	0	0	0	0	0
24	5	1	80	13.6	17.3	157.22	153.17	0.29	0.03	0.1	0.31	13.66	17.56
48	5	1	80	30	32.8	125.61	143.86	1.13	0.11	0.24	0.25	30.26	33.07
72	5	1	80	60.6	57.9	131.2	135.64	6.25	4.32	3.81	8.02	64.37	65.93
96	5	1	80	75.7	74.7	121.87	123.48	4.9	3.23	5.78	9.4	81.52	84.09
120	5	1	80	79.6	81.3	105.44	121.77	12.47	12.3	14.08	15.76	93.72	97.1
144	5	1	80	83.7	94.2	99.02	97.5	13.9	14.02	20.38	19.65	104.1	113.81
168	5	1	80	91.2	101.2	56.64	50.98	15.92	12.64	20.56	19.14	111.75	120.29
0	5	0	80	0	0	240.88	221.07	0	0	0	0	0	0
24	5	0	80	13.6	25.7	192.45	192.63	0.28	0.28	0.34	0.45	13.9	26.19
48	5	0	80	60.6	38	186.71	172.18	8.91	14.5	2.19	3.85	62.75	41.88
72	5	0	80	70.1	67.4	170.37	158.08	10.45	15.01	3.47	3.12	73.55	70.5
96	5	0	80	72.5	88.7	161.54	155.14	17.52	17.47	8.71	11.46	81.18	100.17
120	5	0	80	73.1	77.5	151.71	145.49	21.84	19.87	8.22	10.13	81.34	87.62
144	5	0	80	79.9	95	86.78	73.99	21.26	29.72	13.28	15.09	93.19	110.05
168	5	0	80	93.9	112.4	56.01	37.31	29.72	32.64	14.61	16.5	108.53	128.88

APPENDIX X: Minitab V15 Statistical Analysis Of Fermentation Data

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Welcome to Minitab. press F1 for help.

Results for: Worksheet 5

Full Factorial Design

Factors: 3 Base Design: 3; 8
Runs: 18 Replicates: 2
Blocks: 1 Center pts (total): 2

All terms are free from aliasing.

Design Table (randomized)

Run	A	B	C
1	+	+	+
2	-	+	-
3	+	+	+
4	-	+	-
5	+	-	-
6	0	0	0
7	-	-	-
8	+	-	-
9	+	+	-
10	+	-	+
11	-	-	-
12	+	+	-
13	+	-	+
14	-	+	+
15	0	0	0
16	-	-	+
17	-	+	+
18	-	-	+

Factorial Fit: Citric acid; Res. Fructose; Biomass; Other Acid; TOT Acids

Factorial Fit: Citric acid versus pH; Init. Fructose; Nutrients

Estimated Effects and Coefficients for Citric acid (coded units)

Term	Effect	Coef	SECoef	T	P
Constant		43.881	0.7230	60.69	0.000
pH	21.293	10.647	0.7230	14.72	0.000
Init.Fructose	40.047	20.023	0.7230	27.69	0.000
Nutrients	-2.229	-1.115	0.7230	-1.54	0.158
pH*Init.Fructose	13.318	6.659	0.7230	9.21	0.000
pH*Nutrients	-1.656	-0.828	0.7230	-1.14	0.282
Init.Fructose*Nutrients	1.296	0.648	0.7230	0.90	0.394
pH*Init.Fructose*Nutrients	-3.671	-1.835	0.7230	-2.54	0.032
CtPt		7.249	2.1691	3.34	0.009

S = 2.89215 R-Sq = 99.18% R-Sq(adj) = 98.45%

Analysis of Variance for Citric acid (coded units)

Source	DF	SeqSS	AdjSS	AdjMS	F	P
MainEffects	3	8248.46	8248.46	2749.49	328.71	0.000
2-WayInteractions	3	727.19	727.19	242.40	28.98	0.000
3-WayInteractions	1	53.90	53.90	53.90	6.44	0.032
Curvature	1	93.42	93.42	93.42	11.17	0.009
ResidualError	9	75.28	75.28	8.36		
PureError	9	75.28	75.28	8.36		
Total	17	9198.24				

Obs	StdOrder	Citricacid	Fit	SEFit	Residual	StResid
1	8	76.2400	78.0800	2.0451	-1.8400	-0.90
2	3	39.9900	44.4020	2.0451	-4.4120	-2.16R
3	16	79.9200	78.0800	2.0451	1.8400	0.90
4	11	48.8140	44.4020	2.0451	4.4120	2.16R
5	10	29.1700	28.6000	2.0451	0.5700	0.28
6	18	50.0200	51.1300	2.0451	-1.1100	-0.54
7	9	26.0800	22.6400	2.0451	3.4400	1.68
8	2	28.0300	28.6000	2.0451	-0.5700	-0.28
9	4	84.9600	84.3400	2.0451	0.6200	0.30
10	6	27.9000	27.0900	2.0451	0.8100	0.40
11	1	19.2000	22.6400	2.0451	-3.4400	-1.68
12	12	83.7200	84.3400	2.0451	-0.6200	-0.30
13	14	26.2800	27.0900	2.0451	-0.8100	-0.40
14	15	49.2400	48.7950	2.0451	0.4450	0.22
15	17	52.2400	51.1300	2.0451	1.1100	0.54
16	13	17.5000	17.1000	2.0451	0.4000	0.20
17	7	48.3500	48.7950	2.0451	-0.4450	-0.22
18	5	16.7000	17.1000	2.0451	-0.4000	-0.20

R denotes an observation with a large standardized residual.

Estimated Coefficients for Citric acid using data in uncoded units

Term	Coef
Constant	5.49368
pH	-6.30841
Init. Fructose	0.0888201
Nutrients	-14.0718
pH*Init. Fructose	0.0879092
pH*Nutrients	3.14306
Init. Fructose*Nutrients	0.0976320
pH*Init. Fructose*Nutrients	-0.0242294
Ct Pt	7.24913

Least Squares Means for Citric acid

	Mean	SE Mean
pH		
2	33.23	1.023
5	54.53	1.023
Init. Fructose		
102	23.86	1.023
203	63.90	1.023
Nutrients		
-1	45.00	1.023
1	42.77	1.023
pH*Init. Fructose		
2 102	19.87	1.446
5 102	27.85	1.446

2 203	46.60	1.446
5 203	81.21	1.446
pH*Nutrients		
2 -1	33.52	1.446
5 -1	56.47	1.446
2 1	32.95	1.446
5 1	52.58	1.446
Init. Fructose*Nutrients		
102 -1	25.62	1.446
203 -1	64.37	1.446
102 1	22.10	1.446
203 1	63.44	1.446
pH*Init. Fructose*Nutrients		
2 102 -1	22.64	2.045
5 102 -1	28.60	2.045
2 203 -1	44.40	2.045
5 203 -1	84.34	2.045
2 102 1	17.10	2.045
5 102 1	27.09	2.045
2 203 1	48.79	2.045
5 203 1	78.08	2.045

Mean for Center Point = 51.13

Predicted Response for New Design Points Using Model for Citric acid

Point	Fit	SE Fit	95% CI	95% PI
1	78.0800	2.0451	(73.4538; 82.7062)	(70.0671; 86.0929)
2	44.4020	2.0451	(39.7758; 49.0282)	(36.3891; 52.4149)
3	78.0800	2.0451	(73.4538; 82.7062)	(70.0671; 86.0929)
4	44.4020	2.0451	(39.7758; 49.0282)	(36.3891; 52.4149)
5	28.6000	2.0451	(23.9738; 33.2262)	(20.5871; 36.6129)
6	51.1300	2.0451	(46.5038; 55.7562)	(43.1171; 59.1429)
7	22.6400	2.0451	(18.0138; 27.2662)	(14.6271; 30.6529)
8	28.6000	2.0451	(23.9738; 33.2262)	(20.5871; 36.6129)
9	84.3400	2.0451	(79.7138; 88.9662)	(76.3271; 92.3529)
10	27.0900	2.0451	(22.4638; 31.7162)	(19.0771; 35.1029)
11	22.6400	2.0451	(18.0138; 27.2662)	(14.6271; 30.6529)
12	84.3400	2.0451	(79.7138; 88.9662)	(76.3271; 92.3529)
13	27.0900	2.0451	(22.4638; 31.7162)	(19.0771; 35.1029)
14	48.7950	2.0451	(44.1688; 53.4212)	(40.7821; 56.8079)
15	51.1300	2.0451	(46.5038; 55.7562)	(43.1171; 59.1429)
16	17.1000	2.0451	(12.4738; 21.7262)	(9.0871; 25.1129)
17	48.7950	2.0451	(44.1688; 53.4212)	(40.7821; 56.8079)
18	17.1000	2.0451	(12.4738; 21.7262)	(9.0871; 25.1129)

Factorial Fit: Res. Fructose versus pH; Init. Fructose; Nutrients

Estimated Effects and Coefficients for Res. Fructose (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		76.930	2.813	27.35	0.000
pH	-15.525	-7.763	2.813	-2.76	0.022
Init. Fructose	58.555	29.277	2.813	10.41	0.000
Nutrients	3.298	1.649	2.813	0.59	0.572
pH*Init. Fructose	-13.780	-6.890	2.813	-2.45	0.037
pH*Nutrients	-3.078	-1.539	2.813	-0.55	0.598
Init. Fructose*Nutrients	-12.547	-6.274	2.813	-2.23	0.053
pH*Init. Fructose*Nutrients	22.428	11.214	2.813	3.99	0.003
Ct Pt		11.400	8.438	1.35	0.210

S = 11.2508 R-Sq = 94.17% R-Sq(adj) = 88.98%

Analysis of Variance for Res. Fructose (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	14722.3	14722.3	4907.4	38.77	0.000
2-Way Interactions	3	1427.2	1427.2	475.7	3.76	0.053
3-Way Interactions	1	2012.0	2012.0	2012.0	15.89	0.003
Curvature	1	231.0	231.0	231.0	1.83	0.210
Residual Error	9	1139.2	1139.2	126.6		
Pure Error	9	1139.2	1139.2	126.6		
Total	17	19531.8				

Obs	StdOrder	Res.Fructose	Fit	SEFit	Residual	StResid
1	8	106.430	96.605	7.956	9.825	1.23
2	3	141.520	135.160	7.956	6.360	0.80
3	16	86.780	96.605	7.956	-9.825	-1.23
4	11	128.800	135.160	7.956	-6.360	-0.80
5	10	61.430	51.610	7.956	9.820	1.23
6	18	88.980	88.330	7.956	0.650	0.08
7	9	19.700	27.850	7.956	-8.150	-1.02
8	2	41.790	51.	6107.956	-9.820	-1.23
9	4	73.990	86.505	7.956	-12.515	-1.57
10	6	45.800	41.950	7.956	3.850	0.48
11	1	36.000	27.850	7.956	8.150	1.02
12	12	99.020	86.505	7.956	12.515	1.57
13	14	38.100	41.950	7.956	-3.850	-0.48
14	15	116.430	106.560	7.956	9.870	1.24
15	17	87.680	88.330	7.956	-0.650	-0.08
16	13	69.900	69.200	7.956	0.700	0.09
17	7	96.690	106.560	7.956	-9.870	-1.24
18	5	68.500	69.200	7.956	-0.700	-0.09

Estimated Coefficients for Res. Fructose using data in uncoded units

Term	Coef
Constant	-41.9181
pH	8.69596
Init. Fructose	0.898102
Nutrients	103.199
pH*Init. Fructose	-0.0909571
pH*Nutrients	-23.6014
Init. Fructose*Nutrients	-0.642360
pH*Init. Fructose*Nutrients	0.148036
Ct Pt	11.4000

Least Squares Means for Res. Fructose

	Mean	SE Mean
pH		
2	84.69	3.978
5	69.17	3.978
Init. Fructose		
102	47.65	3.978
203	106.21	3.978
Nutrients		
-1	75.28	3.978
1	78.58	3.978
pH*Init. Fructose		
2 102	48.53	5.625
5 102	46.78	5.625
2 203	120.86	5.625
5 203	91.56	5.625
pH*Nutrients		

2	-1	81.51 5.625
5	-1	69.06 5.625
2	1	87.88 5.625
5	1	69.28 5.625

Init. Fructose*Nutrients

102	-1	39.73 5.625
203	-1	110.83 5.625
102	1	55.58 5.625
203	1	101.58 5.625

pH*Init. Fructose*Nutrients

2	102	-1	27.85 7.956
5	102	-1	51.61 7.956
2	203	-1	135.16 7.956
5	203	-1	86.50 7.956
2	102	1	69.20 7.956
5	102	1	41.95 7.956
2	203	1	106.56 7.956
5	203	1	96.61 7.956

Mean for Center Point = 88.33

Predicted Response for New Design Points Using Model for Res. Fructose

Point	Fit	SE Fit	95% CI	95% PI
1	96.605	7.956	(78.608; 114.602)	(65.434; 127.776)
2	135.160	7.956	(117.163; 153.157)	(103.989; 166.331)
3	96.605	7.956	(78.608; 114.602)	(65.434; 127.776)
4	135.160	7.956	(117.163; 153.157)	(103.989; 166.331)
5	51.610	7.956	(33.613; 69.607)	(20.439; 82.781)
6	88.330	7.956	(70.333; 106.327)	(57.159; 119.501)
7	27.850	7.956	(9.853; 45.847)	(-3.321; 59.021)
8	51.610	7.956	(33.613; 69.607)	(20.439; 82.781)
9	86.505	7.956	(68.508; 104.502)	(55.334; 117.676)
10	41.950	7.956	(23.953; 59.947)	(10.779; 73.121)
11	27.850	7.956	(9.853; 45.847)	(-3.321; 59.021)
12	86.505	7.956	(68.508; 104.502)	(55.334; 117.676)
13	41.950	7.956	(23.953; 59.947)	(10.779; 73.121)
14	106.560	7.956	(88.563; 124.557)	(75.389; 137.731)
15	88.330	7.956	(70.333; 106.327)	(57.159; 119.501)
16	69.200	7.956	(51.203; 87.197)	(38.029; 100.371)
17	106.560	7.956	(88.563; 124.557)	(75.389; 137.731)
18	69.200	7.956	(51.203; 87.197)	(38.029; 100.371)

Factorial Fit: Biomass versus pH; Init. Fructose; Nutrients

Estimated Effects and Coefficients for Biomass (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		14.727	0.8136	18.10	0.000
pH	11.181	5.591	0.8136	6.87	0.000
Init. Fructose	0.016	0.008	0.8136	0.01	0.992
Nutrients	-4.991	-2.496	0.8136	-3.07	0.013
pH*Init. Fructose	4.719	2.359	0.8136	2.90	0.018
pH*Nutrients	-1.834	-0.917	0.8136	-1.13	0.289
Init. Fructose*Nutrients	-0.854	-0.427	0.8136	-0.52	0.612
pH*Init. Fructose*Nutrients	-5.571	-2.786	0.8136	-3.42	0.008
Ct Pt	-3.967	2.4408	-1.63	0.139	

S = 3.25437 R-Sq = 89.99% R-Sq(adj) = 81.10%

Analysis of Variance for Biomass (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P

Main Effects	3	599.73	599.73	199.91	18.88	0.000
2-Way Interactions	3	105.43	105.43	35.14	3.32	0.071
3-Way Interactions	1	124.16	124.16	124.16	11.72	0.008
Curvature	1	27.98	27.98	27.98	2.64	0.139
Residual Error	9	95.32	95.32	10.59		
Pure Error	9	95.32	95.32	10.59		
Total	17	952.61				

Obs	StdOrder	Biomass	Fit	SEFit	Residual	StResid
1	8	10.8600	16.0600	2.3012	-5.2000	-2.26R
2	3	1.9700	6.0050	2.3012	-4.0350	-1.75
3	16	21.2600	16.0600	2.3012	5.2000	2.26R
4	11	10.0400	6.0050	2.3012	4.0350	1.75
5	10	17.9000	18.1500	2.3012	-0.2500	-0.11
6	18	10.3700	10.7600	2.3012	-0.3900	-0.17
7	9	16.3400	15.4250	2.3012	0.9150	0.40
8	2	18.4000	18.1500	2.3012	0.2500	0.11
9	4	29.7200	29.3100	2.3012	0.4100	0.18
10	6	18.7000	17.7500	2.3012	0.9500	0.41
11	1	14.5100	15.4250	2.3012	-0.9150	-0.40
12	12	28.9000	29.3100	2.3012	-0.4100	-0.18
13	14	16.8000	17.7500	2.3012	-0.9500	-0.41
14	15	8.6200	7.5650	2.3012	1.0550	0.46
15	17	11.1500	10.7600	2.3012	0.3900	0.17
16	13	8.6000	7.5500	2.3012	1.0500	0.46
17	7	6.5100	7.5650	2.3012	-1.0550	-0.46
18	5	6.5000	7.5500	2.3012	-1.0500	-0.46

R denotes an observation with a large standardized residual.

Estimated Coefficients for Biomass using data in uncoded units

Term	Coef
Constant	18.2822
pH	-1.02281
Init. Fructose	-0.108853
Nutrients	-18.6953
pH*Init. Fructose	0.0311469
pH*Nutrients	4.99677
Init. Fructose*Nutrients	0.120256
pH*Init. Fructose*Nutrients	-0.0367739
Ct Pt	-3.96688

Least Squares Means for Biomass

	Mean	SE Mean
pH		
2	9.136	1.151
5	20.318	1.151
Init. Fructose		
102	14.719	1.151
203	14.735	1.151
Nutrients		
-1	17.223	1.151
1	12.231	1.151
pH*Init. Fructose		
2 102	11.488	1.627
5 102	17.950	1.627
2 203	6.785	1.627
5 203	22.685	1.627
pH*Nutrients		

2	-1	10.715 1.627
5	-1	23.730 1.627
2	1	7.558 1.627
5	1	16.905 1.627

Init. Fructose*Nutrients

102	-1	16.788 1.627
203	-1	17.658 1.627
102	1	12.650 1.627
203	1	11.813 1.627

pH*Init. Fructose*Nutrients

2 102	-1	15.425 2.301
5 102	-1	18.150 2.301
2 203	-1	6.005 2.301
5 203	-1	29.310 2.301
2 102	1	7.550 2.301
5 102	1	17.750 2.301
2 203	1	7.565 2.301
5 203	1	16.060 2.301

Mean for Center Point = 10.760

Predicted Response for New Design Points Using Model for Biomass

Point	Fit	SE Fit	95% CI	95% PI
1	16.0600	2.3012	(10.8544; 21.2656)	(7.0436; 25.0764)
2	6.0050	2.3012	(0.7994; 11.2106)	(-3.0114; 15.0214)
3	16.0600	2.3012	(10.8544; 21.2656)	(7.0436; 25.0764)
4	6.0050	2.3012	(0.7994; 11.2106)	(-3.0114; 15.0214)
5	18.1500	2.3012	(12.9444; 23.3556)	(9.1336; 27.1664)
6	10.7600	2.3012	(5.5544; 15.9656)	(1.7436; 19.7764)
7	15.4250	2.3012	(10.2194; 20.6306)	(6.4086; 24.4414)
8	18.1500	2.3012	(12.9444; 23.3556)	(9.1336; 27.1664)
9	29.3100	2.3012	(24.1044; 34.5156)	(20.2936; 38.3264)
10	17.7500	2.3012	(12.5444; 22.9556)	(8.7336; 26.7664)
11	15.4250	2.3012	(10.2194; 20.6306)	(6.4086; 24.4414)
12	29.3100	2.3012	(24.1044; 34.5156)	(20.2936; 38.3264)
13	17.7500	2.3012	(12.5444; 22.9556)	(8.7336; 26.7664)
14	7.5650	2.3012	(2.3594; 12.7706)	(-1.4514; 16.5814)
15	10.7600	2.3012	(5.5544; 15.9656)	(1.7436; 19.7764)
16	7.5500	2.3012	(2.3444; 12.7556)	(-1.4664; 16.5664)
17	7.5650	2.3012	(2.3594; 12.7706)	(-1.4514; 16.5814)
18	7.5500	2.3012	(2.3444; 12.7556)	(-1.4664; 16.5664)

Factorial Fit: Other Acid versus pH; Init. Fructose; Nutrients

Estimated Effects and Coefficients for Other Acid (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		14.254	0.7449	19.14	0.000
pH	-0.439	-0.219	0.7449	-0.29	0.775
Init. Fructose	9.269	4.634	0.7449	6.22	0.000
Nutrients	0.914	0.457	0.7449	0.61	0.555
pH*Init. Fructose	-2.174	-1.087	0.7449	-1.46	0.179
pH*Nutrients	-1.814	-0.907	0.7449	-1.22	0.254
Init. Fructose*Nutrients	1.609	0.804	0.7449	1.08	0.308
pH*Init. Fructose*Nutrients	-1.024	-0.512	0.7449	-0.69	0.509
Ct Pt		-1.359	2.2346	-0.61	0.558

S = 2.97946 R-Sq = 83.27% R-Sq(adj) = 68.40%

Analysis of Variance for Other Acid (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P

Main Effects	3	347.749	347.749	115.916	13.06	0.001
2-Way Interactions	3	42.412	42.412	14.137	1.59	0.258
3-Way Interactions	1	4.192	4.192	4.192	0.47	0.509
Curvature	1	3.285	3.285	3.285	0.37	0.558
Residual Error	9	79.895	79.895	8.877		
Pure Error	9	79.895	79.895	8.877		
Total	17	477.533				

Obs	StdOrder	Other Acid	Fit	SE Fit	Residual	St Resid
1	8	21.5500	17.4250	2.1068	4.1250	1.96
2	3	13.9900	17.5150	2.1068	-3.5250	-1.67
3	16	13.3000	17.4250	2.1068	-4.1250	-1.96
4	11	21.0400	17.5150	2.1068	3.5250	1.67
5	10	10.2100	11.2300	2.1068	-1.0200	-0.48
6	18	12.5800	12.8950	2.1068	-0.3150	-0.15
7	9	8.0900	8.7050	2.1068	-0.6150	-0.29
8	2	12.2500	11.2300	2.1068	1.0200	0.48
9	4	15.1000	17.7400	2.1068	-2.6400	-1.25
10	6	9.6900	9.7450	2.1068	-0.0550	-0.03
11	1	9.3200	8.7050	2.1068	0.6150	0.29
12	12	20.3800	17.7400	2.1068	2.6400	1.25
13	14	9.8000	9.7450	2.1068	0.0550	0.03
14	15	21.5500	22.8750	2.1068	-1.3250	-0.63
15	17	13.2100	12.8950	2.1068	0.3150	0.15
16	13	8.2900	8.8000	2.1068	-0.5100	-0.24
17	7	24.2000	22.8750	2.1068	1.3250	0.63
18	5	9.3100	8.8000	2.1068	0.5100	0.24

Estimated Coefficients for Other Acid using data in uncoded units

Term	Coef
Constant	-6.88699
pH	2.04185
Init. Fructose	0.141988
Nutrients	-3.46291
pH*Init. Fructose	-0.0143482
pH*Nutrients	0.42592
Init. Fructose*Nutrients	0.0395792
pH*Init. Fructose*Nutrients	-0.00675743
Ct Pt	-1.35938

Least Squares Means for Other Acid

	Mean	SE Mean
pH		
2	14.474	1.053
5	14.035	1.053
Init. Fructose		
102	9.620	1.053
203	18.889	1.053
Nutrients		
-1	13.798	1.053
1	14.711	1.053
pH*Init. Fructose		
2 102	8.753	1.490
5 102	10.488	1.490
2 203	20.195	1.490
5 203	17.583	1.490
pH*Nutrients		
2 -1	13.110	1.490
5 -1	14.485	1.490

2	1	15.838 1.490
5	1	13.585 1.490
Init. Fructose*Nutrients		
102	-1	9.968 1.490
203	-1	17.628 1.490
102	1	9.273 1.490
203	1	20.150 1.490
pH*Init. Fructose*Nutrients		
2 102	-1	8.705 2.107
5 102	-1	11.230 2.107
2 203	-1	17.515 2.107
5 203	-1	17.740 2.107
2 102	1	8.800 2.107
5 102	1	9.745 2.107
2 203	1	22.875 2.107
5 203	1	17.425 2.107

Mean for Center Point = 12.895

Predicted Response for New Design Points Using Model for Other Acid

Point	Fit	SE Fit	95% CI	95% PI
1	17.4250	2.1068	(12.6591; 22.1909)	(9.1702; 25.6798)
2	17.5150	2.1068	(12.7491; 22.2809)	(9.2602; 25.7698)
3	17.4250	2.1068	(12.6591; 22.1909)	(9.1702; 25.6798)
4	17.5150	2.1068	(12.7491; 22.2809)	(9.2602; 25.7698)
5	11.2300	2.1068	(6.4641; 15.9959)	(2.9752; 19.4848)
6	12.8950	2.1068	(8.1291; 17.6609)	(4.6402; 21.1498)
7	8.7050	2.1068	(3.9391; 13.4709)	(0.4502; 16.9598)
8	11.2300	2.1068	(6.4641; 15.9959)	(2.9752; 19.4848)
9	17.7400	2.1068	(12.9741; 22.5059)	(9.4852; 25.9948)
10	9.7450	2.1068	(4.9791; 14.5109)	(1.4902; 17.9998)
11	8.7050	2.1068	(3.9391; 13.4709)	(0.4502; 16.9598)
12	17.7400	2.1068	(12.9741; 22.5059)	(9.4852; 25.9948)
13	9.7450	2.1068	(4.9791; 14.5109)	(1.4902; 17.9998)
14	22.8750	2.1068	(18.1091; 27.6409)	(14.6202; 31.1298)
15	12.8950	2.1068	(8.1291; 17.6609)	(4.6402; 21.1498)
16	8.8000	2.1068	(4.0341; 13.5659)	(0.5452; 17.0548)
17	22.8750	2.1068	(18.1091; 27.6409)	(14.6202; 31.1298)
18	8.8000	2.1068	(4.0341; 13.5659)	(0.5452; 17.0548)

Factorial Fit: TOT Acids versus pH; Init. Fructose; Nutrients

Estimated Effects and Coefficients for TOT Acids (coded units)

Term	Effect	Coef	SE Coef	T	P
Constant		57.073	1.276	44.73	0.000
pH	18.730	9.365	1.276	7.34	0.000
Init. Fructose	47.190	23.595	1.276	18.49	0.000
Nutrients	-3.440	-1.720	1.276	-1.35	0.211
pH*Init. Fructose	9.020	4.510	1.276	3.53	0.006
pH*Nutrients	-5.595	-2.797	1.276	-2.19	0.056
Init. Fructose*Nutrients	0.780	0.390	1.276	0.31	0.767
pH*Init. Fructose*Nutrients	-6.820	-3.410	1.276	-2.67	0.026
Ct Pt		6.953	3.828	1.82	0.103

S = 5.10427 R-Sq = 97.93% R-Sq(adj) = 96.09%

Analysis of Variance for TOT Acids (coded units)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Main Effects	3	10358.2	10358.2	3452.72	132.52	0.000
2-Way Interactions	3	453.1	453.1	151.03	5.80	0.017

3-Way Interactions	1	186.0	186.0	186.05	7.14 0.026
Curvature	1	85.9	85.9	85.93	3.30 0.103
Residual Error	9	234.5	234.5	26.05	
Pure Error	9	234.5	234.5	26.05	
Total	17	11317.7			

Obs	StdOrder	TOT Acids	Fit	SE Fit	Residual	St Resid
1	8	80.790	87.005	3.609	-6.215	-1.72
2	3	53.980	61.915	3.609	-7.935	-2.20R
3	16	93.220	87.005	3.609	6.215	1.72
4	11	69.850	61.915	3.609	7.935	2.20R
5	10	39.380	39.830	3.609	-0.450	-0.12
6	18	62.600	64.025	3.609	-1.425	-0.39
7	9	34.170	31.345	3.609	2.825	0.78
8	2	40.280	39.830	3.609	0.450	0.12
9	4	100.060	102.080	3.609	-2.020	-0.56
10	6	37.590	36.835	3.609	0.755	0.21
11	1	28.520	31.345	3.609	-2.825	-0.78
12	12	104.100	102.080	3.609	2.020	0.56
13	14	36.080	36.835	3.609	-0.755	-0.21
14	15	70.790	71.670	3.609	-0.880	-0.24
15	17	65.450	64.025	3.609	1.425	0.39
16	13	25.790	25.900	3.609	-0.110	-0.03
17	7	72.550	71.670	3.609	0.880	0.24
18	5	26.010	25.900	3.609	0.110	0.03

R denotes an observation with a large standardized residual.

Estimated Coefficients for TOT Acids using data in uncoded units

Term	Coef
Constant	-4.2530
pH	-2.83620
Init. Fructose	0.258845
Nutrients	-20.3978
pH*Init. Fructose	0.0595380
pH*Nutrients	5.00002
Init. Fructose*Nutrients	0.165281
pH*Init. Fructose*Nutrients	-0.0450165
Ct Pt	6.95250

Least Squares Means for TOT Acids

	Mean	SE Mean
pH		
2	47.71	1.805
5	66.44	1.805
Init. Fructose		
102	33.48	1.805
203	80.67	1.805
Nutrients		
-1	58.79	1.805
1	55.35	1.805
pH*Init. Fructose		
2 102	28.62	2.552
5 102	38.33	2.552
2 203	66.79	2.552
5 203	94.54	2.552
pH*Nutrients		
2 -1	46.63	2.552
5 -1	70.96	2.552
2 1	48.79	2.552
5 1	61.92	2.552

Init. Fructose*Nutrients
102 -1 35.59 2.552
203 -1 82.00 2.552
102 1 31.37 2.552
203 1 79.34 2.552
pH*Init. Fructose*Nutrients
2 102 -1 31.35 3.609
5 102 -1 39.83 3.609
2 203 -1 61.91 3.609
5 203 -1 102.08 3.609
2 102 1 25.90 3.609
5 102 1 36.84 3.609
2 203 1 71.67 3.609
5 203 1 87.01 3.609

Mean for Center Point = 64.03

Predicted Response for New Design Points Using Model for TOT Acids

Point	Fit	SE Fit	95% CI	95% PI
1	87.005	3.609	(78.840; 95.170)	(72.863; 101.147)
2	61.915	3.609	(53.750; 70.080)	(47.773; 76.057)
3	87.005	3.609	(78.840; 95.170)	(72.863; 101.147)
4	61.915	3.609	(53.750; 70.080)	(47.773; 76.057)
5	39.830	3.609	(31.665; 47.995)	(25.688; 53.972)
6	64.025	3.609	(55.860; 72.190)	(49.883; 78.167)
7	31.345	3.609	(23.180; 39.510)	(17.203; 45.487)
8	39.830	3.609	(31.665; 47.995)	(25.688; 53.972)
9	102.080	3.609	(93.915; 110.245)	(87.938; 116.222)
10	36.835	3.609	(28.670; 45.000)	(22.693; 50.977)
11	31.345	3.609	(23.180; 39.510)	(17.203; 45.487)
12	102.080	3.609	(93.915; 110.245)	(87.938; 116.222)
13	36.835	3.609	(28.670; 45.000)	(22.693; 50.977)
14	71.670	3.609	(63.505; 79.835)	(57.528; 85.812)
15	64.025	3.609	(55.860; 72.190)	(49.883; 78.167)
16	25.900	3.609	(17.735; 34.065)	(11.758; 40.042)
17	71.670	3.609	(63.505; 79.835)	(57.528; 85.812)
18	25.900	3.609	(17.735; 34.065)	(11.758; 40.042)

Alias Structure
I
pH
Init. Fructose
Nutrients
pH*Init. Fructose
pH*Nutrients
Init. Fructose*Nutrients
pH*Init. Fructose*Nutrients

Response Optimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Maximum	19.20	48.00	48.00	1	1
Res.Fructos	Target	36.00	135.16	141.52	1	1
Biomass	Target	7.57	15.00	15.43	1	1

Global Solution

pH = 3.5
Init. Fructo = 152.5
Nutrients = 0.0

Predicted Responses

Citric acid = 51.13; desirability = 1.00000
Res. Fructos = 88.33; desirability = 0.52773
Biomass = 10.76; desirability = 0.42934

Composite Desirability = 0.60964

Response Optimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Maximum	17.5	48	48.00	1	1
Res.Fructos	Minimum	88.0	88	88.98	1	1
Biomass	Target	8.6	11	11.50	1	1

Global Solution

pH = 3.5
Init. Fructo = 152.5
Nutrients = 0.0

Predicted Responses

Citric acid = 51.13; desirability = 1.00000
Res. Fructos = 88.33; desirability = 0.66327
Biomass = 10.76; desirability = 0.90000

Composite Desirability = 0.84200

Response Optimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Maximum	19.20	48	48.00	1	1
Res.Fructos	Target	36.00	88	141.52	1	1
Biomass	Target	7.57	11	15.43	1	1

Local Solution

pH = 3.5
Init. Fructo = 152.5
Nutrients = 0.0

Predicted Responses

Citric acid = 51.13; desirability = 1.00000
Res. Fructos = 88.33; desirability = 0.99383
Biomass = 10.76; desirability = 0.93003

Composite Desirability = 0.97410

Response Optimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Target	39.99	84	84.96	1	1
Res.Fructos	Target	73.99	88	141.52	1	1
Biomass	Target	1.97	11	29.72	1	1

Local Solution

pH = 3.5

Init. Fructo = 152.5
Nutrients = 0.0

Predicted Responses

Citric acid = 51.13; desirability = 0.25312
Res. Fructos = 88.33; desirability = 0.99383
Biomass = 10.76; desirability = 0.97342

Composite Desirability = 0.62563

Response Optimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Maximum	16.70	90	90.00	1	1
Res.Fructos	Minimum	27.00	27	141.52	1	1
Biomass	Maximum	1.97	29	29.00	1	1

Global Solution

pH = 5
Init. Fructo = 203
Nutrients = -1

Predicted Responses

Citric acid = 84.340; desirability = 0.92278
Res. Fructos = 86.505; desirability = 0.48040
Biomass = 29.310; desirability = 1.00000

Composite Desirability = 0.76249

Response Optimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Maximum	16.70	90	90	1	1
Res.Fructos	Maximum	19.70	27	27	1	1
Biomass	Maximum	1.97	29	29	1	1

Global Solution

pH = 5
Init. Fructo = 203
Nutrients = -1

Predicted Responses

Citric acid = 84.340; desirability = 0.92278
Res. Fructos = 86.505; desirability = 1.00000
Biomass = 29.310; desirability = 1.00000

Composite Desirability = 0.97357

Response Optimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Maximum	17.70	84.34	84.34	1	1
Res.Fructos	Minimum	27.00	27.00	135.16	1	1
Biomass	Maximum	6.01	29.00	29.00	1	1

GlobalSolution

pH=5
Init.Fructo=203
Nutrients=-1

PredictedResponses

Citricacid = 84.340; desirability= 1.00000
Res.Fructos = 86.505; desirability= 0.44984
Biomass = 29.310; desirability= 1.00000

CompositeDesirability=0.76622

ResponseOptimization
Parameters

Goal Lower Target Upper Weight Import
Citricacid Maximum 17.708 4.348 4.3411
Res.Fructos Maximum 27.853 0.003 0.0011
Biomass Maximum 6.012 9.002 9.0011

GlobalSolution

pH=5
Init.Fructo = 203
Nutrients = -1

PredictedResponses

Citricacid= 84.340 ;desirability = 1
Res.Fructos= 86.505 ;desirability = 1
Biomass= 29.310 ;desirability = 1

CompositeDesirability=1.00000

ResponseOptimization

Parameters	Goal	Lower	Target	Upper	Weight	Import
Citricacid	Maximum	17.70	84.34	84.34	1	1
Res.Fructos	Maximum	27.00	27.00	135.16	1	1
Biomass	Maximum	6.01	29.00	29.00	1	1

GlobalSolution

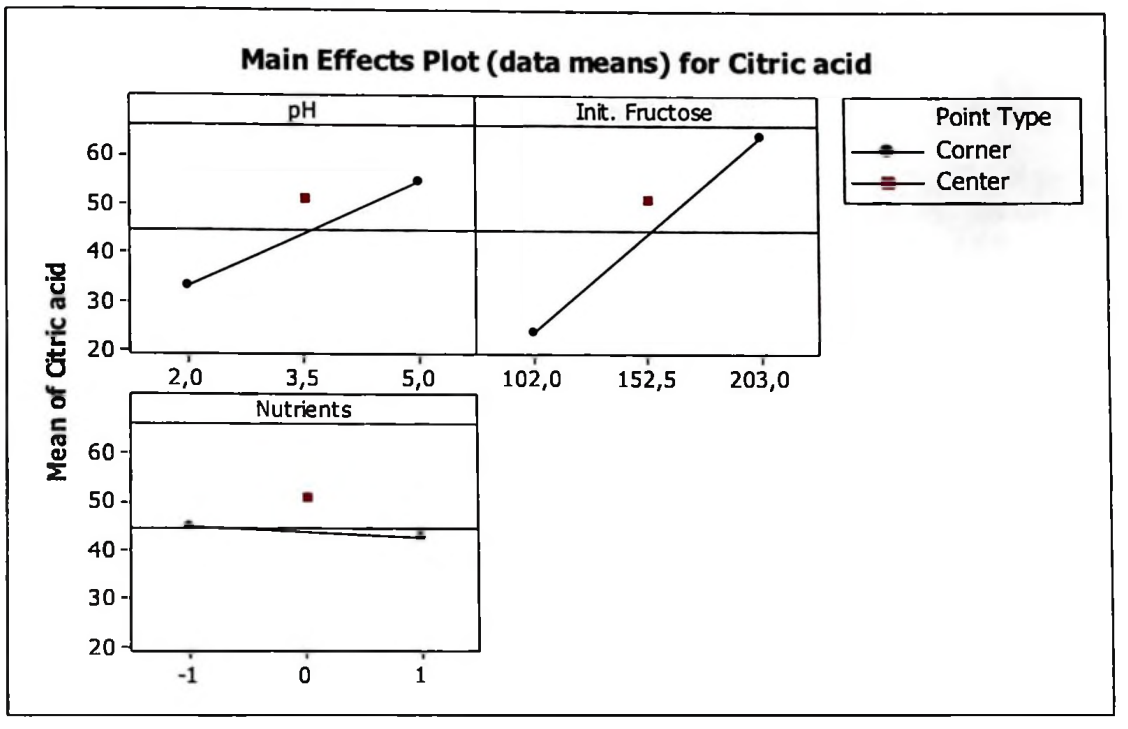
pH=5
Init.Fructo=203
Nutrients=-1

PredictedResponses

Citricacid= 84.340; desirability= 1.00000
Res.Fructos= 86.505; desirability= 0.54659
Biomass= 29.310; desirability= 1.00000

CompositeDesirability= 0.81763

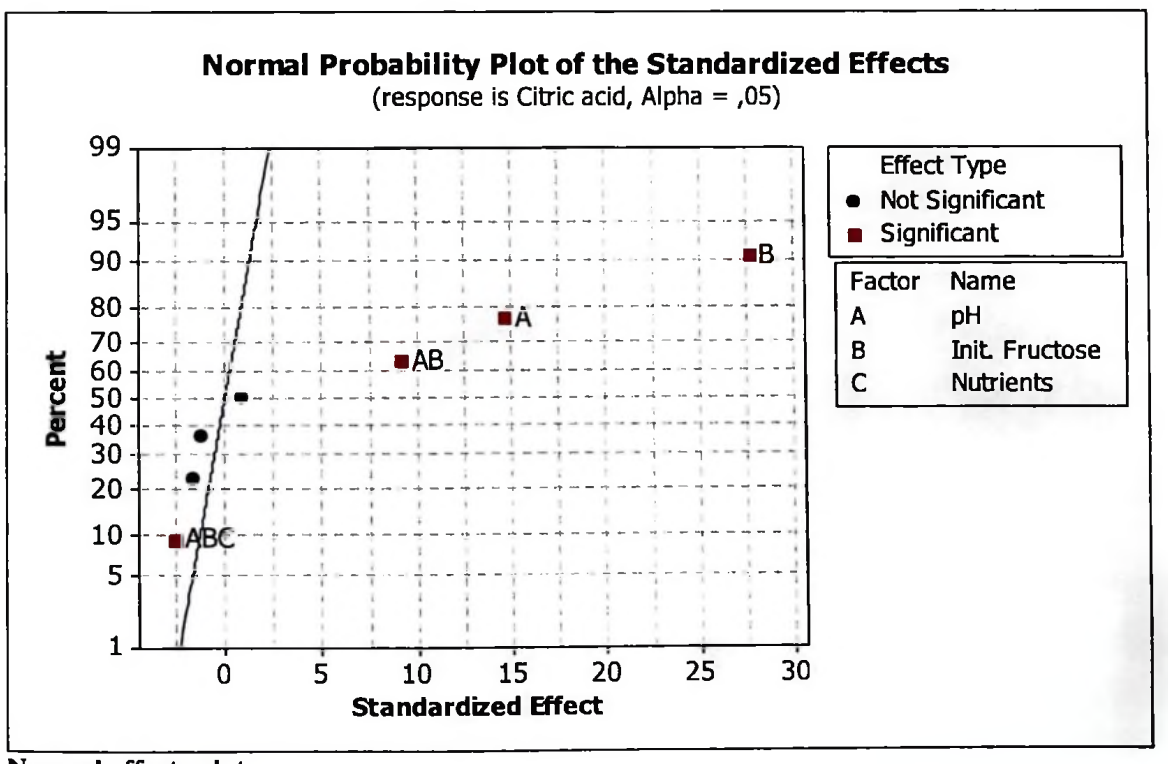
The four-in-one residual plot



main effects plot

Interactions plot

Pareto effects plot



Normal effects plot

cube plot

APPENDIX XI: General Linear Model (GLM): Multiple Comparisons using the Tukey Method

One of the most commonly used methods in statistical decision making is hypothesis testing. In general, a hypothesis test is a process in which you assume an initial claim to be true and then test this claim using sample data. Ordinarily, the initial claim refers to a population parameter of interest such as the population mean (m).

Hypothesis tests include two hypotheses: the null hypothesis (denoted by H0) and the alternative hypothesis (denoted by H1). The null hypothesis is the initial claim and is often specified using previous research or common knowledge. The alternative hypothesis is what you may believe to be true or hope to prove true. The alternative hypothesis is sometimes referred to as the research hypothesis.

The decision-making process for a hypothesis test can be based on the probability value (p-value) for the given test.

- If the p-value is less than or equal to a predetermined level of significance (α-level), then you reject the null hypothesis and claim support for the alternative hypothesis.

- If your p-value is greater than the α-level, you fail to reject the null hypothesis and cannot claim support for the alternative hypothesis.

When you perform a hypothesis test, there are four possible outcomes. The outcomes depend on whether the null hypothesis is true or false and whether you reject or fail to reject the null hypothesis. These outcomes are summarized in the following table:

	Null Hypothesis	
Decision	True	False
fail to reject Ho	correct decision	
$p = 1 - \alpha$		Type II error
$p = \beta$		
reject Ho	Type I error	
$p = \alpha$	correct decision	
$p = 1 - \beta$		

When the null hypothesis is true and you reject it, you make a type I error. The probability of making α type I error is called alpha (α) and is sometimes referred to as the level of significance.
When the null hypothesis is false and you fail to reject it, you make a type II error. The probability of making a type II error is called beta (β).
The probability of rejecting the null hypothesis when it is false is equal to 1 - β. This value is also referred to as the power of the test.

Hypothesis test

Use the adjusted p-values provided with the individual hypothesis tests to determine whether pairs of means are different. These p-values are adjusted to maintain the family error rate at the desired α-level (in this case 0.05):

- If the adjusted p-value for a comparison is less than or equal to your chosen α-level, the difference between the means is significant.

- If the adjusted p-value is greater than your chosen a-level, the difference between means is not significant.

For the salary analysis, comparisons were requested between the mean for subject 4 (the designated control level) and the means for all other levels of the subject factor. The confidence intervals for the comparisons reveal the following:

- The adjusted p-value for the difference between the means for subject 4 and 1 (0.0000) is lower than the chosen α-level of 0.05, indicating that this difference is significant. In addition, the difference (Differences of Means, -0.6900) is negative, indicating that teaching subject 1 paid less than teaching subject 4.

- The adjusted p-value for the difference between the means for subjects 4 and 2 (0.9207) is greater than the chosen α-level, indicating that these means are not significantly different.

The adjusted p-value for the difference between the means for subjects 4 and 3 (0.0017) is lower than the chosen a-level, indicating that the mean for subject 4 is significantly different from that of subject 3. In addition, the difference between the means (0.2733) is positive, indicating that teaching subject 3 paid better than teaching subject 4.

In Depth

Data description
Choosing a comparison method
Hypothesis testing
Choosing an a-level
Statistical_versus_practical_significance

APPENDIX XII: First order decay (FOD) model of IPCC (UNFCCC, 2007).

$$BE_{CH4,y} = \varphi \bullet (1 - f_y) \bullet GWP_{CH4} \bullet (1 - OX_y) \bullet \frac{16}{12} \bullet F \bullet DOC_f \bullet MCF \bullet W \bullet DOC_w \bullet e^{-kw(y-x)} \bullet (1 - e^{-kw}) \quad [33]$$

TABLE 1: Abreviations and calculated values from model of IPCC (UNFCCC, 2007).*(Salum, 2008): **(Ngonyani, 2009)

Symbol	Description	Value		
		HSE(a)	**HSE(b)**	KT
φ	model correction factor to account for model uncertainties,	0.9	**0.9**	0.9
fy	fraction of methane recovered at the disposal site,	0	**0**	0
$GWPCH_4$	global warming potential of methane,	21	**21**	21
OX	oxidation factor	0	**0**	0
F	volume fraction of methane in the emitted gas,	0.5	**0.5**	0.5
DOCf	decomposable degradable organic carbon (fraction),	0.5	**0.5**	0.5
MCF	methane correction factor,	0.4	**0.4**	0.4
Wy	amount of waste avoided from being dumped in the disposal site in year y (tons),	92072	**93002**	341,640
DOCw	weight of decomposable degradable organic carbon in the waste (fraction),	0.4	**0.4**	0.4
kw	decay rate for the sisal waste,	0.045	**0.045**	0.045
y	year for which CH_4 emission is calculated, and	2009	**2009**	2008
e	exponential constant	2.718	**2.718**	2.718
$BECH_{4,y}$	baseline emissions of methane from sisal waste in year y (tCO2e),	**4086	****4128**	*15152

HSE= Highlands sisal estate estimate (a=198 Ha; b= 200Ha); KAT=KATANI LTD- four estates as estimated by Salum, (2008)

APPENDIX XIII: Selected HPLC chromatogram data for inulin extracts (retention values & concentrations)

DaxLite 7.1 Massende 1/31/2000 5:28:41PM

Peaks list:

INUL2451~1.TXT Col 2 * (not saved)

Measured 28/06/2007 4:00:00AM by CJNM

Description: clear juice

Point to point noise (V): 0.021241 (1.01%); RMS Noise (V): 0.0079864 (0.38%)

Peak	Begin/RT(min)	Top (min)	End (min)	Top (V)	Annotation	Component	Actual conc	Rel.Conc.%	RSDev (min)	Area (V.min)	Asymmetry	AppMob	RPeakRel/ Mig %
1	**8.317**	8.667	9.350	0.597	Inu A	0.1764	**2.0**	11.35	0.159	0.2072	2.154	147.54	65.9
2	**18.633**	19.100	19.867	0.944	Inu A	0.3527	**6.4**	18.20	0.164	0.3322	2.231	72.82	66.3
3	**28.950**	29.533	30.383	1.479	Inu A	0.5291	**15.4**	29.19	0.177	0.5326	2.000	48.34	62.9
4	**39.450**	39.967	40.850	1.987	Inu A	0.7055	**29.1**	41.25	0.181	0.7527	2.143	36.17	64.3
4 of 4 peaks										**1.8247**			

DaxLite 7.1 Massende 1/31/2000 5:40:11PM

Peaks list:

INUL2851~1.TXT Col 2 * (not saved)

Measured 28/06/2007 4:00:00AM by CJNM

Description: Dark juice

Point to point noise (V): 0.022093 (0.53%); RMS Noise (V): 0.008034 (0.19%)

Peak	Begin/RT (min)	Top (min)	End (min)	Top (V)	Annotation	Component	Actual Conc	Rel.Conc.%	RSDev (min)	Area (V.min)	Asymmetry	AppMob	RPeakRel/ Mig %
1	**8.318**	9.035	10.318	1.167	Inu B	0.168	**1.904**	11.34	0.281	0.6860	1.708	130.43	59.5
2	**18.317**	19.000	20.367	1.823	Inu B	0.336	**5.996**	17.86	0.280	1.0804	1.783	68.18	62.5
3	**28.323**	29.073	30.573	2.960	Inu B	0.504	**14.625**	29.04	0.304	1.7568	1.640	46.18	58.2
4S	**38.890**	39.523	41.623	4.119	Inu B	0.671	**27.900**	41.55	0.340	2.5132	1.792	34.94	61.1
5s	**41.007**	41.207	41.623	0.039	Inu B	0.003	**0.001**	0.21	0.130	0.0127	2.083	33.62	72.0
5 of 5 peaks										**6.0491**			

APPENDIX XIV: Bacteria colony characteristics and API 20 NE results for non-enteric Gram (-) 24-48 hrs

Table 4: Bacteria colony characteristics

	Biochemical Characteristics Procedures							COMMENTS
ID	13	RTN4a	other	RTC4	RTC6a	WA4	7	Viewed from above
Media	NA	NA	NA	CZ	CZ	WAYE	NA	
Temp ºC	30	30	30	20	20	30	30	
Hrs	24	24	24	24	24	24	24	
Size	2	1.5	2	1.5	3	1.5	3	Average diameter of several c.f.u (mm)
General Shape:	Spr	Ro	Ro	Ro	Ro	Ro	Spr	(Spr)=Spreading; (Ro)=Round, irregular
Gram Staining	G(-) R	G(-) C	G(-) R	G(-) C	G(-) C	G(-) C	G(-) R	G(-) R Gram-negative rods G(-) C Gram-negative cocci - cells appeared pink to red.
Catalase	(+)	(+)	(+)	(+)	(+)	(-)	(+)	The evolution of bubbles after drops of H_2O_2
Oxidase	(+)	(-)	(+)	(-)	(-)	(-)	(-)	Presence of purple coloration
Endospores:	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Endospores stained green, while vegetative cells were stained red
Oxygen demand	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Not conclusive leakage??
Minimal nutrient	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Growth motile flagellates
Maconkey	(-)	(-)	(-)	(-)	(-)	(-)	(-)	
Starch Hydrolysis	(+)	(+)	(+)	(+)	(+)	(+)	(+)	A clear zone indicates hydrolysis.
Lipid Hydrolysis	ND	ND	ND	ND	ND	ND	ND	(not determined)
Gelatin Hydrolysis	(+)	(-)	(+)	(-)	(-)	(-)	(+)	Liquefaction of the gelatin a positive test for gelatin hydrolysis.
Casein Hydrolysis	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Clearing around growth is casein hydrolysis
Litmus milk	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Alkaline positive
Sulfur Indole Motility Test	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Black ppt hydrogen sulfide formation. Growth away stab line is motility
Minimum agar	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Motile flagellate confirmed
MaConkey	(-)	(-)	(-)	(-)	(-)	(-)	(-)	Negative re confirms G (-) for endo-spores
FE	(+)	(+)	(+)	(+)	(+)	(+)	(+)	positive re confirms G (+) for endo-spore
FM	(-)	(-)	(-)	(-)	(-)	(-)	(-)	Pinkish red (+) pH; Red and orange (-)acid

Table 5: Antibiotic tests (AB Biodisk)

		Biochemical Characteristics Procedures							
ID		13	RTN4a	other	RTC4	RTC6a	WA4	7	COMMENTS
	Drug	NA	NA	NA	CZ	CZ	WAYE	NA	
		Temp ºC 30			Temp ºC 20		Temp ºC 30		
Hrs		24	24	24	24	24	24	24	
	Tetracyclines								
	(**CL**)	(R)	(IS)	(R)	(IS)	(R)	(R)	(R)	(**CL**) Chloramphenicol30µg(IS)Intermediate - Sensitive to systemic infection dosage to be concentrated to organs (R); Resistant to
	(**TC**)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(**TC**) Tetracyclin30g (R)Resistant to Tetracyclin30g
	(**RI**)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(**RI**) Rifampicines 2µg(R)Resistant to
	Penicillins								
	(**PG**)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)Resistant to (**PG**) Benzyl penicillin (G) 100µg
	(**AM**)	(S)	(S)	(R)	(S)	(R)	(R)	(R)	(IS)-(S)Intermediate Sensitive to systemic infection dosage to be concentrated to organs (**AM**) Ampicilin10µg
	(**GM**)	(R)	(R)	(IS)	((R)	(IS)	(IS)	(IS)	(IS)-(S)Intermediate Sensitive to systemic infection dosage to be concentrated to organs (**GM**) Gentamycin 30µg
	Aminoglycosides								
	(**SM**)	(S)	(S)	(S)	(S)	(S)	(S)	(S)	Sensitive to (**SM**) Streptomycin 30µg (S)
	Sulphonamides								
	(**SU**)	(R)	(R)	(R)	(R)	(R)	(R)	(R)	(R)Resistant to (**SU**) Sulphonamide Trimethoprim250µg
Inference						(R)	(R)		Bacteria are resistant to most common antibacterial

Table 6: API 20 NE TESTS

	Biochemical Characteristics Procedures							COMMENTS
ID	13	RTN	other	4a	RTC6a	WA4	TN3b	
Media	NA	NA	NA	CZ	CZ	WAYE	NA	
Temp ºC	30	30	30	20	20	30	30	
API 20 NE code-24 hrs	1510004	1430000	1510004	1430000	1600000	0400000	0430000	
code-48 hrs	1510004	1430000	1510004	1430000	1600000	0400000	1430000	NO_3 & N_2 tested +Ve in **13**
Suggested nomenclature	*(B. dim)* synonym *(P. dim) OR (S. put)*	*(B. ves) OR (Pas)*	*(B. dim)*	*(B. ves) OR (Pas)*	*(Bru)*	*(B. ves) OR (Pas)*	*(B. ves) OR (Pas)*	**dominant sp** ***B. diminuta*** ***S. putrefaciens*** ***B. vesicularis pasteurella sp***
(B. dim)=Brevundimonas diminuta sp; (B. Ves)= B. Vesicularis; (P. dim)=Pseudomonas diminuta; (S. put)=Shewanella putrefaciens sp; (B)=Brucella sp (Pas)=Pasteurella sp								