KINETICS OF HYDROLYSIS OF STARCH BY ALPHA- AMYLASE FROM FREE AND IMMOBILISED *Bacillus subtilis* ON *Raphia sudanica* CHIPS

BY

NYAM TARHEMBA TOBIAS DANIEL

M.ENG/SEET/2001/744

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Thesis submitted to Chemical Engineering Department, Federal University of Technology, Minna, in partial Fulfilment of Requirement for Award of Master of Engineering (Chemical Engineering)

August, 2006

DECLARATION

I, Nyam T. Tobias Daniel (M. Eng/SEET/2001/744), declare that this thesis titled: kinetics of hydrolysis of starch by alpha – amylase from free and immobilised *B. subtilis* on *R.sudanica* chips, presented for the award of Master Degree (M.Eng.) of Engineering in Department of Chemical Engineering, is my original work and the references cited have been duly acknowledged.

Nyam ..... 11/09/2006 .....

Signed Date

CERTIFICATION

This thesis titled: **kinetics of hydrolysis of starch by alpha – amylase from free and immobilised *B. subtilis* on *R.sudanica* chips**, by: **Nyam T. Tobias Daniel (M.Eng/SEET/2001/744)** meets the regulations governing the award of Master Degree (Chemical Engineering) of the Federal University of Technology, Minna, and is approved for its contribution to scientific knowledge and literally presentation.

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ABSTRACT

The desire in this work is to determine optimal conditions suitable for the microbe (*Bacillus subtilis)* and enzymes (α amylase) to produce high level of sugar. In this study α amylase from free and immobilized *Bacillus subtilis* on chips of *Raphia sudanica* was used to catalyse the hydrolysis of starch using batch and feed batch processes respectively. *Raphia sudanica* was cut to size and severally boiled for purification and then used for immobilization of *Bacillus subtilis*. The total sugar produced was monitored using the Somogyi-Nelson method. Optimal operating conditions such as temperature and pH were investigated and Michaelis Menten equation was employed to determine the kinetic parameters for both the free and immobilized processes.

The optimum temperature, pH as well as the activation energy were 50°C, 5.0, 6.5kJ/ mol and 70°C, 5.0, 22.6kJ/ mol for the free and immobilized processes, respectively. The Michaelis Menten constant and maximum rate of hydrolysis was 0.03352 mol and 40.0μ mol/min, respectively, for the free process. Similarly, for the immobilized process, the apparent Michaelis Menten constant and apparent maximum rate of hydrolysis were 0.05044 mol and 125.0μ mol/min respectively. The substrate flow rate employed during the immobilized process was 12ml/min.

Regardless of whether the process was free or immobilized, the slight acidic pH medium of 5.0 was most suitable for the bacteria and its enzyme. The α - amylase from immobilized *Bacillus Subtilis* showed its resilience at high temperature by exhibiting optimal activity. The lower

activation energy exhibited by α amylase from free *Bacillus subtilis* shows its efficacy over the α -amylase from immobilized *Bacillus subtilis*, owing to better spatial orientation and in addition an almost zero effect of external mass transfer resistance enjoyed by it.

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ABBREVIATION

ABU: Ahmadu Bello University, Zaria

App: apparent

B.subtilis: *Bacillus subtilis*

Conc.: concentration

Immob: immobilised

NIPRD: Nigeria Institute for Pharmaceutical Research and Development

O.D: optical density

R. hookeri: *Raphia hookeri*

R. sudanica: *Raphia sudanica*

SEDI: Scientific Equipment Development Institute Minna

S.cerevisiae: *Saccharomyces cerevisiae*

NOMENCLATURE

α	Alpha
β	Beta
η	Effective factor
D_a	Damkohler Number
E_a	Activation energy
E	Enzyme
k	Kinetic rate of reaction
K_m	Michealis Menten constant
P	Product
S	Substrate
T	Temperature
V_{max}	Maximum rate of reaction
[E]	Enzyme concentration
[P] = p	Product concentration
[S] = s	Free substrate concentration
k_1, k_2, k_3	Rate constant
k_s	Mass transfer coefficient
S_o	Initial substrate concentration
V	Rate of reaction
x, k_a	Dimensionless variable

CHAPTER ONE

1.0 INTRODUCTION

Starch represents a link with the energy of the sun - the ultimate source of energy in the Universe – only one life form has the divine opportunity, mechanism and device to successfully synthesise some of this energy primarily as its own food reserves during photosynthesis. Apart from serving as food reserves for plants, it provides a mechanism by which non-photosynthesising organisms can utilise the energy provided by the sun. To utilise this energy, starch has to be converted to easily degradable form such as sugar. Sugar has innumerable applications to man, such that investigating new or better ways of its production and understanding their kinetics will go on for some time.

The conversion of starch to sugar involves the hydrolysis of the 1→4 glycosidic bonds found in starch, which is achievable by several catalytic processes. These include:

- acid hydrolysis of starch as reported by Dangana (1995), Audu, et al (1996), Layokun (1985) etc.;
- alkaline hydrolysis of starch as reported by Tsado (2003);
- enzymatic hydrolysis of starch (Alli et al (1998), Peter (1979), Agaie (1997), Ivonye (1998)); and
- freely suspended bacterial *Bacillus subtilis* were reportedly used by Ogunbayo and Bello (1993 a & b).

In the same vein, various hydrolysis of cellulose have been reported, and the results from these processes were to say the least impressive, resulting in production of glucose and cellobiose.

To further improve the gains of enzymatic hydrolysis, which is seen as the best form of hydrolysis, Sitton and Gaddy (1980), Tsado (2003), immobilisation technology evolved with several successes. Peter (1979), Sitton and Gaddy (1980), Ogunbayo and Bello (1986, 1993), Algacyr (2001), reported either immobilising enzymes or cells. These immobilised enzymes or cells have been used to among others hydrolyse cellulose, starch, and lactose etc. to obtain various products e.g. alcohol, L-aspartic acid, galactose, glucose etc.

The need for this work is brought to bear by the following reasons:

- Nigeria is a growing technology that needs to look inward for raw materials and processes that have well defined kinetics. This research findings are a footage in evolving indigenous technology;
- Other workers, Olatunji and Aberuagba (1986), Ogunbayo and Bello (1993) used (*B. subtilis)* from fermenting gari. In this work, the source of *B. subtilis* is different. Ivonye (1998) used fungal α amylase and Agaie (1997) used bacterial α amylase for the study of cassava starch hydrolysis. Alli and Rahman (1998) used fungal amylase to hydrolyse cereal starches;
- Ogunbayo and Bello (1993) reported using *Raphia hookeri* chips as carriers, this work tries to show that another substitute, *Raphia sudanica* which is ubiquitous can be used;

- Ogunbayo and Bello (1986, 1993 a, b) merely established the stability of Lactoczym and *Bacillus subtilis* immobilisation and hence the usability of enzyme and cell in hydrolysis of lactose and starch respectively. This work strives to propose the kinetics of starch using both free and immobilised cells. Algaeyr (2001) used a different strain *Bacillus circulans* GRS 313 in his work while investigating carrier size effect. This work is a stepping-stone to comparative kinetic analysis.

1.1 Aims and Objectives

1. To investigate the optimum conditions, that is pH, temperature, and concentration for hydrolysis of starch by α-amylase from free *Bacillus subtilis* and hence establish the kinetics parameters of the process.
2. To investigate the possibility of immobilising *Bacillus subtilis* on *Raphia sudanica*.
3. To investigate optimal conditions such as flow rate, pH and temperature using previously determined substrate concentration from α-amylase from free *Bacillus subtilis* for hydrolysis of starch by α-amylase from immobilised *Bacillus subtilis* and hence establish the kinetics of the process.

1.2 Limitation

1. Effect of mass transfer is an important parameter to be established for a thorough kinetics of immobilised cell, it is however, a function of equipment specification, it is a constraint in this work.
2. The peristaltic pump could not sustain variation in speed beyond those reported here in.
3. There was an accompany rise in temperature of substrate pumped into the column owing to heating up of the pump at high flow rates.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Carbohydrates

These are primary products of photosynthetic conversion of atmospheric carbondioxide to simple sugar (C_3 – C_9), which are polymerised into various forms. They have a general formula of $(CH_2O)_n$ where $n \geq 3$.

There are different types of sugar this include Monosaccharides, Disaccharides and Polysaccharides.

2.1.1 Monosaccharides

These are simple sugars, which serve as monomeric block. Examples include: glucose, fructose, sucrose, mannose and galactose, which are all hexose (C_6).

2.1.2 Disaccharides

These are formed from the condensation of two monosaccharides, which produce glycosidic bonds with elimination of water via condensation of –OH on α position and fourth position of the two monomers. Examples include lactose and sucrose.

2.1.3 Polysaccharides

These are complex sugar with long chain condensed monosaccharides. They are grouped into

Structural polysaccharides are divided into cellulose and chitin;

i. Cellulose (plants). Made up of β 1- 4 glucose; they form flat sheets with multiple hydrogen bonds between strands. They are digestible by symbiotic microbes.

ii. Chitin (typical of insects and shell fish exoskeleton). It contains N-acetylglucosamine.

b. Storage polysaccharides

Storage polysaccharides which are energy providers in animals and plants are divided into Glycogen and starch;

i. Glycogen (animals): highly branched, one branch per twelve glucose unit, than starch; glucose unit released by the enzyme glycogen phosphorylase producing glucose + - phosphate.

ii. Starch (plants): contains amylose (α, 1, 4 glucose; contains amylopectin which is branched at approximately one per 25 glucose unit; similar in structure to α helix in proteins; form a helix.

2.2 Types of Starch

Twenty percent (20%) of starch is amylose, which are straight chain polymer of glucose that occur in the interior of starch granule. It is insoluble in water.

The bulk of starch is amylopectin – branched D-glucose polymer containing amylose and 1,6 branches. They occur in cell walls, water-soluble forming gel on absorbing water.

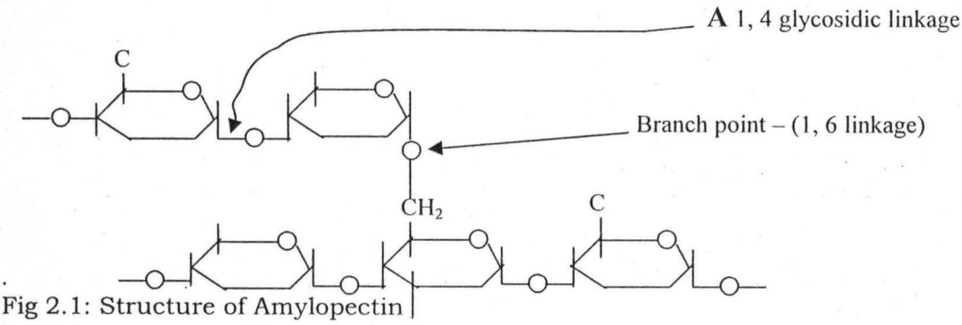

Fig 2.1: Structure of Amylopectin

2.2.2 Properties and Reaction of Starch

Starch exhibits the following physical and chemical properties;

- Starch is tasteless, odourless and insoluble in water at room temperature. However, at elevated temperature, it absorbs water – gelatinization (Nam, 1995).
- It dehydrates when reacted with H_2SO_4 acid.
- It can be esterified to form acetates, nitrates etc.
- It gives intense blue colour with iodine, this colour disappears on heating.
- Partial hydrolysis with acid or enzyme results in dextrin, glucose and maltose.

2.3 Hydrolysis

It is an example of solvolysis, which is a nucleophilic substitution in which the nucleophile is a molecule of the solvent (solvent + lysis: cleavage by the solvent). Where the solvent is water, then the reaction is hydrolysis.

2.3.1 Acid hydrolysis

This is carried out using dilute or concentrated acid. Numerous workers reported using acid hydrolysis of cellulose to obtain sugar e.g. Oyeyemi (1997) reported acid hydrolysis of sawdust recording optimum temperature of 80oC using 2.5M H_2SO_4 and E_a of 57.85kJ/gmol.

Auta (1998), reported acid hydrolysis of rice husk using optimum acid concentration of 3.75M H_2SO_4 and optimum temperature of 75°C with E_a as 9.913kJ/gmol. Other workers successfully carried out acid hydrolysis of starch e.g. Dangana (1995), reported acid hydrolysis of cassava using optimum acid concentration of 7.5M H_2SO_4 and temperature between 70°C – 80°C, E_a was 33.96kJ/gmol. Audu et al, (1996), reported acid catalyzed dextrinization of cassava starch using dilute HCl like all other workers, showed that increased catalyst concentration results in higher yield, obtained 124.7kJ/gmol as E_a.

DEMERITS OF ACID HYDROLYSIS

Though acid hydrolysis is the most researched and commercially applied method of hydrolysis because of the catalyst stability on the feed stock and its being readily available, the following counts against it:

- High energy cost
- High ash content in product
- Product toxicity to microbes in subsequent fermentation.
- Requiring large quantity of acid to wet feed stock.
- Poor acid recovery.

2.3.2 Alkaline hydrolysis

This is done using dilute or concentrated alkaline. Bazo (2000) reported alkaline hydrolysis of maize cobs recording optimum concentration of 0.4M NaOH and temperature of 80°C, E_a was 68.96 kJ/gmol as reported for acid hydrolysis it was first order kinetics. Tsado (2003), reported using dilute NaOH (0.5 – 0.6)M to hydrolyze cassava starch of first order kinetic and optimal temperature range of 70°C - 80°C with E_a between 50.7 – 53.3 kJ/gmol. Jolaiya (2000), reported alkaline hydrolysis of groundnut shell for which optimum temperature was 80°C, a first order kinetics with E_a of 65.64kJ/gmol.

DEMERITS OF ALKALINE HYDROLYSIS

Like acid hydrolysis there is:

- High-energy cost as well as pretreatment requirement in the case of cellulose.
- Requirement of large quantity of alkaline for effective wetting.
- Recovery problem of the alkaline used.

2.3.3 Enzymatic hydrolysis

Ogunbayo and Bello (1986), reported using lactozym in hydrolysis of lactose, Alli and Rahman (1998) reported using crude fungal amylase in hydrolysis of certain cereal starches, the results were impressive. Ivonye (1998), also reported hydrolysis of cassava starch by fungal α-amylase at very moderate temperature of 50°C. Olatunji and Aberuagba (1986), reported hydrolysis of cassava starch using *B.subtilis* α-amylase and Olatunji and Ene (1982) also used bacterial α-amylase at

temperatures of 60°C and 40°C. Similarly, Agaie (1997), also reported use of bacterial α-amylase for hydrolysis of cassava starch the temperature was however on the high side, 75°C.

The process is costly from the perspective of enzymes production and its recovery; however this is compensated by its low energy requirement viza vis lower temperature. The process is friendly to any grade of starch with specific products formation.

2.3.4 Direct microbial conversion

Ogunbayo and Bello (1993) indicated that *B.subtilis* is able to grow on starch and in the process produce sugar. Peter (1979), reported numerous work on starch hydrolysis by various glucoamylase enzymes, including use of source microbes.

Solomon et al (1997), reported direct use of a genetically modified *Saccharomyces cerevisiae (S. cerevisiae)* strain to aerobically and anaerobically degrade starch to ethanol with impressive results. The application of this process to commercial scale is slow; however, the process has stability and enjoys variations.

2.4 Enzymes

These are protein molecules, derived from living sources that act as catalyst for biochemical reactions. They consist of chains of amino acid linked by peptide bonds. The choice of enzyme is determined by various factors. Some catalytic qualities of enzyme include: high catalytic power, specificity, regulation and possession of active sites.

The mechanism of enzyme catalysis requires reactants to contain sufficient energy to surmount potential energy barriers - activation energy. This activation energy may be decreased by a number of mechanisms, Chaplain and Bucke (1990).

2.4.1 Mechanism of glycosidic bond hydrolysis

The active site of an enzyme is divided into two (2) parts, which together determine the type of product formed. These are: the binding site and catalytic site. In endo-enzyme, the active site cleft to the internal part of starch resulting in multiple attacks and lower molecular weight products. Whereas in exo-enzyme, the site pocket in to the non reducing end of the starch chain producing single low molecular weight product. Examples are β maltose, β- D- glucopyranose.

2.4.2 Amylase

These are enzymes that are capable of hydrolysing α – D- (1→4) linkages in starch. They can be classified variously Nam (1995)

- Fungal α amylase: they attack the second linkage from the non-reducing terminal (C_4), which result in an extensive breakage with production of maltose.
- Bacterial α amylase: this is a liquefying enzyme that randomly attacks only α – 1,4 bonds. *B.subtilis* α-amylase is known to be saccharifying enzyme that produces mostly glucose and maltose from starch, Moat and Forster (1988). *B.subtilis* is prokaryotic cell, that is a gram-positive and aerobe. It therefore secretes a variety of hydrolase's in to its environment.

Robyt (1984) proposed that α-amylase have multiple attack mechanism.

2.4.4 Kinetics of enzyme Action (Michealis Menten mechanism)

It is established that enzymes form complex with their substrate in the course of their catalysis and prior to the release of products. This can be summarised thus based on Michealis-Menten mechanism for one substrate reaction.

$$E + S \underset{k_3}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E \qquad (1)$$

$$r = \frac{d[p]}{dt} = k_2[ES] \qquad (2)$$

It has been established that for steady state $\frac{d[ES]}{dt} = 0$

So that $r = k_2[ES] = \frac{k_2[S][E]}{[S] + K_m} = \frac{V_{max}[S]}{[S] + K_m}$ (3)

Using various data and the Lineweaver-Burk plot, which is the most preferred, V_{max} and K_m, can be determined. Note K_m represents the extent of binding between the enzyme and its substrate.

Enzyme action can be affected by inhibition, pH, ionic strength, temperature etc.

2.5 Immobilisation

This is physical or chemical binding of an enzyme or cell to a support matrix, which is not essential to the activity of the enzyme or cell. The history of this technology shows it is old and has a pridal place, (Ikediobi and Ukoha, 1991). The rationale behind the technology has been discussed by several workers, (Chaplain and Bucke, 1990) (Sitton and Gaddy, 1980) (Peter 1979).

Sitton and Gaddy (1980) reported using *S.cerevisiae* on Raschig ring in immobilised cell reactor to convert glucose to ethanol. They reported over 60% conversion, a result 4.2 times better than the stirred reactor, with regeneration ability among other advantages. Ogunbayo and Bello (1986) reported immobilising lactase on *R.hookeri* chips for lactose hydrolysis. The results showed that the system could be stable with a performance improvement 100% better than the use of free enzyme. They also reported testing binder effect Ogunbayo & Bello (1986, 1993b). Isa (2002) reported immobilising *S.cerevisiae* on cane sugar pulp, maize cobs and wood to use in converting glucose to ethanol. Ogunbayo and Bello (1993) reported hydrolysis of cassava starch using *B.subtilis* immobilised on *R.hookeri chips,* the result showed that 15% of the cells were not immobilised while the glucose yield was 6g/l in 5hrs. Immobilised cells have long since found use in process industries.

2.5.1 Mechanism of bacterial immobilisation

Ikediobi and Ukoha (1991) reported that all the conventional methods of enzyme immobilisation are applicable to cell immobilisation. It is proposed that since bacterial cell membranes contain lipoproteins with lipopolysaccharides extending from membrane, the organism uses this glycoalyx structures to enable it attach to inert materials.

2.5.2 *Raphia sudanica*

Our support in this work was sourced from *R.sudanica* (tukuruwa in Hausa). It abound in swampy areas in savanna countries and is characterised by stout trunk 2-3m and can reach 8m high. Its leaves are as long as 10m, stiff and upright; they can have secondary branches (Keary, 1989). The branches (leaves) are mostly used for chairs, bed, food stirrers, mats, hand woven fan and twig for binding. The cortex from debarking the leaves was used as support material in this research.

2.6 Immobilised Enzyme Kinetics

In order to express global rate in terms of bulk properties, expression must be formulated for each of the steps in the overall process of converting reactants to products. In heterogeneous catalysis- these have been enumerated (Smith, 1984).

Two cases are possible i.e.

i. When enzyme is immobilised only on external surface of support, the effect of mass transfer would need to be determined through the use of dimensionless parameter D_a, where

$$D_a = \frac{V_{\max}}{K_s S_o} = \frac{\max imum\ reaction\ rate\ at\ the\ \text{sup}\ port\ surface}{\text{maximum mass transfer rate}} \qquad (4)$$

this is provided for where

$$\eta = \frac{\text{observed reaction rate}}{\text{rate without mass transfer resistance}}$$

$$= \frac{x/(k+x)}{1/(k+1)} \qquad (5)$$

$$For\ D_a \to 0,\ \eta = 1\ \text{then}\ \overline{V} = \frac{V_{\max} S_o}{K_m + S_o} \qquad (6)$$

$$and\ D_a >> 1,\ \eta = \frac{1+k}{D_a},\ \overline{V} = K_s S_o \qquad (7)$$

ii. A case where enzymes are entrapped within permeable matrix, which necessitates solute and product diffusion through pores. The rate of diffusion of all species is under the influence of certain factors, defined with operating equation in Bailey and Ollis (1990).

Algacyr (2001), showed that immobilized enzyme kinetics (activity) is affected by the size of the carrier matrix.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Materials

The following are the materials under various categories used in this work.

Table 3.1: Chemicals and Reagents

Chemical Reagents	Source	Comments
Basal medium and nutrient agar	Analar	commercial reagent grade
Calcium chloride	Analar	commercial reagent grade
Sodium Hydroxide	Analar	commercial reagent grade
Copper reagent	Analar	compounded
Arsenomolybdate reagent	Analar	compounded
Hydrogen chloride	Analar	commercial reagent grade
Acetic acid	Analar	commercial reagent grade
Starch	BDH	commercial reagent grade

Table 3.2: Equipment

Equipment	Source	Comments
Peristaltic pump	Masterflex USA	for feeding of substrate to column reactor.
Column reactor	SEDI, Minna	Reaction vessel for holding *R. hookeri* chips.
Spectronic 2D Spectrophotometer	Miltonroy USA	Optical density determination
Jenway 6051 Spectrophotometer	Jenway USA	Optical density determination
Water bath	Grant instruments England	Cooling purposes
Oven	Gallen Kamp	heating substrate and drying
Flask shakers	Gallen Kamp	Inoculums preparation
Centrifuge	Gallen Kamp	
R. hookeri chips	Aliade, Benue state	immobilization support
Auto clave	Indian Co. USA	sterilization
pH meter	Crison Barcelona Spain	
Microscope	Carl Zeiss Jena	

3.2.1 Microbial morphology

Nutrient Agar powder weighing 3.6g was dissolved in 100ml of distil water, the solution was sterilised in an autoclave at 121°C for 15 minutes, then allowed to cool to 45°C – 50°C. It was thereafter dispensed into sterile petri dishes which were earlier sterilized at 160°C for 1hr.

and incubating for 24hrs at 37°C. Grams morphology was carried out for the micro-organism by using Grams Staining Technique (see appendix A).

3.2.2 Inoculum preparation

Three conical flasks were sterilised at 15 psia and 121°C for 15 minutes with each containing 200ml of nutrient broth. Each of the flasks was inoculated with *B. subtitlis* and allowed to grow for 24hrs on a shaker. This was the inoculum culture.

One litre of basal medium was prepared and transferred into the conical flask and sterilised at 121°C and 15 psia for 15minutes. The medium was then inoculated with the inoculum culture and refrigerated as stock culture.

3.2.3 Bacteria cell standard calibration curve

An aliquot of cell suspension was taken from stock and attuned to a temperature of 37°C for about 10 minutes, then centrifuged for 15 minutes, washed, filtered, dried and the weight of the cell per millilitre determined. Five millilitre of the cell suspension was taken and made up to 100ml from which 5ml were taken to prepare serial dilution of 15ml, 20ml, 25ml, 50ml and 100ml. The optical density of each dilution was determined at 420nm against water blank and calibration curve was then prepared.

3.2.4 Sugar assay

The method used was Somogyi-Nelson where 0.5g of dextrose was dissolved in 1000ml distil water. 1ml of the solution was variously taken for a serial dilution of 1000ml, 500ml, 250ml, 100ml and 50ml. From each dilution 20ml was taken and 2ml of copper reagent added to it. The solution was boiled for 12 minutes and allowed to cool. Two millilitre of Arsenomolybdate was added to the solution followed by addition of 5ml water. The optical density of each solution was determined at 520nm against a blank.

3.2.5 Optimum substrate concentration determination

Two gram of starch was gelatinised by using boiling water and bringing up the volume to 700ml. From this volume 35ml of the gel was taken and put into a conical flask and placed in a water bath at 40°C. Separately, 0.5ml of cell was taken and made to 10ml by adding water while 0.01g of $CaCl_2$ was dissolved in 10ml of water. This was added to the prepared cell solution and mixed thoroughly. This mixture was used to inoculate the starch gel in the water bath. From the inoculated gel 2ml of the mixture was taken at interval of 5, 10, 15, 20, 25 and 30 minutes, each of which was terminated with 2ml Acetic acid and allowed to cool, then filtered.

From the filtrate 1ml was pipetted into a 25ml flask and 1ml of copper reagent was added to it and mixed. The solution was

covered with foil paper and boiled for 12 minutes after which it was cooled under running tap water. To the now cooled solution 1ml of Arsenomolybdate was added followed by 5ml of water to brighten the colour developed. The optical density of each solution was determined at 520nm against a blank. The total sugar concentration was determined from the Glucose Standard Calibration Curve. The procedure was repeated for 4.0g, 6.0g, 8.0g, and 10.0g of starch.

3.2.6 pH and temperature effect on starch hydrolysis

Starch concentration of 0.0528g/dm^3 was chosen to determine the effects of pH and temperature on the hydrolysis. 0.1molar NaOH and 0.1 molar HCl were used and the pH of the gel attuned to pH 4.0. The process of hydrolysis as in 3.3.5.1 was repeated and appropriate measurements taken at various temperatures of 50°C, 60°C and 70°C. The process was repeated for the pH of 5.0, 6.0, 7.0, and 8.0.

3.3. Immobilisation

The cell support i.e. *R.sudanica* chip was treated by boiling until decolouration of water stopped to extract soluble contaminants after which it was sun dried for 2–3 days. The column reactor was randomly packed with the cell support and its top plugged with cotton wool. 50ml of cell suspension was made up to 1000ml by adding water. This was placed on a shaker. Using the peristaltic pump, the cell solution was transferred into the reactor at a flow rate of 12ml/min for 24hrs. The cell solution was continuously recirculated and the extent of retention

monitored hourly by taking optical density of the effluent at 420nm against water blank.

3.3.1 Stability of immobilised cell

Using a portion of the chips and the pump, a substrate concentration of 0.0528 mol (8.571g/dm^2) at pH 5.0 and temperature of 40^{0}C was transferred up the reactor at 12ml/min. After the transfer, samples were taken at 10, 20, and 30 minutes and the total sugar determined as in 3.3.5.1. Several other runs were carried out using the same cell support. This was to determine how many effective runs a particular support charge could sustain.

3.3.2 Optimal flowrate

The reactor charged with cells on carrier was used for starch hydrolysis. The substrate as in 3.3.6.1 was fed in, samples were collected and analysed. This was repeated for flow rates of 15ml/min, 20ml/min and 24ml/min using fresh carriers of immobilised cells in each case.

3.3.3 pH and temperature effect

The reactor was lagged using foam then charged with the carrier. The substrate as in 3.3.5.2 was placed in a water bath at 40^{o}C and pumped into the reactor for hydrolysis. Samples were taken at 5, 10, 20 and 30 minutes and analysed as in 3.3.5.1. The process was repeated

for 50°C, 60°C and 70°C at the same pH and for pH of 5.0, 6.0, 7.0 and 8.0.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION OF RESULTS

4.1 Results

The results is presented as follows:

4.1.1 Effect of Substrate Concentration on Hydrolysis of Starch by α- *Amylase*

Figure 1 shows a plot of sugar concentration vs. time at various concentrations of starch. From the figure, it is seen that with increase in concentration of starch, there is a corresponding increase in sugar produced. The plots of each concentration show characteristics typical of enzyme reactions.

4.1.2 Effect of temperature on hydrolysis of starch by α-*amylase*

Figure 2 shows the plot of sugar concentration vs. time at various temperatures and pH 5.0. The figure shows the best production at 50°C; however the plot is typical of that for an enzyme reaction. Figure 3 shows a plot of initial velocity, V_O vs. temperature in which there is a rise in velocity climaxing at 50°C for the free process.

4.1.3 pH Effect on hydrolysis of starch by α- *amylase.*

Figure 4, a plot of sugar concentration vs pH at 50°C, shows the sugar concentration rapidly rising until it climaxed at pH 5.0. This was followed by a rapid decline until pH 7.0.

4.1.4 Retention of *Bacillus subtilis* on *Raphia sudanica* chips during immobilisation.

Figure 5, a plot of *B. subtilis* in effluent vs time showed that the cell declined bihourly until the 19th hour from which there was stability (no decline).

4.1.5 Flow rate effect on hydrolysis of starch

Figure 6, which is a plot of initial sugar concentration Vs flow rate, indicates that concentration continually declined with increased flow rate.

4.1.6 Temperature effect on hydrolysis of starch by α amylase from immobilised *B. subtilis*

Figure 7 shows similar characteristics with Figure 2. At a pH of 5, Figure 3 shows initial velocity, V_o vs Temperature where there is rapid rise in velocity until 50°C. After this, the rise is slower until climaxed at 70°C.

4.1.7 pH effect on hydrolysis of starch by α amylase from immobilised *B. subtilis*.

Figure 4 shows a rather gentle rise in sugar concentration, which climaxed at pH 5.0 and was maintained until pH 6.0 after which a rapidly declining productivity set in.

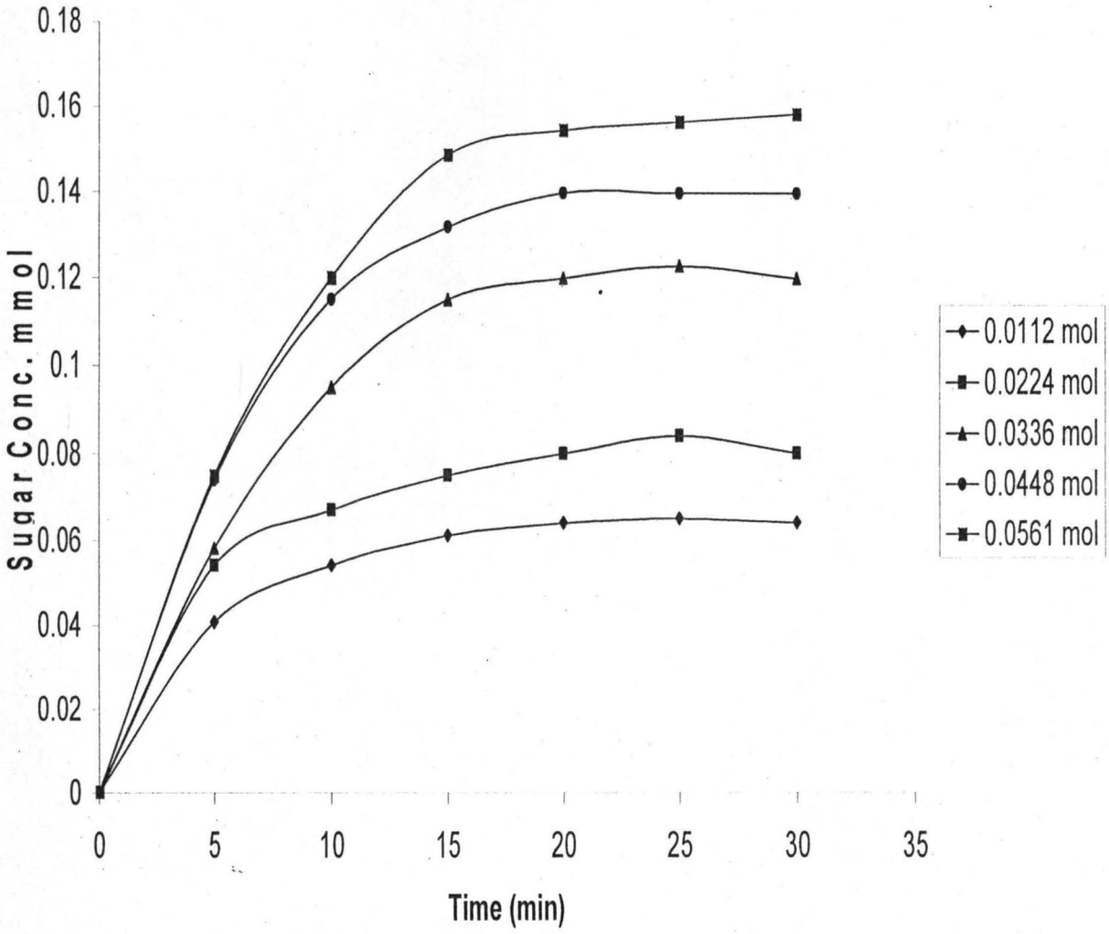

Figure 1: Starch Concentration Effect on Hydrolysis

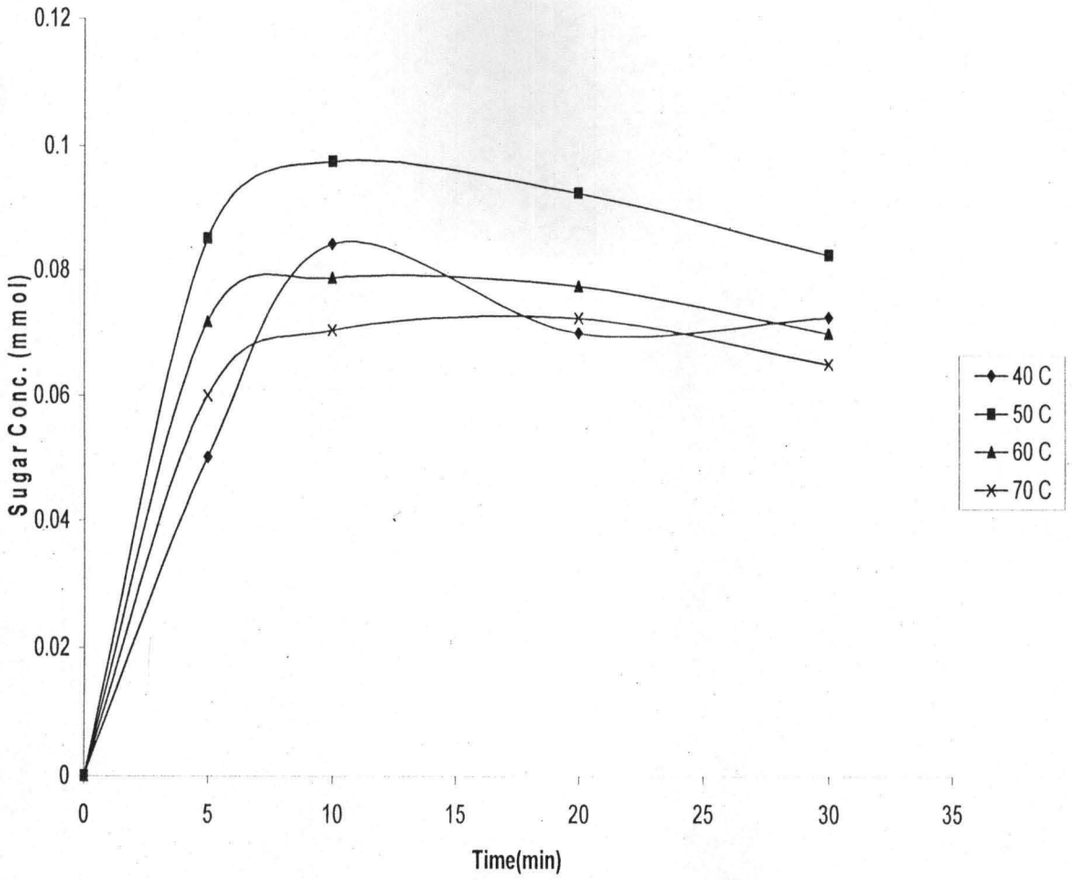

Figure 2: Temperature Effect on Starch Hydrolysis at pH 5.0

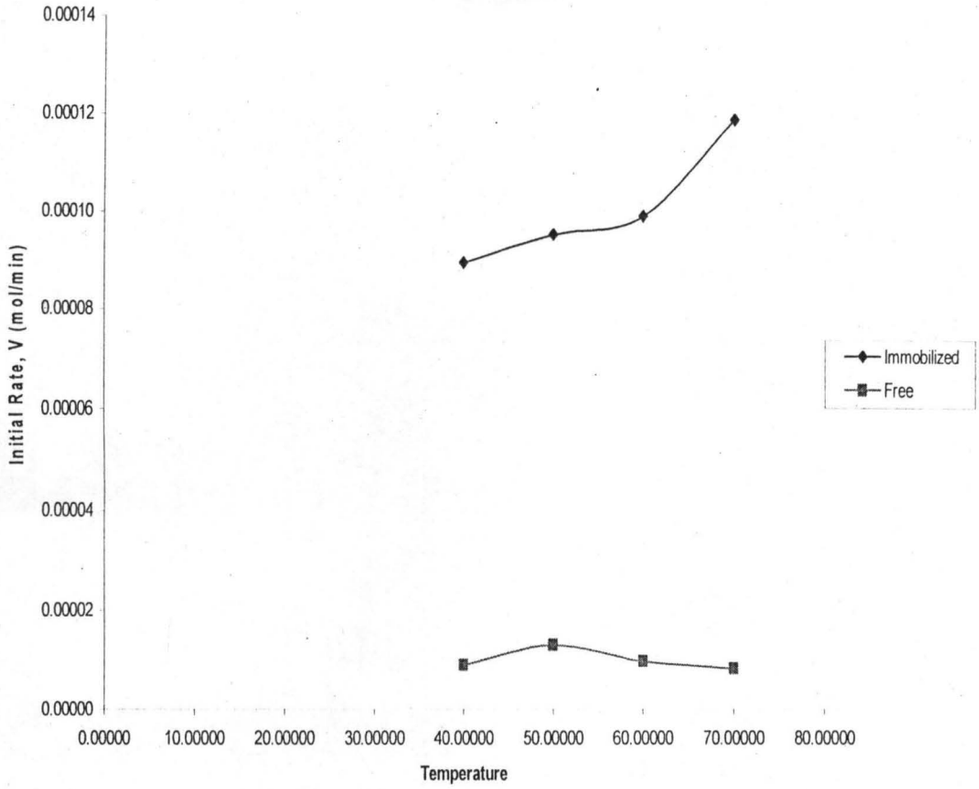

Figure 3: Effect of Temp. on Starch Hydrolysis

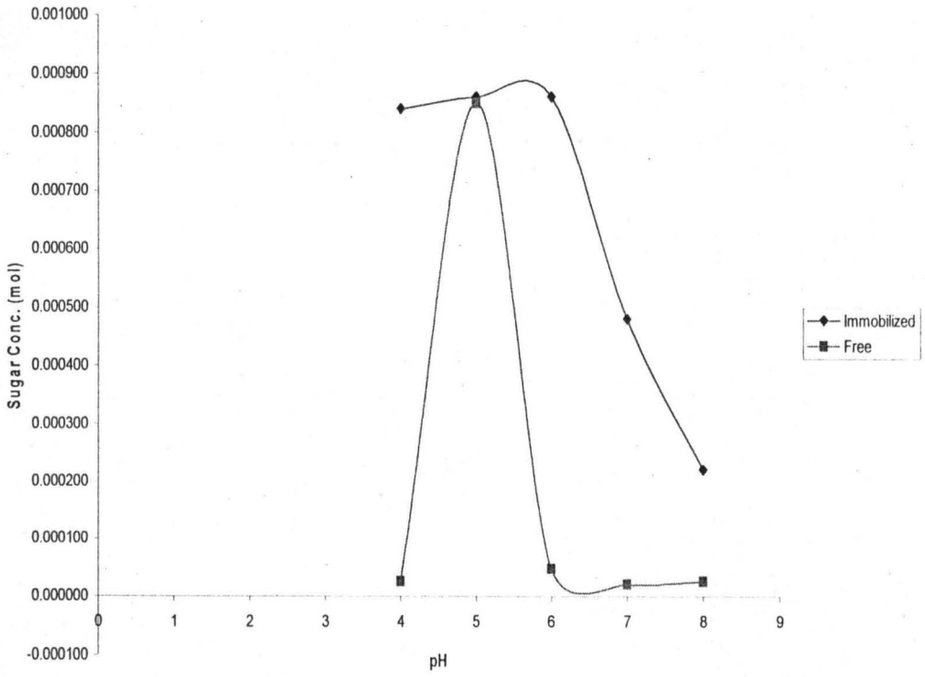

Figure 4 : Effect of pH on Starch Hydrolysis

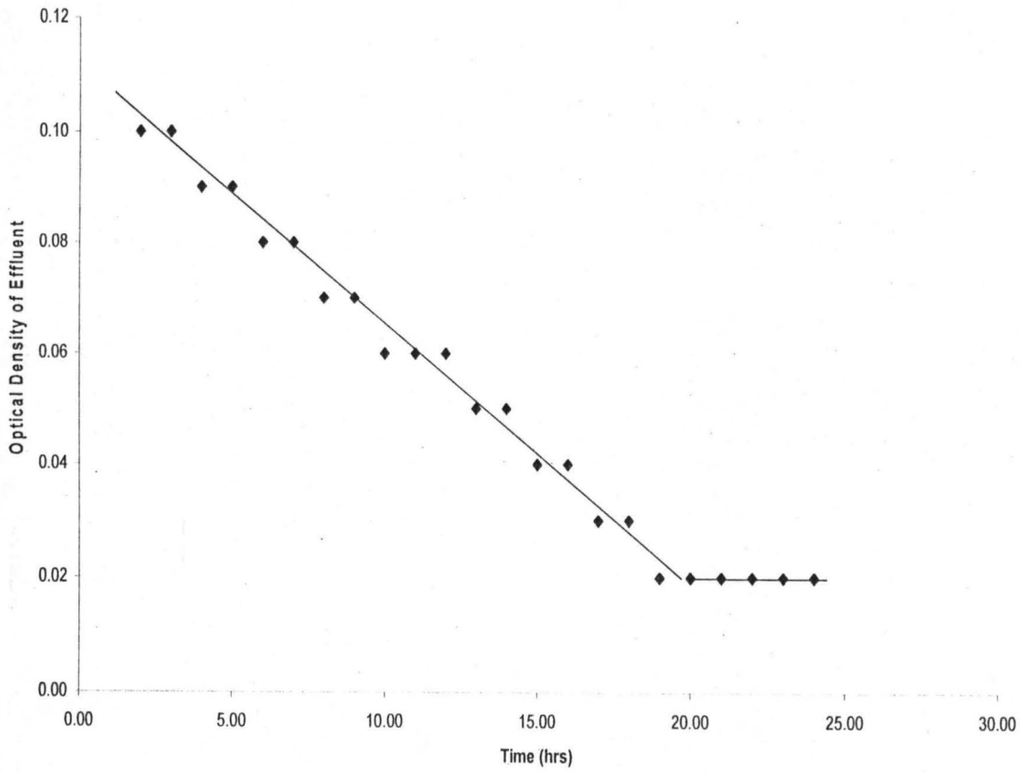

Figure 5: Retension of B.subtilis in Packed Reactor During Immobilisation

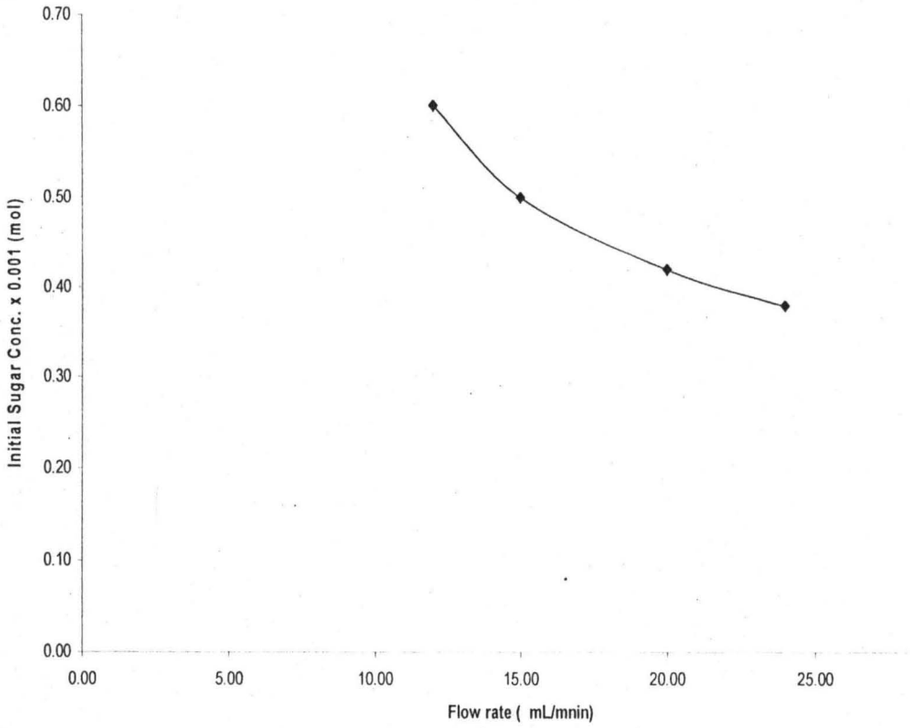

Figure 6: Flow Rate Effect on Starch Hydrolysis

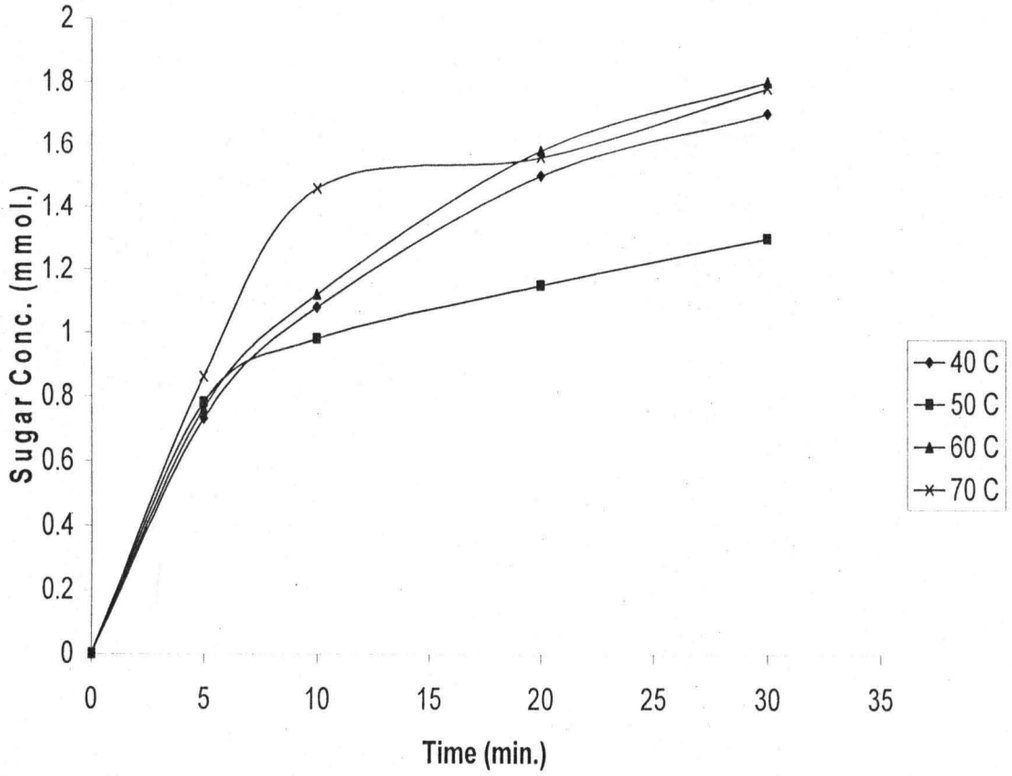

Figure 7: Temperature Effect on Starch Hydrolysis by Immobilized α Amylase at pH 5.0

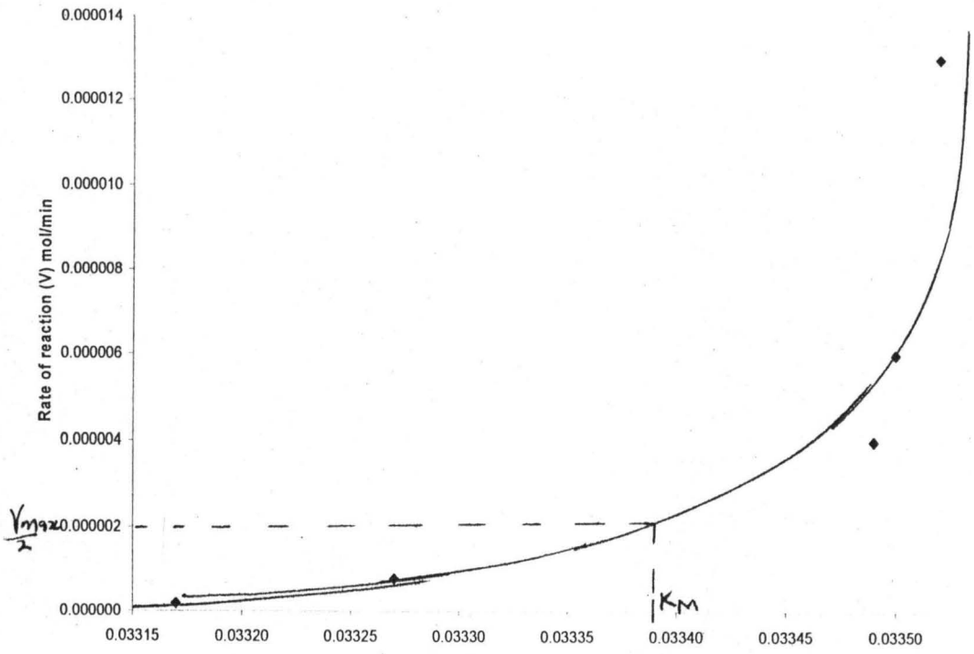

Figure 8: V versus S

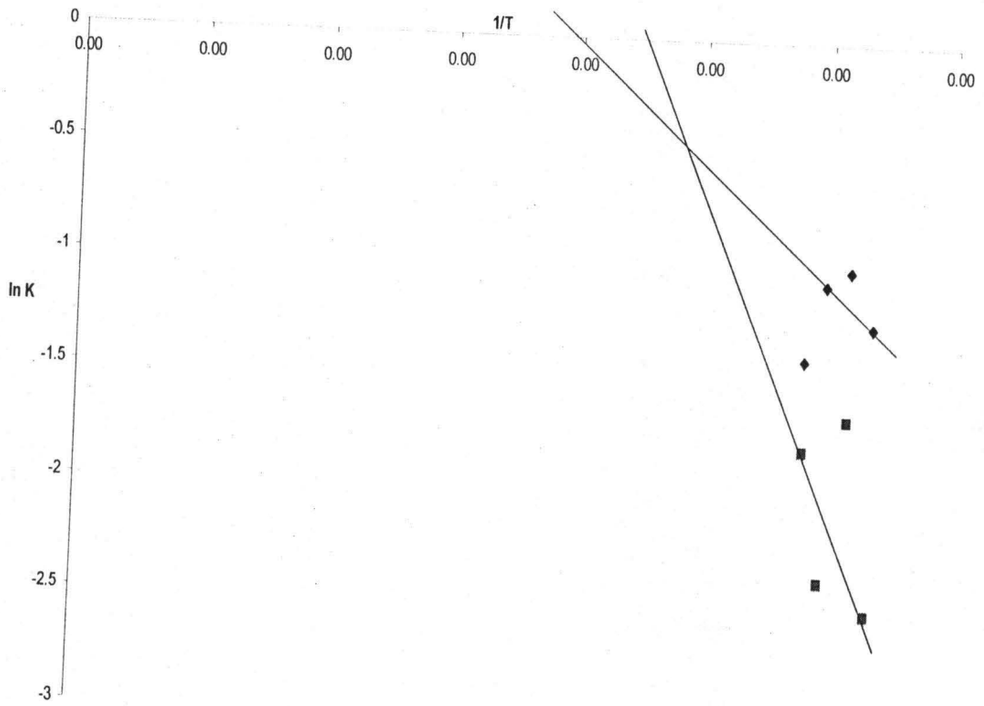

Figure 9 :ACTIVATION ENERGY PLOT

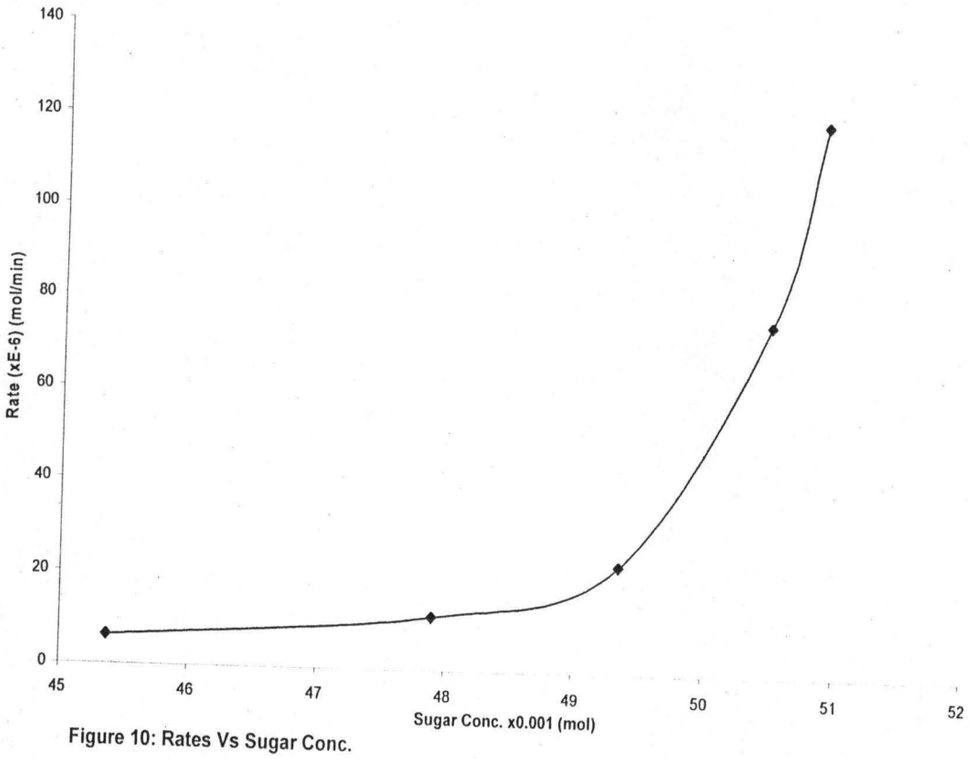

Figure 10: Rates Vs Sugar Conc.

4.2 Discussion of Results

4.2.1 Optimum substrate concentration

In this work the optimum substrate concentration was 0.0561mol. Figure 1 show the effect of starch concentration on *B.subtilis* $\propto$ amylase activity at 40^0c and pH of 5.0 which agrees with the usual Michaelis Menten curve which was reported. Olatunji and Ene (1982), as well as Olatunji and Aberuagba (1986), and Ivonye (1998) reported a highest substrate concentration of 0.01232 mol (2.0 mg/ml) and 0.0502 mol respectively. However, the concentration of 0.0528mol was used for subsequent studies. Figure 2 shows the effect of temperature on hydrolysis of starch by *B.subtilis* $\propto$ amylase at optimum temperature and pH. The result showed the kinetic characteristic where the Michaelis Menten curve of rate of reaction, V vs substrate concentration, S was obtained (Figure 8).

4.2.2 Use of Michaelis Menten equations

From the Lineweaver- Burk plot of $\frac{1}{v}$ vs $\frac{1}{s}$, Figure F12, V_{max} was found to be 40.00μ mol/min while Michaelis Menten constant, K_m was 0.03352mol (5.44mg/ml). Olatunji and Ene (1982), Agaie (1997) and Ivonye (1998) reported 947.99μ mol/min (153.85 μg/ml min), and 0.03623 mol (5.88mg/ml), 919.46μ mol/min (149.22μg/ml min) and 0.03232mol (5.245mg/ml), and 13.99μ mol/min [2.27μ g/ml min] and 0.445m mol respectively as value for V_{max} and Michaelis Menten constant K_m. Olatunji and Aberuagba (1986) reported 2200μ mol/min

(357.14μg/ml min) as V_{max}. These results show that K_m is within the range of 10^{-1}-10^{-5} mol (Chaplain and Bucke, 1990).

To confirm the Km value the direct linear plot of V vs S was carried out as recommended by Henderson, (Eisenthal and Danson, 1992) K_m was ≈ 33.53mmol. The occurrence of the intercept in the third quadrant indicates that the starch concentration was small as compared to K_m. There is a need to evaluate the effect of several starch concentration at optimum pH and temperature to ascertain the K_m and V_{max}, which are interdependent.

The K_m value obtained in this work suggests that the affinity of ∝-amylase and starch may be slightly affected by other factors beyond source of Bacterial ∝ amylase and starch, since this result is consistent with others.

The proposed mechanism for starch – *B. subtilis* ∝ amylase is based on the multiple attack mechanism (Robyt, 1984) that involve

i Mass transport of *B. subtilis* ∝ - amylase and starch with formation of E-S complex at cleavage.

ii Chemical reaction involving hydrolysis of 1,6 and ∝ 1,4 bonds (saccharification)

iii Diffusion of the cleavage products and subsequently enzyme and substrate.

4.2.3 Effect of temperature on starch hydrolysis by *B. subtilis* α amylase.

Figure 2 show that the optimum temperature for starch hydrolysis was 50°c. Figure 3.0 shows the effect of temperature on initial velocity V_o at pH 5.0. For the free process, there was a rise in V up till 50°C. Olatunji and Ene (1982) reported optimum hydrolysis temperature of 60oC (maize) and 40°C (potatoes). Olatunji and Aberuagba (1986), Agaie (1997) and Ivonye (1998) reported temperatures of 60°C, 75°C and 50°C respectively for cassava. This temperature 50°C falls within the range of temperature for maximum activity by *B. subtilis* (40 – 60°C), regardless of pH. This range was favourable and possibly subject to substrate type. It is likely that at temperatures higher than 50°C there may begin to be bacterial mortality, or denaturation of enzyme produced, or the bacterial physiology is affected.

Figure 9, a plot of lnk vs $\frac{1}{T}$ with k values obtained from Figure F14 shows a negative slope for the enzyme reaction, and give an E_a value of 6.593kJ/mol. In earlier work Agaie (1997), Ivonye (1998), Olatunji and Aberuagba (1986) and Olatunji and Ene (1982) reported 9.56kJ/gmol, 18.165kJ/gmol, 46.68kJ/gmol and 18.548KJ/g mol (4.43kcal/gmole) respectively. Olatunji and Ene (1982) quoted a recommended range of 8.8-44.03kJ/gmol for enzyme catalyzed reactions. The value of 6.593kJ/mol is outside the lower limit; it implies that the reaction should proceed at a fast rate at the reactive sites with the presence of the α amylase catalyst.

4.2.4 Effect of pH on starch hydrolysis by *B. subtilis* ∝ amylase

Figure 4 shows the effect of pH on enzyme reaction at optimum temperature of 50°C. The result indicates that the optimum pH is 5.0. Olatunji and Ene (1982) reported optimum pH of 6.5 for maize starch and 6.8 for potatoes starch; Olatunji and Aberuagba (1986), Agaie (1997) and Ivonye (1998) reported 6.0, 7.0 and 6.0 respectively as pH values. All these values conform to reportedly established range of pH 5.85-7 (Wong, 1995) for ∝ amylase from bacteria. This result is typical of enzymes, as they are affected by ionic effect and carboxylic acids, which are denatured at low or high pH. At the optimum pH, maximum number of active sites is available. The result in this work was obtained whilst substrate pH was attuned to the desired level, perhaps the results may be verified with both substrate and innoculums attuned to desired pH before inoculation.

4.3 Immobilization of *B. subtilis* on *R. sudanica* Chips

Figure 5 shows a continual decline in cell concentration though not rapidly- perhaps because of the colorimeter used at this stage –the bihourly decline stopped after the nineteenth hour. Ogunbayo and Bello (1993) reported the same trend while using an average flow rate of 5ml/min and stability after 18 hours. This result indicates that at higher flow rate more time will be required to attain stability.

Figure 6 which is a plot of sugar concentrations with time vs flow rates shows that the optimum flow rate is 12ml/min. This flow rate

allowed for maximum enzyme – substrate contact time in the column. The higher velocity perhaps do not allow for enough ∝ amylase – starch cleavage. The stability of this system was tested at pH 5.0 and 40°C. The result shows that one charge stood 5 runs, declination however started after the third run. Ogunbayo and Bello (1986) reported that lactozym enzyme immobilized on palm woodchips stood 3 runs without binder, while in bounded form the enzyme stood 3 and 16 runs with various binders.

4.3.1 Use of Michaelis – Menten equation

Figure 7 shows the kinetics characteristic where the Michaelis – Menten curve of V vs. S was obtained (Figure F17) from the Lineweaver – Burk plot V_{max} was found to be 125μ mol/min while K_m was 0.05044mol (8.19mg/ml). This value of K_m is at variance with earlier submission. However Olatunji and Ene (1982) noted that V_{max} and K_m increases with increase in ∝ amylase concentration. High K_m translates to lower ∝ amylase – starch affinity (Chaplain and Bucke, 1990). Visual inspection of Figure F16 indicates non linearity and seemingly scattered data, though a line of best fit was employed, this indicates that perhaps Michaelis – Menten equation is inadequate or the assay needs to be improved or there was a systematic error (equipment) or excessive variability in velocity measured. This value of K_m was confirmed by the direct linear plot, and found from this plot to be 0.0552mol. There was a

1.51% deviation; once again the intercept was in the third quadrant. The cause is as previously discussed.

The immobilization of *B. subtilis* implies there will be more *B. subtilis* in the system and by implication more α amylase produced. If only external diffusion of starch towards the surface and sugar away from it in series with the catalytic conversion of starch to sugar occurring at the surface. K_m has been shown to be independent of S_s, which varies with D_a.

4.3.2 Effect of temperature on starch hydrolysis by α-amylase from immobilised *B. subtilis*.

Figure 3 shows the effect of temperature on initial velocity Vo at pH deduced as optimum. There was a rapid rise in V_o which slowed down after 50°C until it peaked at 70°C. Ogunbayo and Bello (1986) and Algacyr (2001) reported optimum temperatures of 40°C and 57°C respectively for their processes. This result cannot be unconnected with the heat transfer coefficient for the chips in use, so further experiments would be needed to confirm that the reaction temperature in the column was exactly 70°C as well as effect of chips size on the mechanism. However, Wong (1995) reported that the temperature optimum for activity values between 70 and 72°C for the α amylase produced by *B. subtilis*. This goes to say that the enzyme in this experiment, in present of Ca^{++} was greatly favoured by the temperature of 70°C.

The plot of lnk vs. $\frac{1}{T}$ in Figure 9 shows a negative slope for the enzyme reaction, E_a was 22.6kJ/mole. This result is well within the range suggested by Wang et al, as reported by Olatunji and Aberuagba (1986). It indicates the enzyme in its immobilized form and assumed high quantity, required three times the energy in its free state to overcome potential energy barrier.

4.3.3 Effect of pH on starch hydrolysis by ∝-amylase from immobilised *B. subtilis*.

Figure 4 shows a plot of [S] vs. pH at the determined optimum temperature. The result shows optimum productivity at pH of 5.0 and 6.0 for the immobilised process, however from Figure 7 at pH 5.0 there is sustained high sugar production. Ogunbayo and Bello (1986) and Algacyr (2001) reported optimum pH of 7.0 and 4.9 respectively for their processes. The absurdity is that at low pH of 4.0 [S] was appreciably high indicating less intense denaturation on the part of the enzyme and mortality on the part of the bacteria. The alkaline content of our support chips is perhaps responsible, in that it is capable of reducing the acidity of the system to a range tolerable for the bacteria and consequently for enzyme production. Furthermore, the pH optimum for ∝ amylase from *B. subtilis* is 5.85 – 6.0 which is close to the pH optimum for *B.subtilis*, 7.0 (Wong, 1995). This may have been a reason for the high production

of sugar witnessed for pH of 5.0 and 6.0 in the immobilised process. This needs to be investigated as previously opined.

It is likely that there is a low mass transfer resistance and this is likely because of non-isothermal operation, which only lends credence to the call for improved temperature monitor both at inlet and outlet or within the column; therefore regime is a reaction limited regime. There could be abrasion arising from fluid forces which may result in denaturation of the enzyme, which is probably responsible for drastic drop in V after the initial high V of 120 x 10^{-6}mol/min (see Figure 3).

Hanson (Olatunji and Ene 1982) has shown that not many enzymes and degradation of linear substrate, having more than n = 1 cleavage, have their kinetics correctly predicated by Michaelis – Menten equation.

Comparatively, the optimum pH suited for optimal activity of α-amylase from both free and immobilized *B.Subtilis* is the same as catalyst in starch hydrolysis and their denaturation is similar at pH higher than 6.0. The immobilized *B.Subtilis* showed a range within which much sugar can be produced i.e. pH. 5.0 – 6.0.

The best catalytic activity of free *B.Subtilis* α-amylase in starch hydrolysis is achievable at lower temperature of 50°C, while that by α-amylase from immobilized *B.Subtilis* is at higher Temperature of 70°C. This could be at an extra cost to a production process.

From Figure 8, the K_m of 0.03352mol for the hydrolysis of starch by α-amylase from free *B.Subtilis* indicates a high α amylase – starch affinity, that is evident in the

lower activation energy of the system. Whereas from Figure 10 the hydrolysis of starch by ∝ amylase from immobilized *B.Subtilis* has a high K_m 0.0504mol and by implication lower ∝ amylase starch affinity consequently higher activation energy.

From Figure 9 the lower E_a shows that the free enzyme active sites are available in correct orientation and specificity thus using their binding energy maximally to reduce free energy of transition state. On the other hand the higher E_a of 2.26 kJ/mol for the immobilized system indicates the effect of mass transfer resistance, active site non availability because it is wrongly oriented – therefore a lower affinity. It is evident too that K_m is dependent on substrate concentration. In spite of the lower affinity exhibited by the immobilized system, its rate of reaction V_{max} ensures a higher sugar production within a short time.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Results of kinetic experiment show that the hydrolysis of starch by free *B. subtilis* α amylase is within the limit of this experiment predictable using the Michealis Menten equation. Under defined conditions, it was observed that K_m is 33.52 x 10^{-3} mol while the maximum rate of hydrolysis is 40.0 x 10^{-6} mol/min. The optimum temperature was observed to be 50°C for the hydrolysis of starch by free *B.subtilis* α amylase. The activation energy of the enzyme reaction was 6.593kJ/mol. The optimum pH of 5.0 agrees with appropriate operating conditions of *B.subtilis.*

On the other hand, during the hydrolysis of starch by immobilized *B.subtilis* α amylase, within the limit of this experiment using Michealis Menten equation, it was observed that the Michealis constant K_m^{app} is 50.44 x 10^{-3} mol and the maximum rate of hydrolysis V_{max}^{app} is 125.0 x 10^{-6} mol/min. For this process, the optimum temperature observed was 70°C while the enzyme reaction activation energy was 22.6kJ/mol.

It was observed that a charge of immobilised *B.subtilis* α amylase can be used effectively for three (3) hydrolysis for best results, however it can sustain 5 runs. The optimum flow rate is 12mL/min.

The D_a of the immobilised process is 0.00158 indicating a reaction-limited regime while the effectiveness factor η is 1.996 showing that the velocity observed with mass transfer resistance is high.

A catalyst efficiency is defined by its ability to considerably lower the reaction activation energy, in order words, use small energy to overcome the potential energy barrier of the reaction. The free *B.subtilis* for the reasons previously discussed shows an over 70% efficiency over the immobilised enzyme in serving as catalyst for starch hydrolysis by α amylase.

5.2 Recommendation

1. The purification process for the *R.sudanica* need be improved by investigating the number of boilings necessary to obtain pure chips, and the possible use of solvent other than water.
2. The effect of temperature on immobilisation of *B.subtilis* can be investigated for improved immobilisation time.
3. The stability test needs to be conducted when there would be 50% drop in production.
4. The temperature within the column need be monitored along with the substrate stock temperature during hydrolysis using immobilised cells.
5. The effect of external mass transfer resistance need to be investigated using better performing peristallic pump that give both lower and higher flow rates.
6. The optimum pH in both the free and immobilised processes need to be confirmed by incubating both substrate and innoculum (chips) at desired pH before use.

7. The effect of substrate concentration at optimum pH and temperature should cover a range of at least $K_m/2$ to $5K_m$.

8. The effect of support chip size on the mechanism parameters needs to be investigated.

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APPENDIX A

GRAM STAINING TECHNIQUE (GST)

Make a smear on a slide with a loopful of bacterial suspension fixing it and air-drying. The fixed bacteria was then stained with crystal violet (Grams iodine) for 30seconds then, washed with distil water and decolourised with 95% ethanol. It was counter stained with Safranine for 1 minute, washed with distil water, air dried and viewed out under a microscope at x100 objective oil immersed.

APPENDIX B

COLONIAL MORPHOLOGY AND RESULT OF GST

Colour: dirty white or milk colour (mucoid)

Shape: spherical

GST Result: Grams +ve

APPENDIX C

I. COMPOUNDING COPPER REAGENT (SOMOGY-NELSON METHOD).

SOLUTION A: Weigh 2.5g of anhydrous Na_2CO_3, 2.5g KNaTartrate, 2.0g of $NaHCO_3$, 20g of anhydrous sodium sulphate dissolve all in water and make up volume to 100ml with distil water.

SOLUTION B: Dissolve 3.75g of anhydrate $CuSO_4$ in 25ml of water; add 1 – 2 drops of concentrated H_2SO_4

Mix 25 parts of solution A and 1 part of solution B

II. ARSENOMOLYBDATE REAGENT

Dissolve 25g of Ammonium molybdate in a mixture of 45ml of water and 21.0ml concentrated H_2SO_4. Add 3.0g of disodium hydrogen Arsenate ($Na_2HASO_4.7H_2O$) in 25ml of water mix and leave for 23 – 48 hrs at 37°C.

APPENDIX D

Table D1: Reading for Glucose Calibration Curve

Concentration (g/l) $X10^{-3}$	0.D
0.250	0.126
0.300	0.152
0.400	0.202
0.476	0.242

Table D2: Reading for *B. subtilis* Cell Calibration Curve

Concentration (g/ml) $X10^{-4}$	0.D
50.0	0.61
100.0	0.119
200.0	0.234
250.0	0.290
333.3	0.384
500.0	0.522

NB: From cell suspension, the cell weight (mass) per millilitre of Aliquot is 0.001g/ml

Table D3: Substrate Concentration Effect on Hydrolysis of Starch by Free *B. subtilis* α – Amylase at pH 5.0, 40°C

Starch Conc. mole/dm³	(*Sugar* Conc. X10⁻³ *mol*) 5min	10min	15min	20min	25min	30min	*B. subtilis* Conc.
0.0112	0.021	0.025	0.035	0.031	0.032	0.031	0.011
	0.0407	0.054	0.074	0.064	0.065	0.064	0.010
0.0224	0.025	0.033	0.037	0.040	0.041	0.040	0.014
	0.054	0.067	0.075	0.080	0.084	0.080	0.0125
0.0336	0.034	0.040	0.057	0.060	0.061	0.060	0.018
	0.070	0.080	0.115	0.120	0.123	0.120	0.0150
0.0448	0.035	0.057	0.062	0.069	0.069	0.069	0.019
	0.074	0.115	0.125	0.140	0.140	0.140	0.0160
0.0561	0.036	0.060	0.073	0.078	0.079	0.080	0.021
	0.0749	0.120	0.149	0.155	0.157	0.159	0.0175

Table D4: Temperature Effect on Hydrolysis of Starch by Free *B. subtilis* α – Amylases

H	Temp °C	Conc. Of Sugar (g/l) x10^{-3} 5 Min	10 Min	20 Min	30 Min	*B. subtilis* Conc. (g/ml) x10^{-6}
.0	40.0	0.025	0.044	0.034	0.036	0.051
		0.050	0.0875	0.070	0.0725	0.0425
.0	50.0	0.042	0.048	0.046	0.041	0.045
		0.085	0.0975	0.0925	0.0825	0.0375
.0	60.0	0.037	0.047	0.039	0.034	0.062
		0.075	0.095	0.0775	0.070	0.0525
.0	70.0	0.030	0.038	0.033	0.032	0.071
		0.060	0.075	0.0675	0.065	0.060
.0	40.0	0.037	0.050	0.038	0.035	0.0525
0	50.0	0.025	0.033	0.025	0.0225	0.0515
0	60.0	0.0225	0.025	0.020	0.020	0.065
0	70.0	0.0075	0.020	0.0175	0.010	0.0665
0	40.0	0.0425	0.050	0.055	0.0375	0.0425
0	50.0	0.0475	0.050	0.060	0.0525	0.04375
0	60.0	0.0125	0.0225	0.055	0.035	0.04825
0	70.0	0.035	0.040	0.050	0.0225	0.0475
0	40.0	0.020	0.030	0.0325	0.0325	0.0225
0	50.0	0.020	0.025	0.025	0.0225	0.03675
0	60.0	0.0125	0.015	0.020	0.020	0.040
0	70.0	0.0075	0.0100	0.010	0.0175	0.04175
0	40.0	0.0225	0.0225	0.025	0.0225	0.0325
0	50.0	0.025	0.025	0.0375	0.0325	0.0375
0	60.0	0.010	0.0125	0.025	0.0125	0.0350
0	70.0	0.0125	0.020	0.0225	0.0175	0.03675

Table D5: Temperature Effect on Initial Rates at Optimum ph 5.0 for Free Process

V_O x10^{-6} mol/min	T°C
9.0	40.00
13.0	50.00
9.8	60.00
8.33	70.00

Table D6: Immobilization of *B.subtilis* on Palmwood Chips

Optical Density	Time (Hr)
0.11	1
0.10	2
0.10	3
0.09	4
0.09	5
0.08	6
0.08	7
0.07	8
0.07	9
0.06	10
0.06	11
0.06	12
0.05	13
0.05	14
0.04	15
0.04	16
0.03	17
0.03	18
0.02	19
0.02	20
0.02	21
0.02	22
0.02	23
0.02	24

Table D7: Stability Of Immobilization on Palmwood Chips at pH 5.0, Temp 40°C

	Optical Density		
No of Runs	10min	20min	30min
1	0.20	0.75	0.85
2	0.50	0.79	0.83
3	0.56	0.88	0.88
4	0.48	0.70	0.72
5	0.30	0.57	0.60

Table D8: Optimal Flowrate Determination for Hydrolysis of Starch by Immobilized *B. subtilis* at pH 5.0, Temp 40°C

Flow Rate ml/min	Total Sugar		
	10min	20min	30min
12.0	0.60	1.17	1.50
15.0	0.50	0.86	1.36
20.0	0.42	0.81	1.20
24.0	0.38	0.80	1.14

Table D9: Temperature Effect on Hydrolysis of Starch by α Amylase from Immobilized *B.subtilis*

pH	Temp. °C	Total Sugar (g/l) x10^{-3} 5min	10min	20min	30min
5.0	40.0	0.19	0.20	0.75	0.85
		0.38	0.40	1.50	1.70
5.0	50.0	0.37	0.49	0.51	0.66
		0.78	0.98	1.02	1.30
5.0	60.0	0.41	0.56	0.61	0.90
		0.82	1.12	1.22	1.80
5.0	70.0	0.43	0.73	0.78	0.89
		0.86	1.46	1.56	1.78
4.0	40.0	0.54	0.64	0.68	0.76
4.0	50.0	0.54	0.56	0.80	0.96
4.0	60.0	0.86	0.86	1.00	1.28
4.0	70.0	0.84	0.94	1.04	1.08
6.0	40.0	0.58	0.62	0.84	0.96
6.0	50.0	0.84	0.88	0.98	1.20
6.0	60.0	0.88	0.98	1.08	1.40
6.0	70.0	0.86	1.20	1.24	1.28
7.0	40.0	0.28	0.38	0.56	0.72
7.0	50.0	0.46	0.54	0.62	0.86
7.0	60.0	0.54	0.68	0.78	1.04
7.0	70.0	0.48	0.58	0.72	0.96
8.0	40.0	0.28	0.34	0.46	0.50
8.0	50.0	0.46	0.74	0.74	0.78
8.0	60.0	0.46	0.58	0.60	0.96
8.0	70.0	0.22	0.34	0.38	0.44

Table D10: Temperature Effect on Initial Rates at Optimum PH 5.0 for Immobilised Process

V_O x10^{-6} mol/min)	T°C
90.0	40.00
96.0	50.00
100.0	60.00
120.0	70.00

APPENDIX E

E1: CALCULATIONS

SUBSTRATE CONCENTRATION

Relative molecular of starch = 162.0g

Number of moles in 6g = $\frac{6}{162} = 0.037\ moles$

Concentration of 0.037 moles in 700ml water is $\frac{0.037\ \text{moles}}{0.700\ \text{dm}^3} = 0.0528\ \text{mol}$

35ml of starch gel contains 0.0528 mol

With addition of 10ml of cell suspension and 10ml of $CaCl_2$ to 35ml of starch gel i.e. V_2 = 55ml

From $C_1V_1 = C_2V_2$

$$C_2 = \frac{0.0528 moles\ \text{dm}^{-3} \times 35ml}{55ml} = 0.0336\ mol$$

E2: RATE OF REACTION (V) FOR FREE *B. subtilis* α AMYLASE

Taking slope at various points on the [P] vs. time plot, Figure 2 say at [P]=0.06 x 10^{-3} mol.

$$V = \frac{d[P]}{dt} = \frac{(0.0592 - 0.0560)10^{-3}}{(22 - 1.8)\text{min}\,s} mol = 13 \times 10^{-6}\ \text{mol/min}$$

Substrate concentration [S] is also determined at that point. This is repeated for four (4) other points on the curve and the various temperatures. The result for V at 50°C is summarised thus:

Table E1: Rates and Concentration of Product and Substrate for Free Process at 323.15K

V x 10^{-6} mol/min	[P] x10^{-3} mol	[S] x10^{-3} mol	1/V x10^{3} (min/mol)	1/S (mol^{-1})
13	0.06	33.52	76.923	29.83
6	0.079	33.50	166.670	29.85
4	0.085	33.49	250.000	29.86
0.769	0.0935	33.27	1300.390	30.06
0.181	0.0975	33.17	5524.860	30.15

E3: DETERMINATION OF V_{max} AND K_m

From Figure. F12 $\left(\frac{1}{V} \text{ vs} \frac{1}{S}\right)$, From Intercept = $\frac{1}{V_{max}} = 25 \times 10^{-3}$ min/Mol

$V_{max} = 40.0 \times 10^{-6}$ Mol/min

$\frac{V_{max}}{2} = 20.0 \times 10^{-6}$ Mol/min

From Figure F13, $K_m = S_{1/2} = 33.522 \times 10^{-3}$ Mol

E4: DETERMINATION OF K AND E_{act}

From plots of V vs [P] for various temperatures, the slope of each line is determined as K and thus the table below:

Table E2: Values for Determination of E_a Free

Table E2: Values for Determination of E_a Free

K (Min)	ln K	Temp. (K)	1/Temp x 10^{-3} (K^{-1})
0.2862	-1.251	313.15	3.19
0.3667	-1.003	323.15	3.096
0.3435	-1.069	333.15	3.00
0.2450	-1.406	343.15	2.92

From graph F15 (ln K vs 1/Temp), from Arhenius equation,

$K = Ae^{-Ea/RT}$

Intercept A = 2.39 x 10^{-3} K^{-1}

$$\text{Slope} = \frac{-E_{act}}{R} = \frac{-0.93-(-1.43)}{(3.46-2.88)\times 10^{-3}}$$

$$-E_{act} = 793.10 \times 8.314\ \text{JK}^{-1}mol^{-1}$$
$$E_{act} = -6.5938\ kJ/mol$$

CALCULATIONS FOR IMMOBILIZED *B.subtilis* α AMYLASE

Substrate concentration used: 0.0528 mol

E5: RATE OF REACTION FOR IMMOBILIZED *B.subtilis* α AMYLASE

As previously determined in appendix E2, the procedure is repeated on figure 5. The following tables are arrived at:

Table E3: Rates and Concentrations of Product and Substrate for Immobilised at 343.15K

V x 10^{-6} (mol/min)	[P] x 10^{-3} (mol)	[S] x 10^{-3} (mol)	1/V x 10^{3} (min/mol)	1/S (1/mol)
120.00	0.86	50.90	8.333	19.65
76.00	1.346	50.50	13.16	19.80
23.00	1.57	49.35	43.48	20.26
11.43	1.74	47.90	87.49	20.88
6.15	1.79	45.37	162.60	22.04

E6: DETERMIANTION OF V_{max}^{app} AND K_m^{app}

From Figure F16

Intercept = $\frac{1}{V_{max}^{app}} = 8\times10^{3}$ min/mol

$V_{max}^{app} = 125.0\times10^{-6}$ mol/min

$\frac{V_{max}^{app}}{2} = 62.5\times10^{-3}$ mol/min

From figure F17 $K_m^{app} = S_{1/2} = 50.44\times10^{-3}$ mol

E7 DETERMINATION OF K AND $E_{(act)immob}$

From the plot of V vs [P] at various temperatures, the slope of each line is determined as K and thus the following table.

Table E4: Values for Determination of E_a Immobilised

K (min)	ln K	Temp (K)	1/Temp x 10^{-3} (K^{-1})
0.08017	-2.5220	313.15	3.19
0.1895	-1.6630	323.15	3.09
0.09227	-2.3830	333.15	3.00
0.1652	-1.8010	343.15	2.92

From the Arrhenius equation, $K = Ae^{-Ea/RT}$, a plot of ln K vs 1/Temp. (Figure F15)

The intercept A = 2.44 x 10^{-3} K^{-1}

Slope = $\frac{E_a}{R} = \frac{-1.46-(-2.41)}{(3.15-2.8)\times 10^{-3}} = -2.714\times 10^{3}$

$-E_a$ = -2.714 x 10^3 * 8.314 J/K/mol

E_a = 22.566 kJ/K/mol

E8 DETERMINATION OF DIMENSIONLESS VARIABLES

$$K_L = K_s = \frac{Flow\ rate}{\text{surface area of chips}} = \frac{0.0012/60}{8\times(0.01)^2} = 0.025m/s = = 1.5\text{m/min}$$

$$D_a = \frac{V_{\max}}{K_s S_o} = \frac{125\mu mol/\min}{1.500m/\min \times 0.0528mol} = 0.00158$$

$$K = \frac{K_m}{S_o} = \frac{0.05044}{0.0528} = 0.955$$

$$\beta \equiv D_a + K_a - 1 \quad \text{----------(4)}$$

$$= 0.00158 + 0.955 - 1 = -0.043$$

$$\text{from x} = \frac{\beta}{2}\left(\pm\sqrt{1+\frac{4K_a}{\beta^2}} - 1\right) \text{----------(5)}$$

$$\text{since } \beta = -0.043 < 0 \text{ then equation (5) becomes}$$

$$\text{x} = \frac{\beta}{2}\left(-\sqrt{1+\frac{4K_a}{\beta^2}} - 1\right) = \frac{-0.043}{2}\left(-\sqrt{1+\frac{4\times 0.955}{(-0.043)^2}} - 1\right)$$

$$\text{x} = \frac{-0.043}{2} - \sqrt{2066.98} - 1 = -46.49$$

$$\eta = \frac{x/(K_a + x)}{1/(K_a + 1)} = \frac{-46.49/(0.955 + (-46.49))}{(0.955+1)^{-1}} = \frac{1.021}{(1.955)^{-1}} = 1.996$$

ASSUMPTIONS

The above calculation is done on the basis of the following assumptions where steady state operation holds:

- No electrostatic effects
- The surface of the chip is flat.
- The rate of sugar production is equal to the rate of substrate diffusion to surface layer and equal to rate of catalysis which equal rate of product diffusion from surface layer.

APPENDIX F

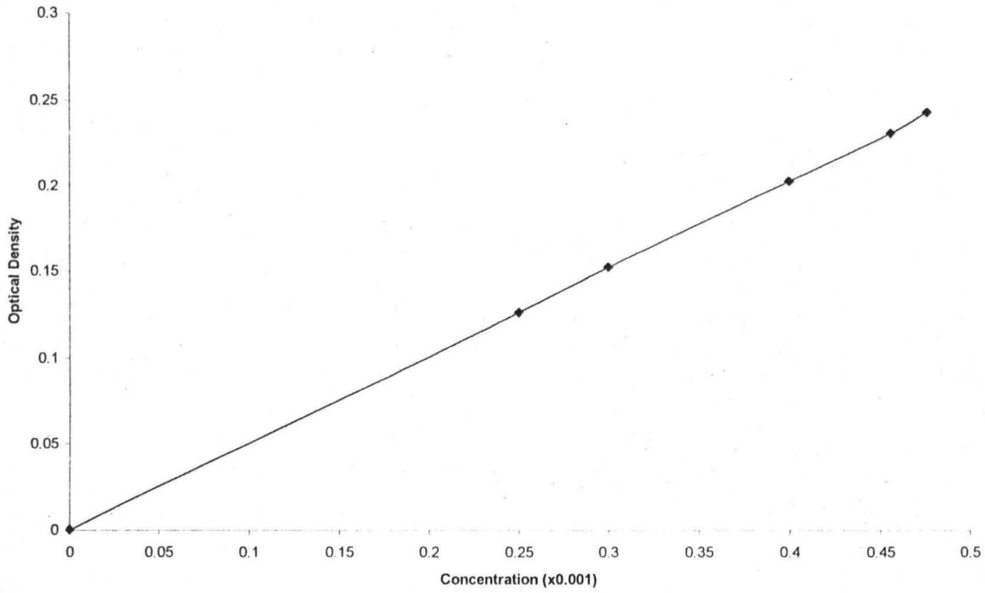

Figure F1: Glucose Calibration Curve

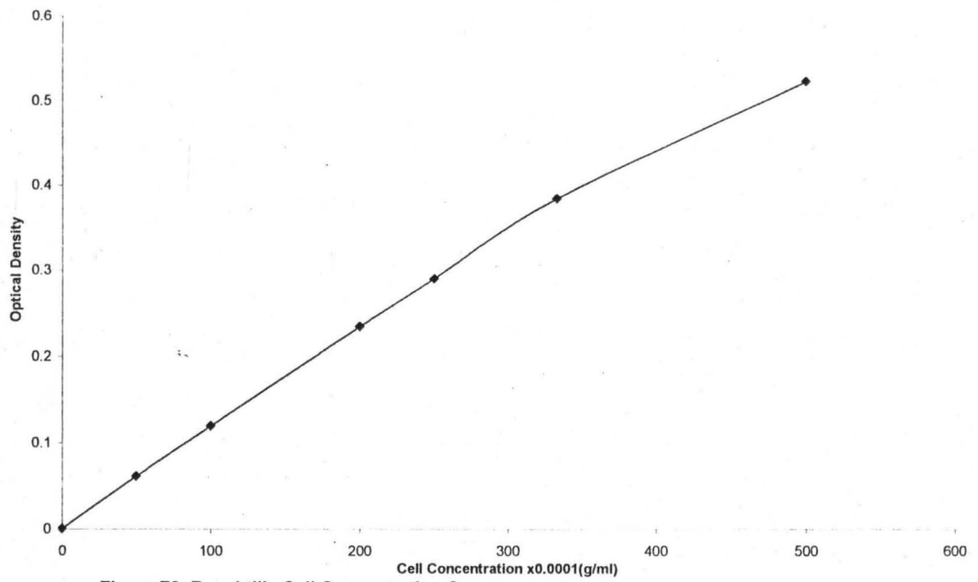

Figure F2: B. subtilis Cell Concentration Curves

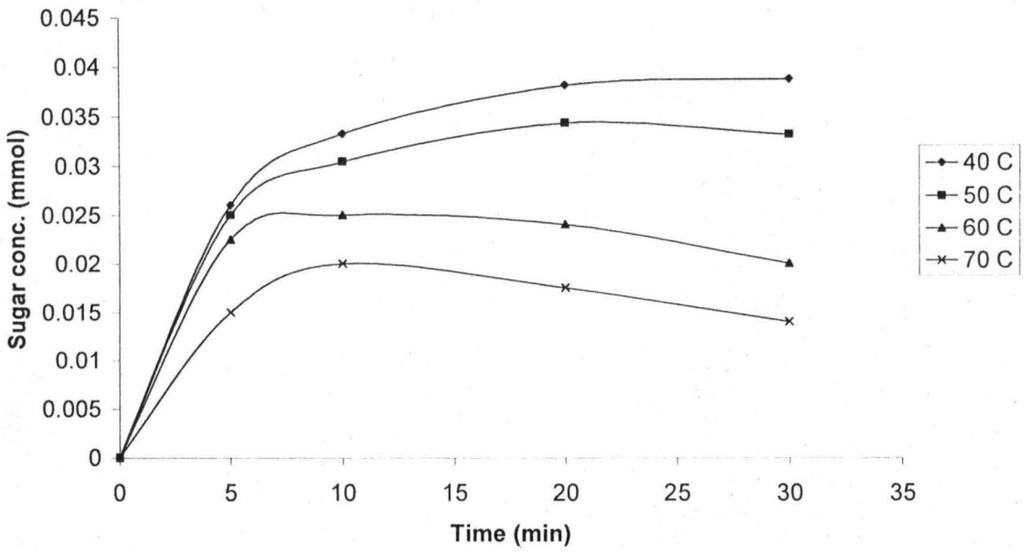

Figure F3: Temperature Effect on Starch Hydrolysis at pH 4.0

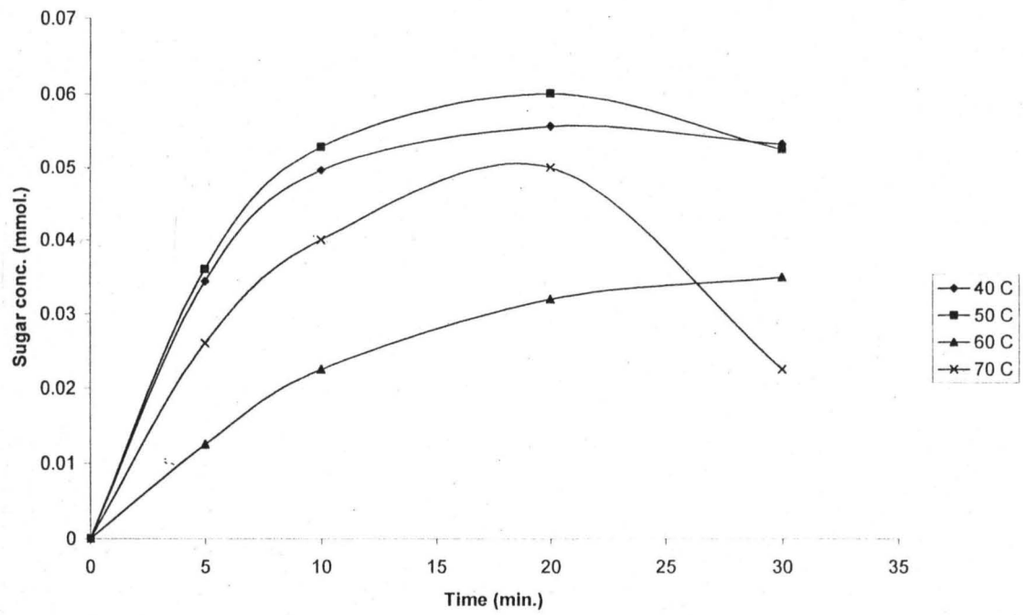

Figure F4: Temperature Effect on Starch Hydrolysis at pH 6.0

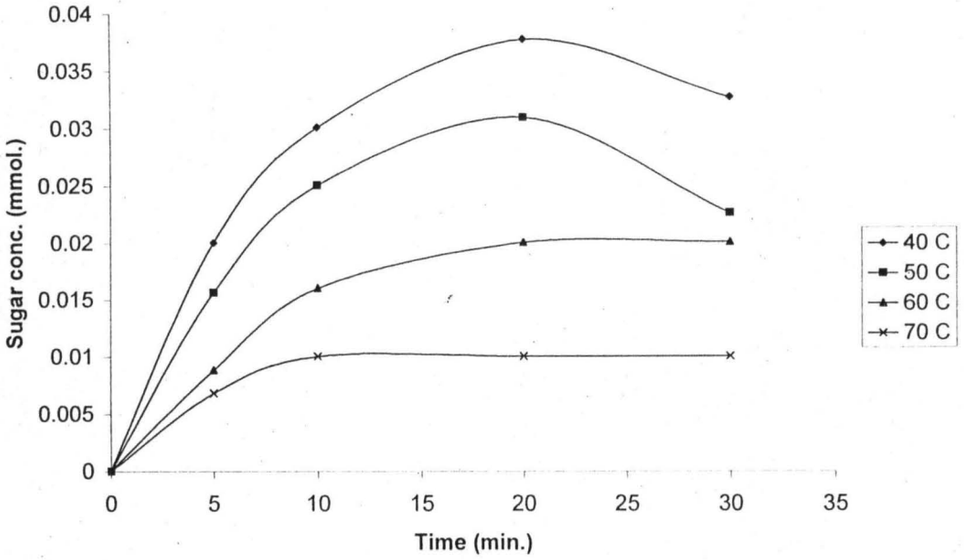

Figure F5: Temperature Effect on Starch Hydrolysis at pH 7.0

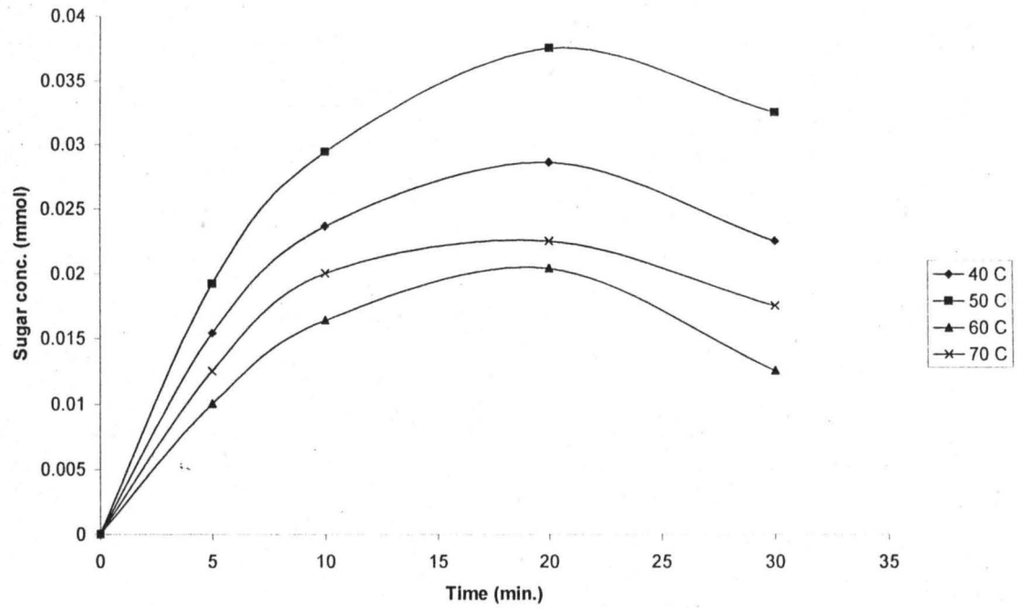

Figure F6: Temperature Effect on Starch Hydrolysis at pH 8.0

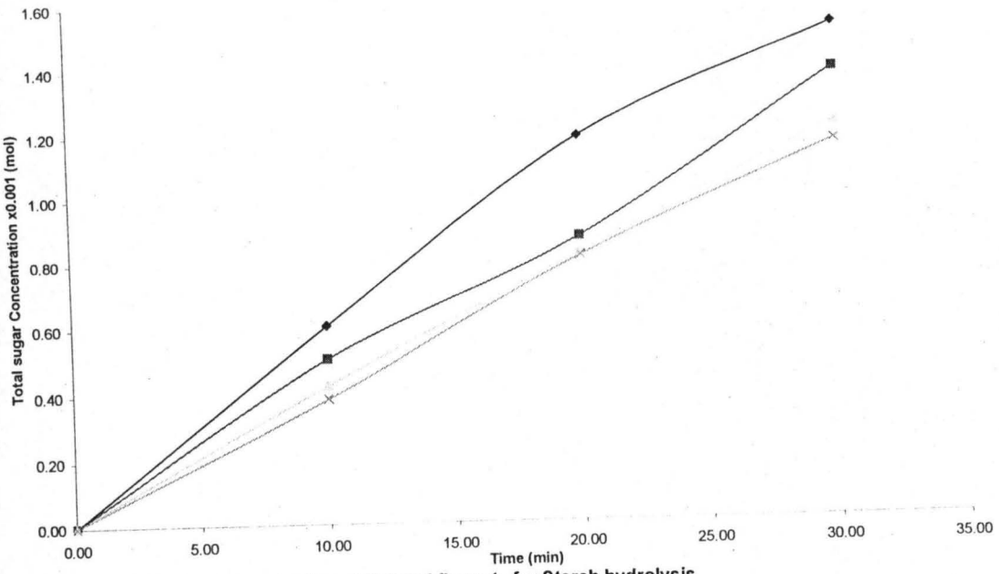

Figure F7; Determination of Optimal flowrate for Starch hydrolysis

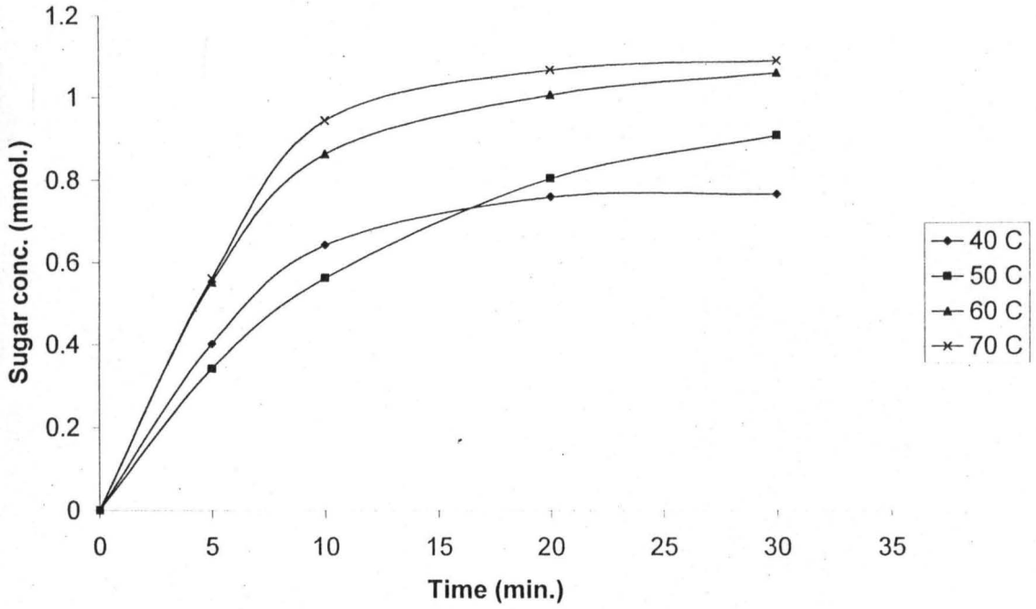

Figure F8: Temperature Effect on Starch Hydrolysis at pH 4.0

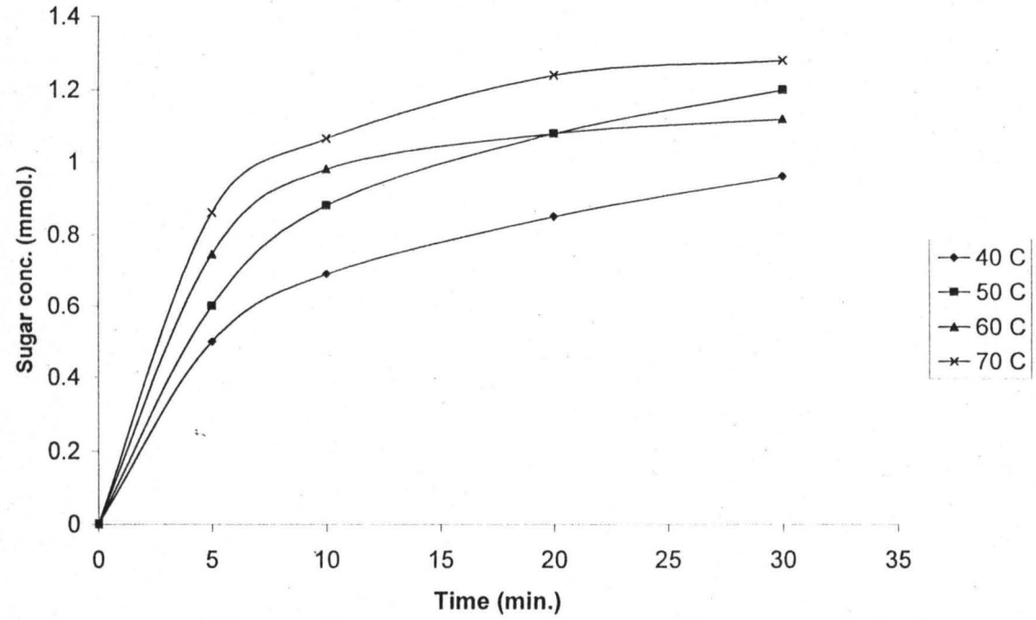

Figure F9: Temperature Effect on Starch Hydrolysis at pH 6.0

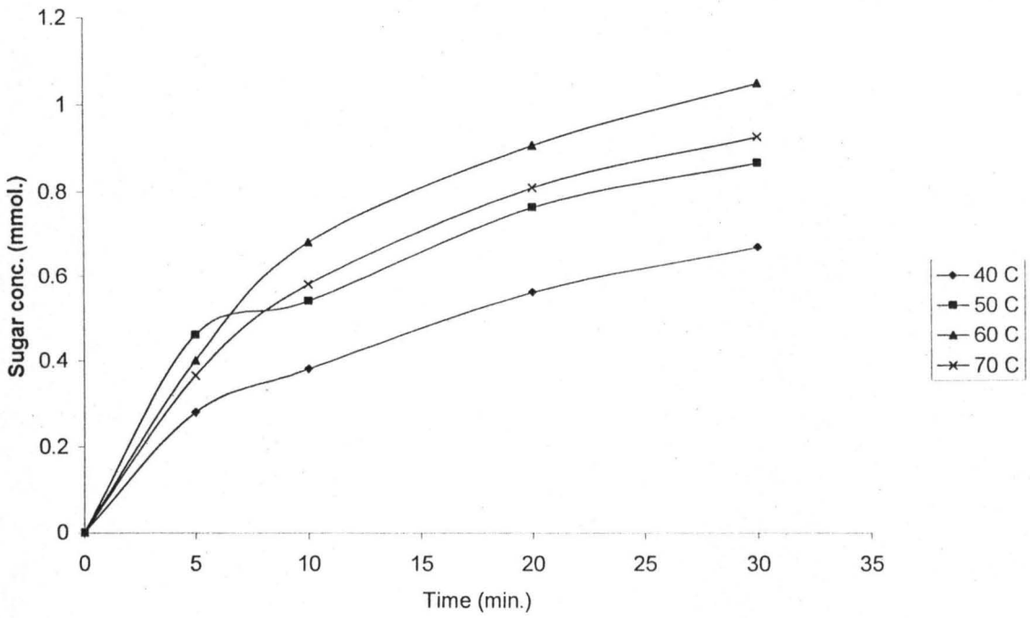

Figure F10: Temperature Effect on Starch Hydrolysis at pH 7.0

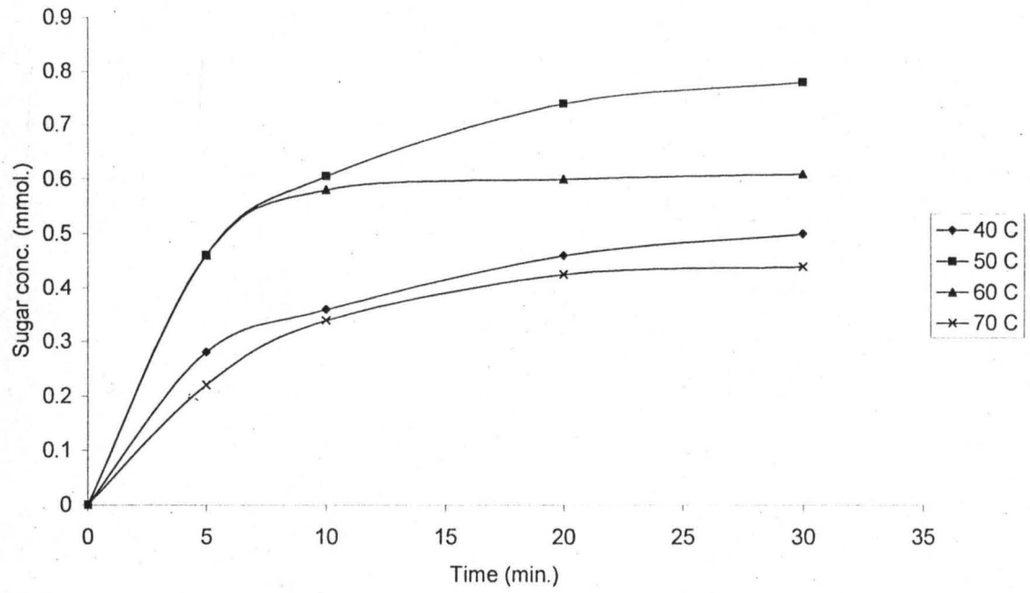

Figure F11: Temperature Effect on Starch Hydrolysis at pH 8.0

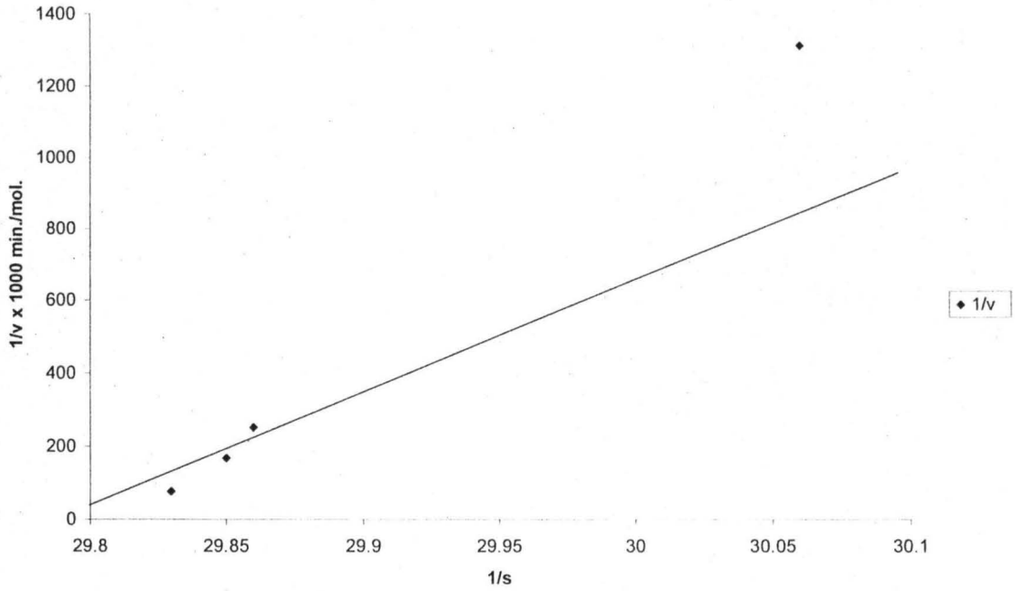

Figure F12: Lineweaver-Burk plot

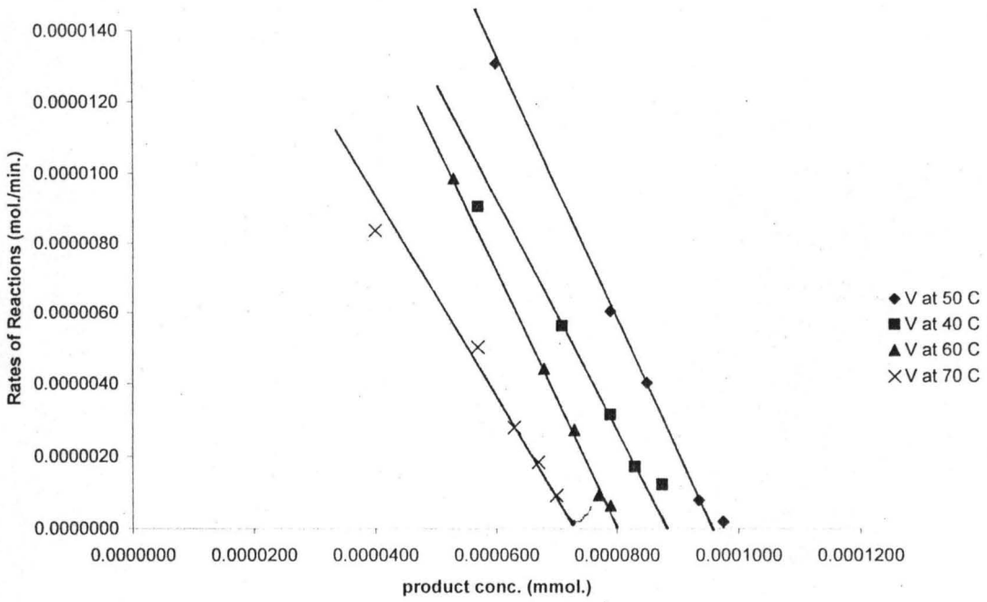

Figure F13: Rates versus Product Concentration

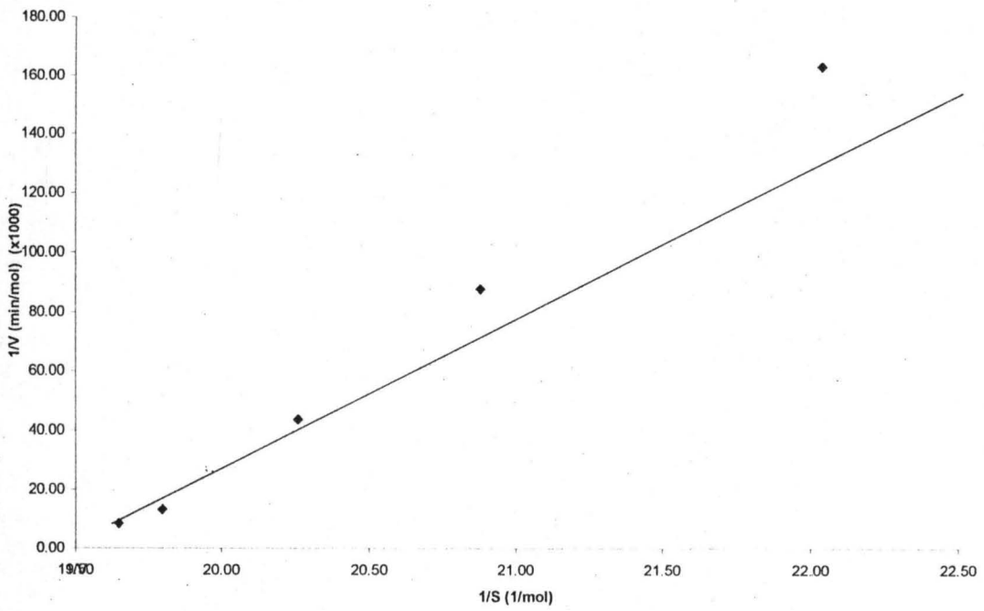

Figure F14: Line weaver-Burk plot for Immob.

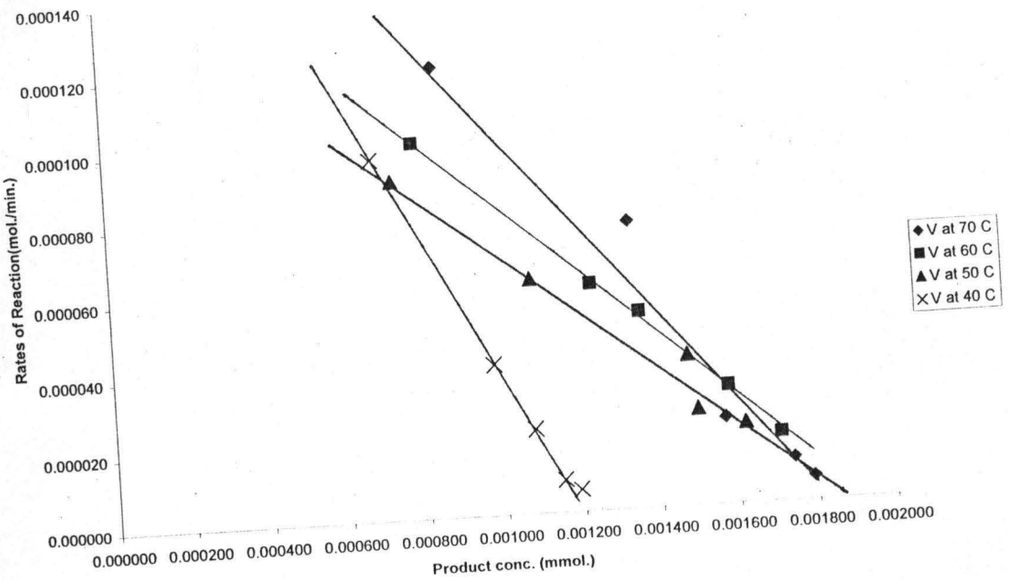

Figure F15: Rates versus Product Conc.

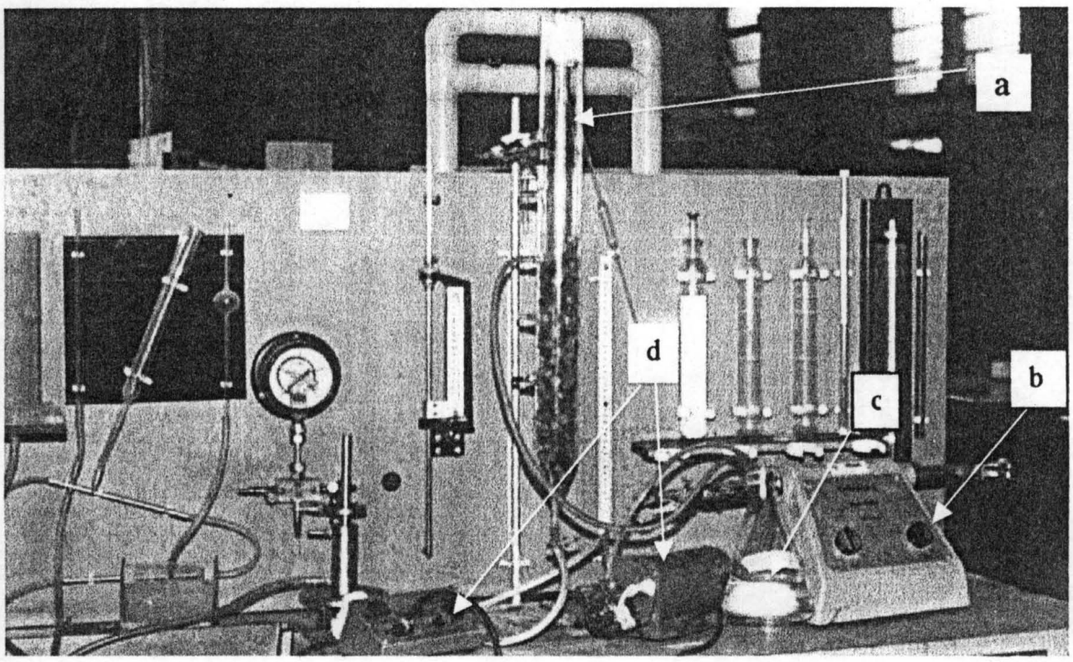

Plate 1 Packed colum connected to a peristaltic pump and substrate stock on Gallen Kemp Shaker

a: Packed column containing chips plugged with cotton wool used for immobilisation and subsequent hydrolysis.

b: Gallenkemp shaker

c: Conical flask containing substrate/cell suspension

d: Peristaltic pump