INVESTIGATION OF BIOPROCESS PARAMETERS FOR GLUCOSE ISOMERASE PRODUCTION by *Bacillus thermoantarcticus*

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ABSTRACT

INVESTIGATION OF BIOPROCESS PARAMETERS FOR GLUCOSE ISOMERASE PRODUCTION by *Bacillus thermoantarcticus*

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In this study, the effects of bioprocess parameters on glucose isomerase production by *Bacillus thermoantarcticus* (DSMZ 9572) were investigated. For this purpose, firstly, in laboratory scale shake-bioreactors, a defined medium was designed in terms of its carbon and nitrogen sources, to achieve the highest glucose isomerase activity. Optimum concentrations of medium components were determined by a statistical approach, namely Response Surface Methodology (RSM). RSM defined the relationship between the response, glucose isomerase activity and the independent variables, medium components. The highest glucose isomerase volumetric activity was obtained as 1630 U L^{-1} in the optimized medium containing 10.64 kg m^{-3} xylan, 5.66 kg m^{-3} yeast extract, 5.92 kg m^{-3} $(NH_4)_2SO_4$, 0.25 kg m^{-3} $MgSO_4.7H_2O$, 0.001 kg m^{-3} $FeSO_4.7H_2O$, 0.001 kg m^{-3} $ZnSO_4.7H_2O$, 0.000075 kg m^{-3} $MnSO_4. H_2O$, and 0.00001 kg m^{-3} $CuSO_4.5H_2O$ at conditions: pH_0= 6.0, T=55°C, N=200 min^{-1}. Then by using the designed medium, pH and oxygen transfer conditions of the bioprocess were investigated in 3.0 dm^3 pilot scale bioreactor. The effect of pH was analyzed at pH=6 uncontrolled and controlled operations with the following conditions: air inlet rate of Q_0/V_R=0.5 vvm and agitation rate of N=500 min^{-1}. The effects of oxygen transfer parameters were examined, at pH=6 controlled condition, at an air inlet rate of Q_0/V_R=0.5 vvm and the agitation rates of N=300,500,750 min^{-1}. The variations in glucose isomerase activity, cell, amino acid and organic acid concentrations with the cell cultivation time, specific cell growth rate, the oxygen uptake rate, the liquid phase coefficient by using the dynamic method, maintenance coefficient for oxygen and yield coefficients were determined. The highest glucose isomerase volumetric activity and cell concentration, 1838 U L^{-1} and 2.26 kg m^{-3} at the 24th of the cell cultivation were attained at 0.5 vvm, 500 min^{-1} and pH=6 uncontrolled operation.

Keywords: Glucose isomerase, production, thermophilic *Bacillus*, response surface methodology (RSM), bioprocess operation parameters

ÖZ

GLİKOZ İZOMERAZ ÜRETİMİ İÇİN BİYOPROSES PARAMETRELERİNİN *Bacillus thermoantarcticus* KULLANILARAK ARAŞTIRILMASI

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Bu çalışmada, *Bacillus thermoantarcticus* (DSMZ 9572) ile biyoproses işletim parametrelerinin glikoz izomeraz üretimine etkileri incelenmiştir. Bu amaçla, ilk olarak, laboratuvar ölçekli biyoreaktörlerde, en yüksek glukoz izomeraz aktivitesini sağlayacak karbon ve azot kaynaklarının etkileri araştırılarak tanımlanmış ortam tasarlanmıştır. En uygun ortam bileşenlerini belirlemek amacıyla, istastik bir yöntem olan Cevap Yüzey Metodu kullanılmıştır. Cevap Yüzey Metodu, bu çalışmada cevap olarak nitelendirilen glukoz izomeraz aktivitesi ile bağımsız değişkenler olarak nitelendirilen ortam bileşenleri arasındaki ilişkiyi tanımlamıştır. En yüksek glukoz izomeraz aktivitesi, 10.64 kg m^{-3} ksilan, 5.66 kg m^{-3} maya özütü, 5.92 kg m^{-3} $(NH_4)_2SO_4$, 0.25 kg m^{-3} MgSO4.7H2O, 0.001 kg m^{-3} FeSO4.7H2O, 0.001 kg m^{-3} ZnSO4.7H2O , 0.000075 kg m^{-3} MnSO4. H2O, and 0.00001 kg m^{-3} CuSO4.5H2O içeren ortamda ve pH_0= 6.0, T=55°C, N=200 min^{-1} koşullarında, 1630 U L^{-1} olarak elde elmiştir. Daha sonraki aşamada tasarlanmış üretim ortamı kullanılarak, 3.0 dm^3 hacimli pilot ölçekli biyoreaktörde, pH ve oksijen aktarımı koşulları incelenmiştir. pH'ın etkisi, hava giriş hızının Q_0/V_R=0.5 vvm ve karıştırma hızının N=500 min^{-1} olduğu oksijen aktarım koşulunda, pH=6 kontrollü ve kontrolsüz işletim koşullarında incelenmiştir. Oksijen aktarım etkileri, başlangıç pH'ının 6 olduğu kontrollü pH işletim koşullarında hava giriş hızının Q_0/V_R=0.5 vvm ve karıştırma hızının N=300,500,750 min^{-1} olduğu üç farklı koşulda incelenmiştir. Glukoz izomeraz aktivitesi, hücre, amino asit, organik asit konsantrasyonlarının kalma süresi ile değişimi; dinamik yöntem kullanılarak , biyoprosesin büyüme evresi süresince , oksijen tüketim hızı ve sıvı faz kütle aktarım katsayısı; verim ve yaşam katsayıları belirlenmiştir. En yüksek glukoz izomeraz aktivitesi ve hücre konsantrasyonu, 0.5 vvm, 500 min^{-1}, pH=6 kontrolsüz koşullarında, biyoprosesin 24. saatinde sırasıyla 1838 U L^{-1} ve 2.26 kg m^{-3} olarak elde edilmiştir.

Anahtar Kelimeler: Glikoz izomeraz, üretim, termofilik *Bacillus*, cevap yüzey metodu, biyoproses işletim koşulları

To my Family

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a	The gas liquid interfacial area per unit liquid volume, $m^2\ m^{-3}$
A	Glucose Isomerase volumetric activity, $U\ L^{-1}$
A_x	Glucose Isomerase specific activity, $U\ g^{-1}DW$
A_λ	Absorbance
C_{AA}	Amino acid concentration, $kg\ m^{-3}$
C_{OA}	Organic acid concentration, $kg\ m^{-3}$
C_O	Dissolved oxygen concentration, $mol\ m^{-3}$; $kg\ m^{-3}$
Co_0	Initial dissolved oxygen concentration, $mol\ m^{-3}$; $kg\ m^{-3}$
C_O^*	Oxygen saturation concentration, $mol\ m^{-3}$; $kg\ m^{-3}$
C_X	Cell concentration, kg dry cell m^{-3}
C_{XYLAN}	Xylan concentration, $kg\ m^{-3}$
C_{YE}	Yeast extract concentration, $kg\ m^{-3}$
$C_{(NH4)2SO4}$	$(NH_4)_2SO_4$ concentration, $kg\ m^{-3}$
C_{as}	$(NH_4)_2SO_4$ concentration, $kg\ m^{-3}$
Da	Damköhler number (=OD / OTR_{max}; Maximum possible oxygen utilization rate per maximum mass transfer rate)
E	Enhancement factor (=K_La / K_La_o); mass transfer coefficient with chemical reaction per physical mass transfer coefficient
HOT	High Oxygen Transfer Condition, Q_0/V_R = 0.5 vvm and N=750 min^{-1} with controlled pH=6
K_La_0	Physical overall liquid phase mass transfer coefficient; s^{-1}
K_La	Overall liquid phase mass transfer coefficient; s^{-1}
K_m	Michealis constant for the substrate
LOT	Low Oxygen Transfer Condition, Q_0/V_R = 0.5 vvm and N=300 min^{-1} with controlled pH=6
MOT-pHuc	Medium Oxygen Transfer Condition, Q_0/V_R = 0.5 vvm and N=500 min^{-1} with uncontrolled pH=6
MOT-pHuc	Medium Oxygen Transfer Condition, Q_0/V_R = 0.5 vvm and N=500 min^{-1} with controlled pH=6
N	Agitation or shaking rate, min^{-1}
pH_0	Initial pH
Q_0	Volumetric air feed rate, $m^3\ min^{-1}$
q_o	Specific oxygen uptake rate, kg kg^{-1} DW h^{-1}
q_p	Specific product formation rate, U kg $cell^{-1}\ h^{-1}$

r	Volumetric rate of reaction (mol m^{-3} s^{-1})
r_{max}	Maximum rate of reaction at infinite reactant concentration
r_0	Oxygen uptake rate, mol m^{-3} s^{-1}; kg m^{-3} h^{-1}
r_P	Product formation rate, U L^{-1} h^{-1}
t	Bioreactor cultivation time, h
T	Bioreaction medium temperature, °C
T_{AA}	Total amino acid concentration, kg m^{-3}
T_{OA}	Total organic acid concentration, kg m^{-3}
U	One unit of an enzyme
V	Volume of the bioreactor, m^3
V_R	Volume of the bioreaction medium, m^3
$Y_{X/O}$	Yield of cell on oxygen, kg kg^{-1}
$Y_{P/O}$	Yield of product on oxygen, U kg O_2^{-1}
$Y_{P/X}$	Yield of product on cell, U kg $cell^{-1}$

Greek Letters

η	Effectiveness factor (=OUR/OD; the oxygen uptake rate per maximum possible oxygen utilization rate)
μ	Specific cell growth rate, h^{-1}
μ_{max}	Maximum specific cell growth rate, h^{-1}
λ	Wavelength, nm

Abbreviations

Ac	Acetic acid
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
But	Butyric acid
CCCD	Central Composite Circumscribed Design
Cys	Cysteine
DO	Dissolved oxygen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

EC	Enzyme Commission
Gln	Glutamine
Gluc	Gluconate
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Mal	Malate
Met	Methionine
OA	Oxaloacetic acid
Orn	Ornithine
OD	Oxygen demand ($=\mu_{max} C_X / Y_{X/O}$; mol m^{-3} s^{-1})
OTR	Oxygen transfer rate, mol m^{-3} s^{-1}
OTR_{max}	Maximum possible mass transfer rate ($=K_L a C_O^*$; mol m^{-3} s^{-1})
Phe	Phenylalanine
Pro	Proline
RSM	Response Surface Methodology
Ser	Serine
Suc	Succinate
Val	Valine
Tyr	Tyrosine
Thr	Threonine
Trp	Tyrptophan

CHAPTER 1

INTRODUCTION

Enzymes are protein, glycoprotein, or RNA molecules that have a key role in catalyzing biologically important chemical transformations; and thus, the essential components of all living systems (Lehninger, 2000). They are used in a multitude of processes, in industry as well as in the daily household, and enzymatically based processes are replacing many classical processes due to their mild reaction conditions and environmentally friendly outcome. The market (approximately 1.5 billion USD in 2000) is expanding, and new applications of enzymes are constantly being explored as can be seen in the yearly reports of the enzyme producers (Nielsen, Villadsen and Liden, 2003).

Enzyme catalyzed reactions are relatively fast at milder conditions and free of by-products .The rapid rate, mild reaction conditions, and high selectivity are the same desirable qualities of good industrial catalysts (Adler et al.,1979) .Distinct advantages of enzymes over chemical catalysts can be summarized as :

1. *Enzymes are very efficient catalysts.* Typically the rates of enzyme mediated processes are accelerated, compared to those of the corresponding non-enzymatic reactions, by a factor of 108-1010, which is far above the values that chemical catalysts are capable of achieving.
2. *Enzymes are environmentally acceptable.* Unlike heavy metals, biocatalysts are environmentally benign reagents since they are completely degradable.
3. *Enzymes act under mild conditions.* Enzymes act in a pH range of about 5-8, typically around 7, and in a temperature range of 20–40oC, preferably at around 30oC. This minimizes problems of undesired side-reactions such as decomposition, isomerization, racemization and rearrangement, which often plague traditional methodology.
4. *Enzymes are compatible with each other.* Since enzymes generally function under the same or similar conditions, several biocatalytic

reactions can be carried out in a reaction cascade in one flask. Thus sequential reactions are feasible by using multi-enzyme systems in order to simplify reaction processes (Faber, 2000).

However, enzymes, being proteins are delicate molecules that are not always stable in or especially out their native environment. Therefore, it is important to develop methods for improving enzymes so that they can be used for long periods of time. One such industrially used enzyme is glucose isomerase in which several targets are present for improving the enzyme.

Glucose isomerase (D-xylose ketol-isomerase EC 5.3.1.5) catalyzes the reversible isomerization of D-xylose to D-xylulose and D-glucose to D-fructose (Belfaquih et al., 2000). Interconversion of xylose to xylulose serves a nutritional requirement in saprophytic bacteria that thrive on decaying plant material and also aids in the bioconversion of hemicellulose to ethanol (Bhosale et al., 1996). However, glucose isomerase gains its importance by being one of the important enzymes in the food industry. Its function in the conversion of glucose to fructose serves an important step in the production of high fructose corn syrup HFCS). While, D-glucose (dextrose) has 70-75% of the sweetening ability of sucrose, D-fructose (levulose), the other monosaccharide moiety of sucrose, has twice the sweetening power of sucrose (Barker, 1976).

The discovery of the glucose-isomerizing capacity of the enzyme from *Pseudomonas hydrophila* by Marshall and Kooi in 1957 was the starting point of the exploitation of glucose isomerase for the manufacture of HFCS as a substitute for cane sugar. Glucose isomerase gained commercial importance in the United States because of the lack of supply of sucrose after the Cuban Revolution in 1958, and continues to be one of the most important industrial enzymes to this day. Enzymatic glucose isomerization was first accomplished on an industrial scale in 1967 by Clinton Corn Processing Co. in the United States. The demand for HFCS in the food industry increased, and by 1980 practically all major starch processing companies in the western world were resorting to GI technology (Bhosale et al., 1996).

HFCS syrup is produced from cornstarch in which process comprises three major steps including several enzymatic reactions. The use of enzymes is preferred as it offers a number of advantages including improved yields and favorable economics. Enzymatic hydrolysis allows greater control over amylolysis, the specificity of the reaction, and the stability of the generated products. The milder reaction conditions involve lower temperatures and near-neutral pH, thus reducing unwanted reactions. Enzymatic methods are favoured because they also lower energy requirements and eliminate neutralization steps. The enzymes employed in the industrial starch hydrolysis market are estimated to occupy 10–15% of the total world enzyme market. This industrial sector is the second biggest consumer of enzymes (Satyanarayana et al., 2004).

Table 1.1 Commercial producers of GI (Bhosale et.al.,1996)

Organism	Trade name	Manufacturer
Actinoplanes missousriensis	Maxazyme	Gist Brocades and Anheuser-Busch Inc.
Bacillus coagulans	Sweetzyme	Novo-Nordisk
Streptomyces rubiginosus	Optisweet Spezyme	Miles Kali-Chemie Finnsugar
Streptomyces phaeochromogenes	Swetase	Nagase
Arthrobacter sp.		Reynolds Tobacco
Streptomyces olivaceus		Miles Laboratories Inc.

Table 1.1 shows the commercial producers of glucose isomerase. For the industrial production of HFCS, the enzyme is immobilized in various ways, including:

- Covalent binding to an insoluble carrier
- Adsorption to an insoluble carrier
- Entrapping in a matrix
- Immobilization within the cells

Glucose isomerases are commercially produced mostly by *Bacillus* species. Novo-Nordisk, being one of the world's biggest producers of enzyme, produces glucose isomerase by *Bacillus coagulans*. Sweetzyme is a glucose isomerase used in the starch industry to convert glucose into fructose during isomerisation and displays a linear decay curve under steady operating conditions. This effective conversion of glucose to fructose for sweeteners provides several benefits:

- Continuous operation – convenience and low by-product formation
- High productivity – column lifetime up to 200-400 days
- Low cost of conversion – up to 18000 kg fructose per kg Sweetzyme IT
- High average flow rate – minimizing column output variation (http://www.novozymes.com/en/MainStructure/ProductsAndSolutions/Starch/Isomerisation/Sweetzyme/Sweetzyme.htm)

Conversion of glucose to fructose is also available chemically. However chemical conversion of glucose to fructose is carried out at high pH and temperature. The reaction is nonspecific and leads to the formation of non-metabolizable sugars such as psicose and other undesirable colored products. It is difficult to attain a fructose concentration of more than 40% by this method. Moreover, chemically produced fructose has off flavors and reduced sweetness, which cannot be easily remedied. Therefore, it cannot be used commercially. On the other hand, enzymatic conversion of glucose to fructose offers several advantages, such as (i) specificity of the reaction, (ii) requirement of ambient conditions of pH and temperature, and (iii) no formation of side products (Bhosale et al., 1996). These results point out the importance of glucose isomerase, being one of the most important food processing enzyme.

Due to the importance of this enzyme in the food industry, various studies have been done for its improvement. The researches were mainly focused on its induction, stability, and purification. In the HFCS process, the final position of the fructose:glucose equilibrium is highly temperature dependent, favoring fructose at higher temperature. Thus attempts have been made to improve the thermostability of enzyme in order to increase the maximum attainable fructose level (Goldberg and Williams, 1991).

One approach to develop glucose isomerase with an increased temperature optimum is to use thermophilic microorganisms. Indeed, glucose isomerases with increased thermostability are produced by the thermophilic bacteria such as *Bacillus stearothermophilus, Clostridium thremosulfurogenes, Thermoanabacter B6A, Thermus aqauticus, Thermus Thermophilus* and *Thermotoga maritima*, thus conforming the validity of this approach (Brown et al.,1993).

Some important literature studies concerning the production of thermostable glucose isomerases are summarized below. The common point in these studies is that the thermostability of glucose isomerase from the source microorganism is coupled with the high production capacity of the host microorganism by genetic engineering techniques.

- Dekker et al. (1992) cloned *Thermus thermophilus HB8* gene in *E. coli* and *Bacillus brevis*. Glucose isomerase from *Thermus thermophilus HB8* is stable at high temperatures and processes at pH around 7.0 where by-product formation is minimal. By using the efficient GI production *E. coli* and *Bacillus Brevis*, their production capacities were determined and compared (Dekker et al., 1992).

- An extremely thermophilic marine anaerobe *Thermotoga maritima* was used to investigate the thermostability of glucose isomerase in several conditions (Brown et al., 1992).

- *Clostridium thermosulfurogenes* xylA gene was cloned in *Saccharomyces cerevisiae* which is known for its high alcohol tolerance, rapid fermentation rate and high end-product yield (Moes et al., 1996).

- The stability of *Bacillus licheniformis* glucose isomerase was improved by the contribution of bivalent cations by cloning in *E. coli* (Vieille et al., 2001).

In literature, efforts were mainly towards the effects of bioprocess parameters on the production of glucose isomerase and investigation of physico-chemical properties of the enzyme. Some of these literature studies are summarized below:

- Deshmukh et al. (1994) performed medium optimization for the production of glucose isomerase from thermophilic *Streptomyces* *thermonitrificans.* An activity of intracellular glucose isomerase (12 U/ml) was achieved in a medium containing 10 kg m^{-3} xylose, supplemented with 2 kg m^{-3} sorbitol, as the second carbon source, at 50°C for 16 h.

- Ensari et al. (1995) investigated the effects of temperature and xylose concentration on the glucose isomerase producing *Thermus thermophilus* and induction time on glucose isomerase activity. 65°C and 5 kg m^{-3} of xylose were found as the optimum temperature and substrate concentration for the growth of *Thermus thermophilus*. The optimum induction time was found as the t=12 h of cultivation.

- Wang et al.(1998) studied the effects of growth medium, aeration and temperature on glucose isomerase production by *Streptomyces lividans*. The highest glucose isomerase activity was found as 2.018 GIU ml^{-1} in a medium containing tryptic soy broth and glucose at an air inlet rate of 1.6 L min^{-1}. The optimum temprature for growth and glucose isomerase production was attained as 37°C.

- In 2000, a bifunctional β-xylosidase-xylose isomerase from *Streptomyces sp. EC10* was reported. Effects of various carbon source on activity were investigated (Belfaquih et al., 2000). They found that birchwood xylan attained the highest GI activity as 8.78 U mg^{-1}.

- The only literature study about glucose isomerase production from *Bacillus thermoantarcticus* was reported by Lama et al (2001). They investigated the physico-chemical properties of the enzyme from *Bacillus thermoantarcticus.* They found that fermentation broth containing 5 kgm^{-3} of xylose attained the highest GI activity as 0.18 U mg^{-1}. They also found Mg+2, and Co+2 as the necessary metal ions, pH=7 and 80°C as the optimum pH and temperature values essential for obtaining the highest enzyme activity. They investigated the variation of GI activity with time and obtained the highest GI activity at approximately t=24 h of the cultivation.

In this present work, production of glucose isomerase by a thermophilic microorganism was investigated. A novel thermophile namely, *Bacillus thermoantarcticus* was used as the potential producer of glucose isomerase. Due to the thermostability of glucose isomerase from *Bacillus thermoantarcticus*, this study could be an alternative way of the production of glucose isomerase for the starch industry. However, the researches were made to optimize the fermentation medium of thermostable glucose isomerase producing *Bacillus thermoantarcticus* rather than improving its thermostability. In the literature studies, a complete medium optimization has not been performed in order to develop an economically viable fermentation process for the production of the enzyme. So aim of this study is to optimize to fermentation medium by all means of medium components, pH and oxygen transfer parameters. This study was performed in two parts.

Primarily, the fermentation medium was analyzed in terms of its carbon and nitrogen sources in laboratory scale bioreactors. Medium components were determined by carrying out individual medium design experiments. These experiments resulted in a medium containing birchwood xylan, yeast extract and ammonium sulfate as carbon and nitrogen sources respectively. Then in order to maximize glucose isomerase activity, appropriate concentrations of these medium components were analyzed by the statistical approach called Response Surface Methodology (RSM). RSM enabled to obtain the optimum combination of medium components that yield the highest glucose isomerase production. After obtaining the optimized medium, effects of initial pH of the fermentation broth, base type in order to adjust the pH of the fermentation medium, xylan types and carbon sources on glucose isomerase activity was determined.

Finally, using the designed medium obtained in the laboratory scale bioreactor, production of glucose isomerase proceeded in the pilot scale bioreactors with batch operation mode. The effects of four combinations of fermentation conditions on glucose isomerase production were investigated in terms of pH and oxygen transfer parameters. Firstly, the effects of uncontrolled and controlled pH operations on glucose isomerase production were determined. After deciding on the suitable pH strategy, effects of oxygen transfer parameters namely, agitation rate(N) and air inlet rate(Q_0/V_R) on glucose isomerase activity were evaluated.

CHAPTER2

LITERATURE SURVEY

2.1 Enzymes

2.1.1 General characteristics

Enzymes are usually proteins of high molecular weight (15,000<MW) that act as catalysts (Shuler and Kargi, 2002). Enzymes have extraordinary catalytic power, often far greater than that of synthetic or inorganic catalysts. They have a high degree of specificity for their substrates, they accelerate chemical reactions tremendously, and they function in aqueous solutions under very mild conditions of temperature and pH (Nelson, Lehninger and Freeman, 2004).

Many enzymes have more than one subunit. Some protein enzymes require a nonprotein group for their activity. This group is either a cofactor, such as metal ions, Mg, Zn, Mn, Fe, or a coenzyme, such as a complex organic molecule, NAD,FAD,CoA, or some vitamins (Shuler and Kargi,2002).

With the exception of a small group of catalytic RNA molecules, all enzymes are proteins. Their catalytic activity depends on the integrity of their native protein conformation. If an enzyme is denatured or dissociated into its subunits, catalytic activity is usually lost. If an enzyme is broken down into its component amino acids, its catalytic activity is always destroyed. Thus the primary, secondary, tertiary, and quaternary structures of protein enzymes are essential to their catalytic activity (Nelson, Lehninger and Freeman, 2004).

With the exception singular exception of solid-phase enzyme synthesis, all enzymes are derived from living sources. Although all living cells produce enzymes, one of the three sources –plant, animal, or microbial-may be favored for a given enzyme or utilization (Bailey, 1986).

2.1.2 Classification of Enzymes

Many enzymes have been named by adding the suffix "-ase" to the name of their substrate or to a word or phrase describing their activity. Sometimes the same enzyme has two or more names, or two different enzymes have the same name. Because of such ambiguities, and the ever increasing number of newly discovered enzymes, biochemists, by international agreement, have adopted a system for naming and classifying enzymes. This system divides enzymes into six classes, each with subclasses, based on the type of reaction catalyzed. Each enzyme is assigned a four-part classification number (enzyme commission, EC) and a systematic name, which identifies the reaction it catalyzes (Nelson,Lehninger and Freeman, 2004). As an example, the EC number of glucose isomerase is 5.3.1.5, in which the first number (5) denotes the class name (isomerases); the second number (3), the subclass (intramolecular oxidoreductases); the third number (1), a intramolecular oxidoreductase interconverting aldoses and ketoses; and the fourth number (5), hydrogen-atom transfer conversion of D-glucose to D-fructose. Table 2.1 shows the international classification of enzymes.

Table 2.1 International classifications of enzymes

No	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C-C, C-S, C-O, and C-N bonds by condensation reactions coupled to ATP cleavage

2.1.3 Enzyme Activity

When the enzyme is part of a crude preparation, its concentration is in terms of 'units'. A unit is the amount of enzyme that gives a predetermined amount of catalytic activity under specific conditions (Shuler and Kargi, 2002).

By international agreement, 1.0 unit of enzyme activity is defined as the amount of enzyme causing transformation of 1.0 µmol of substrate per minute at under optimal conditions of measurement.

One unit of glucose isomerase activity was defined as the formation of 1 µmol of D-fructose per minutes at 80°C (Lama et al., 2001). The method for glucose isomerase activity measurement and fructose calibration curve are given in section 3.5.2 and Appendix B respectively.

2.2 Thermostable enzymes

Thermophilic microorganisms are the source of thermostable proteins and enzymes. Thermostable enzymes enable the producer to work at higher temperatures, which results higher reaction rates, higher equilibrium concentrations of products and decrease of operation time. Moreover, thermophiles offer minimal risk of contamination, decreased density, surface tension, viscosity of culture and easy recovery of volatile products during the fermentation process, thereby reduction of production cost. Hence, usage of thermophiles gains an importance in industrial biotechnology.

One prominent example is in the starch industry, where starch is gelatinized at elevated temperature to make the polysaccharide accessible for enzymatic attack. Thermostable enzymes of interest for starch conversion include amylases, pullulanase, glucoamylase, glucose isomerase, branching enzyme, and cyclodextrin glycosyltransferase (Niehaus et al., 1999; Zeikus et al., 1998). Another example is in the paper industry, which is increasingly aware of environmental risks caused by pollution of wastewater with hazardous chemicals. Therefore, paper manufacturers are aiming to reduce the amount of chlorine-containing chemicals needed for the bleaching of pulp. One of the strategies at trial is to remove residual xylan from the pulp with xylanases, which can significantly reduce the amount of the subsequently applied bleaching agents

(Viikari et al., 1994). Xylanases useful for this prebleaching process should optimally be active at elevated temperature (>70°C) and at alkaline pH (Sterner et al., 2001).

A highly advantageous aspect of extremely thermostable enzymes is their easy purification after recombinant production in mesophilic hosts, because simple heat treatment of a crude cell extract typically removes more than 90% of the heat-labile host proteins (Sterner et al., 2001).

D-xylose (glucose) isomerase from *Bacillus thermoantarcticus* was remarkably stable and resistant to high temperatures. Therefore, this enzyme, stable at acidic pH values, may increase the efficiency of the process and may reduce the possibilities of by-product formation (Lama et al., 2001). Table 2.2 shows the physico-chemical properties of glucose isomerase from thermophilic microorganisms.

Table 2.2 Physico-chemical properties of glucose isomerase from thermophilic microorganisms (Lama et al., 2001)

Microorganisms	Molecular weight (kDa)	Optimum temperature (°C)	Optimum pH	Thermostability
B.thermoantarcticus	200	90	7.0	90°Cx30 min
B.stearothermophilus	130	80	7.5-8.0	75°Cx10 min
Thermot. maritime	200	105-110	6.5-7.5	100°Cx10 min
Thermot. nepolitana	200	95	7.1	half-life 95°Cx24 min
Thermus thermophilus	200	95	7.0	85°Cx8 h
Thermus aquaticus	196	85	7.5	70°Cx1 month
Thermoanaerobacterium saccharolyticum B6A-R1	200	80	7.0-7.5	85°Cx1 h
Thermoanaerobacterium JW/SL-YS 489	200	80	6.8	80°Cx1 h
Clostridium thermosulfurogenes	200	80	7.0-7.5	85°Cx1 h
Streptomyces sp. (PLC)	183	80	7.0	53°Cx10 days
Streptomyces sp. SK	185	95	6.0	80°Cx5 h

2.2.1 Glucose Isomerase

Glucose isomerase catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively (Figure 2.1). Interconversion of xylose to xylulose serves a nutritional requirement in saprophytic bacteria that thrive on decaying plant material and also aids in the bioconversion of hemicellulose to ethanol. Isomerization of glucose to fructose is of commercial importance in the production of high-fructose corn syrup (Bhosale et al., 1996).

a)In vivo

b)In vitro:

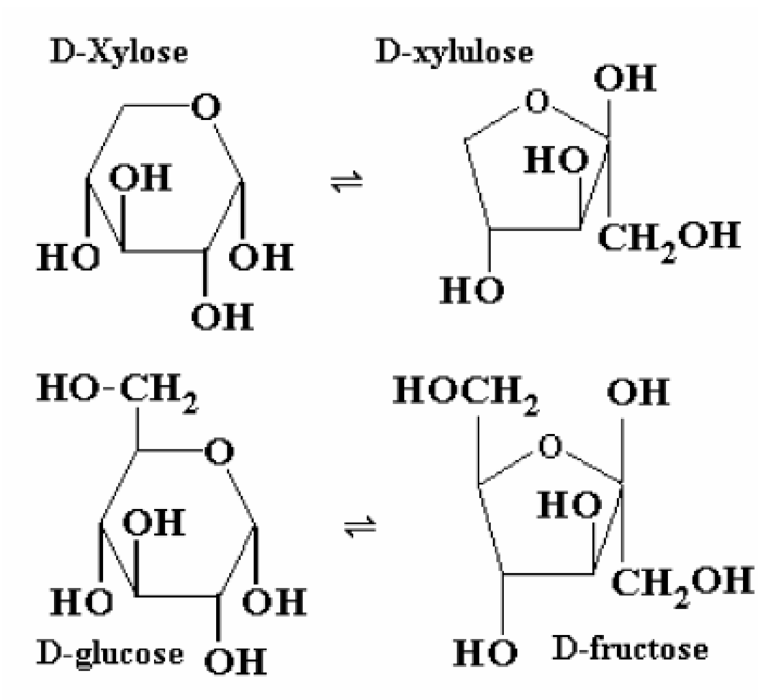

Figure 2.1 Reactions catalyzed by glucose isomerase. (a)In vivo reaction (b)In vitro reaction

Xylose has the same configurations as glucose on C-1 to C-5, and merely lacks C-6. Although xylose is a pentose sugar, it forms a glucose-like pyran ring.

Similarly xylulose resembles fructose without C-6. As a result the catalytic reaction works perfectly well with glucose as substrate, and humans use the enzyme as glucose isomerase (Bhosale et al., 1996).

GI is widely distributed in prokaryotes. Table 2.3 summarizes the GI producing microorganisms. Intracellular production of glucose isomerase is common. *Bacillus thermoantarcticus, Thermus thermophilus, Streptomyces fusant D3, Thermus aquaticus, S. phaeochromogenes, Thermotoga napolitana* are some of the source microorganisms that produce glucose isomerase intracellularly. Reports on extracellular production of GI are not common. Extracellular GI has been reported to be produced by *Streptomyces glaucescens, S. flavogriseus*, *Chainia* sp. and an alkalothermophilic *Bacillus* sp. for which the release of the enzyme from the cells was attributed to a change in the cell wall permeability and partial lysis of the cells (Bhosale et. al., 1996).

The occurrence of GI in a few yeasts such as *Candida utilis* and *Candida boidinii* has been documented. *Aspergillus oryzae* is the only fungus, which is reported to possess GI activity. The existence of GI in barley malt and wheat germ has been reported (Bhosale et. al., 1996).

Table 2.3 GI-producing microorganisms (Bhosale et.al.,1996)

Species
Actinomyces olivocinereus, *A. phaeochromogenes*
Actinoplanes missouriensis
Aerobacter aerogenes, *A. cloacae*, *A. levanicum*
Arthrobacter spp.
Bacillus stearothermophilus, *B. megabacterium*, *B. coagulans*
Bifidobacterium spp.
Brevibacterium incertum, *B. pentosoaminoacidicum*
Chainia spp.
Corynebacterium spp.
Cortobacterium helvolum
Escherichia freundii, *E. intermedia*, *E. coli*
Flavobacterium arborescens, *F. devorans*
Lactobacillus brevis, *L. buchneri*, *L. fermenti*, *L. mannitopoeus*, *L. gayonii*, *L. fermenti*, *L. plantarum*, *L. lycopersici, L. pentosus*
Leuconostoc mesenteroides
Microbispora rosea
Microellobosporia flavea
Micromonospora coerula
Mycobacterium spp.
Nocardia asteroides, *N. corallia*, *N. dassonvillei*
Paracolobacterium aerogenoides
Pseudonocardia spp.
Pseudomonas hydrophila
Sarcina spp.
Staphylococcus bibila, *S. flavovirens*, *S. echinatus*
Streptococcus achromogenes, *S. phaeochromogenes*, *S. fracliae*, *S. roseochromogenes*, *S. olivaceus*, *S. californicos*, *S. venuceus*, *S. virginial*
Streptomyces olivochromogenes, *S. venezaelie*, *S. wedmorensis*, *S. griseolus*, *S. glaucescens*, *S. bikiniensis*, *S. rubiginosus*, *S. achinatus*, *S. cinnamonensis*, *S. fradiae*, *S. albus*, *S. griseus*, *S. hivens*, *S. matensis*, *S. nivens*, *S. platensis*
Streptosporangium album, *S. oulgare*
Thermopolyspora spp.
Thermus spp.
Xanthomonas spp.
Zymononas mobilis

The molecular weights of GI vary from 52,000 to 191,000. The subunit structure and amino acid composition of GI reveal that it is a tetramer or a dimer of similar or identical subunits associated with noncovalent bonds and is devoid of interchain disulfide bonds (Bhosale et al.,1996). Figure 2.2 shows tetrameric and dimeric structures of glucose isomerase. Table 2.4 summarizes the microorganisms producing different structures of glucose isomerase.

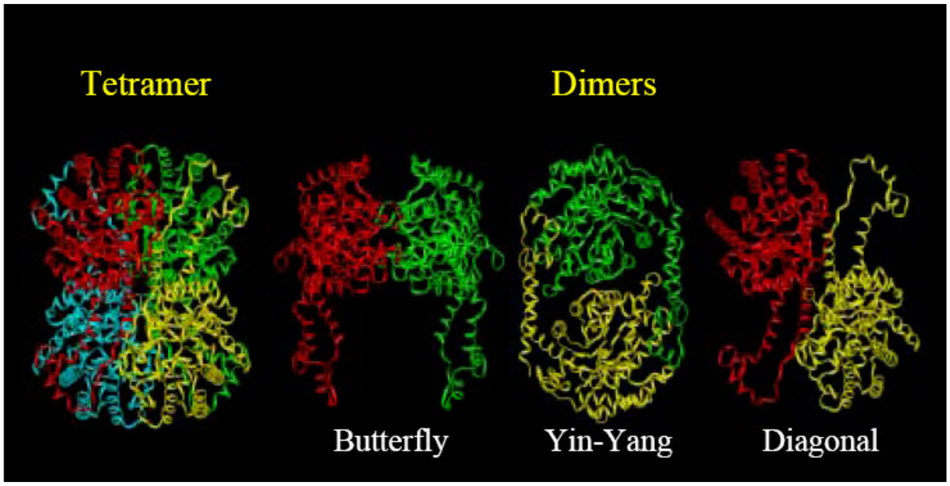

Figure 2.2 Subunit structure of Xylose Isomerase (Vieille,1999)

Tetramer and theoretical dimer configurations
Red = Subunit A Green = Subunit B*
Blue = Subunit A* Yellow = Subunit B

Glucose isomerase from *Bacillus thermoantarcticus* is a homotetramer with a native molecular mass of 200kDa and a subunit molecular mass of 47 kDa, with an isoelectric point 4.8 (Lama et. al., 2001).

Table 2.4 Microorganisms producing glucose isomerase based on its subunit structure (http://www.brenda.uni-koeln.de/php/result_flat.php4?ecno=5.3.1.5)

SUBUNITS	ORGANISM	REFERENCE
Dimer	*Bacillus sp.*	Kwon et al.,1987
Dimer	*Escherichia coli*	Tucker et al.,1988
Dimer	*Hordeum vulgare*	Kristo et al.,1996
Dimer	*Streptomyces olivochromogenes*	Suekane et al.,1978
Dimer	*Thermotoga neapolitana*	Hess et al.,1998
Homotetramer	*Streptomyces diastaticus No. 7*	Zhu et al.,2000
Homotetramer	*Thermus caldophilus*	Chang et al.,1999
Homotetramer	*Thermus thermophilus*	Chang et al.,1999
Monomer	*Geobacillus stearothermophilus*	Suekane et al.,1978
Tetramer	*Arthrobacter sp.*	Smith et al.,1991
Tetramer	*Bacillus coagulans*	Danno et al.,1973
Tetramer	*Lactobacillus xylosus*	Yanmanka et al.,1977
Tetramer	*Streptomyces albus*	Hogue-Angeletti,1975
Tetramer	*Streptomyces chibaensis*	Joo et al.,2001
Tetramer	*Streptomyces flavogriseus*	Chen et al.,1979
Tetramer	*Streptomyces rubiginosus*	Karimaki et al.,2004
Tetramer	*Streptomyces rubiginosus*	Hanson et al.,2004
Tetramer	*Streptomyces sp.*	Inyang et 0al.,1995
Tetramer	*Thermoanaerobacterium sp.*	Liu et al.,1996
Tetramer	*Thermotoga neapolitana*	Hess, et al.,1998
Tetramer	*Thermus aquaticus*	Lembacher et al.,1990
Trimer	*Bacillus coagulans*	Danno et al.,1973
Trimer	*Bacillus sp.*	Kitada et al. 1989
Trimer	*Bacillus sp.*	Chauthaiwale, et al.,1994
Trimer	*Bifidobacterium adolescentis*	Kawai et al.,1994
Trimer	*Thermotoga neapolitana*	Epting et al.,2005

Maximum isomerization was obtained with the substrates having hydroxyl groups at carbons 3 and 4 in the equatorial position, as in glucose and xylose (Bhosale et al., 1996).

GI requires a divalent cation such as Mg^{+2}, Co^{+2}, or Mn^{+2}, or a combination of these cations, for maximum activity. Although both Mg^{+2} and Co^{+2} are essential for activity, they play differential roles. While Mg^{+2} is superior to Co^{+2} as an activator, Co^{+2} is responsible for stabilization of the enzyme by holding the ordered conformation, especially the quaternary structure of the enzyme (Bhosale et al.,1996). The effect of various metal ions on the activity of glucose isomerase activity from *Bacillus thermoantarcticus* were investigated by Lama et al. (2001) and found that use of 10mM of Mg^{+2} and 1 mM of Co^{+2} yielded the highest glucose isomerase activity.

The optimum temperature of GI ranges from 60 to 80°C and increases in the presence of Co+2. The optimum pH range of GI is generally between pH 7.0 and 9.0 (Bhosale et al., 1996). Glucose isomerase from *Bacillus thermoantarcticus* was found to give the highest activity with the reaction temperature and pH of 90°C and 7.0 respectively (Lama et al., 2001).

The conversion ratios of D-glucose to D-fructose catalyzed by GI from various organisms in soluble or immobilized form were in the range of 26 to 59%. Lama et al. (2001) investigated the effect of temperature on equilibrium of isomerization of glucose isomerase as shown in Table 2.5.

Table 2.5 Effect of temperature on equilibrium of isomerization of glucose isomerase from *Bacillus thermoantarcticus* (Lama et al., 2001)

Temperature(°C)	**Conversion(%)**
60	15
70	33
80	48

The Km values of the enzyme for D-glucose were in the range of 0.086 to 0.920 M (Bhosale et al.,1996). Glucose isomerase from *Bacillus thermoantarcticus* has K_m and V_{max} of 167 mM and 6.3 U mg^{-1} respectively. Table 2.6 shows the catalytic properties of glucose isomerases from thermophilic microorganisms.

Table 2.6 Kinetic parameters for glucose isomerases from thermophilic microorganisms (Brown et al., 1993)

Organism	K_m (mM)	V_{max} (μmol/min per mg)
Arthrobacter B3728	210	27.4
Streptomyces olivochromogenes	250	5.3
Bacillus stearothermophilus	220	6.0
Clostridium thermosulfurogenes	140	5.2
Thermoanabacter	120	6.3
Thermus aquaticus	93	9.1
Thermotoga maritima	118	16.2

2.2.1.1 Production of HFCS

Increasing demands for refined sugar, coupled with its high cost of production and awareness of the adverse effects of sucrose and invert sugar consumption on human health, have necessitated the search for acceptable sucrose substitutes. A large number of noncalorific and noncarbohydrate artificial sweeteners such as saccharine, cyclamate, acesulfame-K, aspartame, and thaumatin have been discovered and dismissed on the basis of health concerns or other drawbacks. For example, aspartame, which is used to sweeten soft drinks, has a shorter shelf life because it is slowly hydrolyzed at low pH. HFCS is 1.3 times sweeter than sucrose and 1.7 times sweeter than glucose, which allows manufactures to achieve the same level of sweetness using a smaller amount, thus reducing the amount of calories. The price of HFCS is 10 to 20% lower than that of sucrose on the basis of its sweetening power. HFCS is preferred by the food industry since it does not pose the problem of crystallization, as is the case with sucrose. Moreover, D-fructose plays an important role as a diabetic sweetener because it is only slowly reabsorbed by the stomach and does not influence the glucose level in blood. The major uses of HFCS are in the beverage, baking, canning, and confectionery industries (Bhosale et al., 1996).

While several starch sources such as corn, potatoes, wheat, and tapioca can be used for producing fructose syrup, corn is the most common choice due to its low cost and abundant supply (Venkatasubramanian et al., 1979).

The HFCS process consists of three main steps (Bhosale et al., 1996):

(i) Liquifaction of starch by α-amylase

(ii) Saccharifcation of starch by the combined action of amyloglucosidase anda branching enzyme

(iii) Isomerisation of glucose by GI.

Figure 2.3 shows the large scaled HFCS manufacturing process for a corn wet milling operations. A typical plant may process up to 150,00 bushels of corn per day. For isomerisation, the 95.5% glucose solution is adjusted to pH 7-8 and run through an immobilized column containing glucose isomerase. Temperature and column flow rate are controlled to yield approximately 42% fructose is prepared by large scale chromatographic separation of the monosaccharide (Crabb et al., 1999).

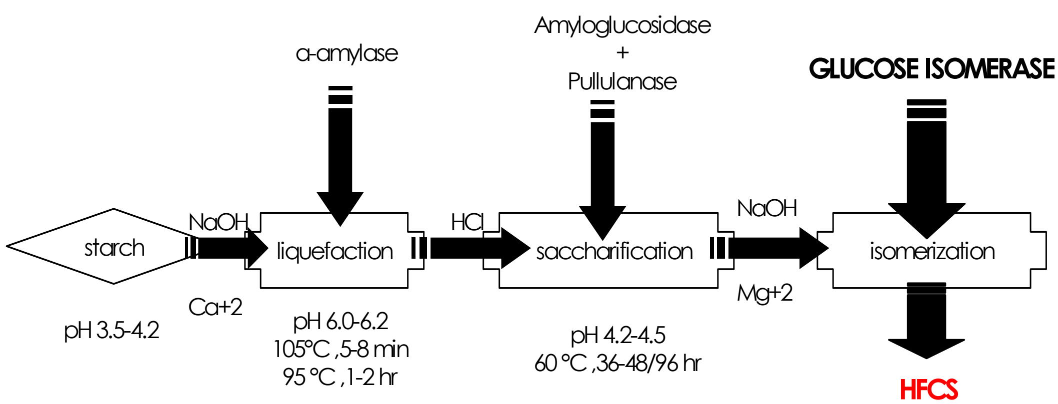

Figure 2.3 HFCS manufacturing process (modified from Crabb et al., 1999)

2.1.1.2 Conversion of Glucose to Fructose

Glucose isomerization to fructose by the enzyme glucose isomerase (GI) is carried out traditionally in a packed bed reactor using immobilized GI. In order to achieve high fructose productivity, the immobilized enzyme packed bed reactor should be operated optimally. Temperature is one of the most important factors affecting the performance of glucose isomerase reactor. While an increase in reaction temperature shifts the equilibrium conversion of glucose to fructose to higher fructose ratios, it can inactivate glucose isomerase. As a result, literature studies were mainly focused on the ways to prevent the thermal inactivation of the enzyme. In literature, glucose isomerase kinetics is generally described (as it is seen below) supposing that the inactivation of the enzyme, both in complexed or free forms, follows first order kinetics:

$$G + E \underset{k-1}{\overset{k+1}{\rightleftharpoons}} X \underset{k-2}{\overset{k+2}{\rightleftharpoons}} F + E$$

$$E \xrightarrow{kd} e$$

$$X \xrightarrow{(1-n)kd} x$$

where E is the active free enzyme, G is the glucose, F is the fructose, X is the intermediate complex between the enzyme and glucose or fructose, e and x are the inactivated forms of the free and the complexed enzyme, respectively. Constants k_{+1}, k_{-1}, k_{+2} and k_{-2} are the rate constants of the elementary reactions, kd and $(1-n)k_d$ the decay constant of the active free enzyme and that of the enzyme complexed with the substrate.

Converti et al. (1998) studied the optimization of an immobilized glucose isomerase column for HFCS production. They investigated the concertic effect of temperature and substrate protection on the kinetic parameters such as r_{max}, Michaelis-Menten constants, equilibrium constants and, also productivity and enzyme half-life. They concluded that substrate protection effect partially counterbalanced the temperature effect on enzyme activity.

Lin (1986) evaluated the substrate protection effect against deactivation of glucose isomerase. Enzyme half-life was calculated at various substrate concentrations and found to be increased slowly at high sugar concentrations.

Faqir et al. (1998) investigated the optimal temperature mode required to achieve constant outlet glucose conversion. They used reversible Michaelis-Menten kinetics for enzyme inactivation and substrate protection and found that an increasing temperature profile is required to obtain a constant glucose conversion. Glucose conversion of 45% was obtained using 2 hours of residence time in all operating periods. The maximum temperature to achieve 45% conversion was 70°C.

2.2.1.3 Determination of Glucose Isomerase Activity

There is a widespread interest in the commercial application of D-glucose-isomerase for the large-scale conversion of D-glucose into fructose-containing syrups. As such, there is a need for a rapid and reliable method for the routine analysis of glucose-isomerising activity in fermentation broths. The analytical methods currently in use include the incubation of the enzyme preparation with a solution of D-glucose followed by colorimetric analysis of the fructose produced (Roy and Buccafuri, 1980).

Although several reactions have been described for differentiation of ketohexoses from aldohexoses in which characteristic differences in the quality of the color appear, ketoses other than ketohexoses have not been tested in these reactions. The only reaction in which reactivity of various classes of ketoses have been investigated systematically is the reaction with L-cysteine and carbazole in sulfuric acid, which can be used for detection of various ketoses as well as for their quantitative determination. In this reaction, however, aldoses also react, although much more slowly and with extinction coefficients very much smaller so that, in general , an accurate determination of ketoses with this reaction is possible (Whistler and Wolfrom, 1962).

As a conclusion in this study, the spectrophotometric assay described by Dische and Borenfreund (1951) namely, cysteine-carbazole-sulfuric acid method was used to determine glucose isomerase activity. This method offers a selective and sensitive analysis of fructose in the presence of glucose.

2.2.2 Xylanase

In this present work, for the production of glucose isomerase, a thermophilic *Bacillus* namely, *Bacillus thermoantarcticus* was used. Besides glucose isomerase, production of xylanase by *Bacillus thermoantarcticus* was reported by Lama et al.(1996). Xylanase is another industrially important thermostable enzyme whose production was also analyzed in the designed medium for glucose isomerase.

Xylanases are hydrolases depolymerising the plant cell wall component-xylan, the second most abundant polysaccharide. The importance of xylanases is not bound to the paper and pulp industry and there are other industries with equal importance of applicability. Potential applications of xylanases also include bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in consistency of beer and the digestibility of animal feed stock. Application of xylanase in the saccharification of xylan in agrowastes and agrofoods intensifies the need of exploiting the potential role of them in biotechnology (Subramaniyan et al., 2002).

Xylanases are used in the hydrolysis and assimilation of xylan. Xylanases, cleaves β-1,4 linkages in the backbone of xylans. Besides xylanase, β-xylosidase is the other xylanolytic enzyme playing an important role in the hydrolysis of xylan. It releases xylosyl residues by endwise attack of xylooligosaccharides. Figure 2.4 shows the xylan degradation mechanism of xylanase, β-xylosidase and other xylanolytic enzymes.

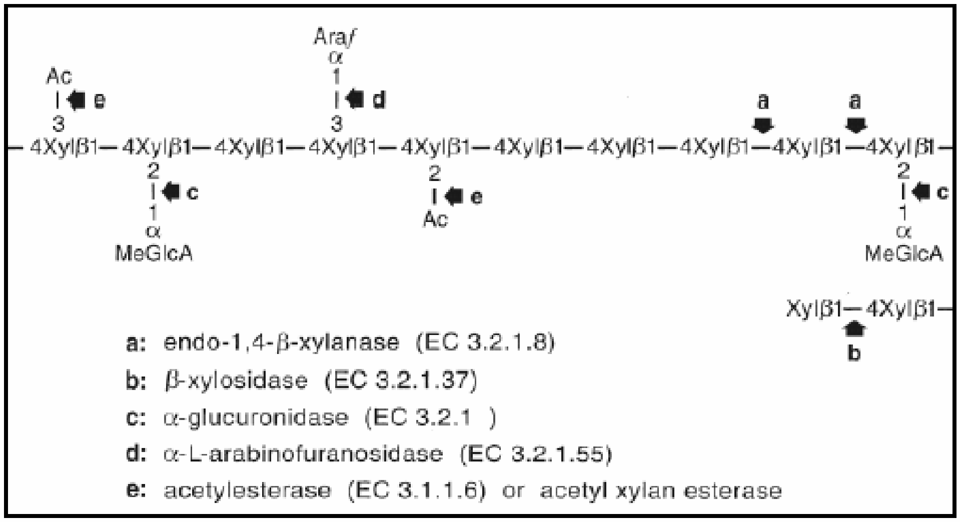

Figure 2.4 Representative plant xylan and attack sites of xylan hydrolyzing enzymes for xylan degradation (Saha, 2000).

Xylanases have been reported in *Bacillus*, *Streptomyces* and other bacterial genera that do not have any role related to plant pathogenicity. Since the introduction of xylanases in paper and pulp and food industries, there have been many reports on xylanases from both bacterial and fungal microflora (Subramaniyan et al., 2002). Table 2.7 shows physicochemical properties of bacterial xylanases.

Certain strains of *Bacillus polymyxa*, *Bacillus pumilus, Bacillus subtilis, Cellulomonas fimi*, *Clostridium acetobutylicum, Streptomyces lividans, Streptomyces flavogriseus, Aspergillus fumigatus, Neurospora crassa, Trichoderma viride, Pichia stipitis,* and *Candida shehatae* secrete xylanases under mesophilic growth conditions (Gosalbes et al., 1991; Wong et al., 1988).

Moreover, xylanases from thermophilic organisms, *Thermomyces lanugiosus* (DSM 5826) (Schlacher *et al*., 1996), *Clostridium thermocellum* (DSM 1237) (Royer et al., 1989), and actinomycetes *Thermomonaspora alba* ULJB1 (Blanco et al., 1997) have been investigated.

Hyperthermophilic eubacteria that grow at temperatures above 80°C have also been isolated. These microbes include *Thermotoga maritima* (Winterhalter and Liebl, 1995), *Caldocellum saccharolyticum* (Lüthi et al., 1990), and *Rhodothermus marinus* (Dahlberg et al., 1993).

Xylan has been shown to be the best inducer of xylanase production (Nakamura et al., 1992) but, few organisms show constitutive production of the enzyme (Debeire et al., 1990). Hemicellulosic substrates like corn cob, wheat bran, rice bran, rice straw, corn stalk and bagasse have also been found to be most suitable for the production of xylanase in certain microbes.

Xylanase production from *Bacillus thermoantarcticus* was reported by Lama et al. (2004). They investigated the characterization of thermostable xylanase and β-xylosidase. The production of enzymes were investigated in the medium containing 1 kg m^{-3} yeast extract, 3 kg m^{-3} NaCl and 5 kg m^{-3} of different carbon sources: xylose, xylan, starch, cellobiose, glucose, maltose, yeast, sucrose, fructose. Medium containing xylan showed the highest activity of xylanase as 1.32 U mg^{-1}.

The optimum temperature and pH of xylanase from *Bacillus thermoantarcticus* was obtained as 80°C and 5.6 respectively. Km of xylanase for xylan was calculated as 1.6 mg ml^{-1}. The isoelectric point and molecular mass were obtained as 4.5 and 45 kDa respectively (Lama et al., 2004).

Table 2.7 Physicochemical properties of bacterial xylanases

			Optimum pH and Temperature		Catalytic properties	
MICROORGANISMS	**Reference**	**Mol. Wt. (KDa)**	**pH**	**T(°C)**	**Km (mg/ml)**	**Vmax (µmol/min/mg)**
Aeromonas caviae ME1	Viet et al., 1991	20	7	50	9.4	4330
Bacillus amyloliquefaciens	Breccia et al., 1998	18.5-19.6	6.8-7.0	80	-	-
Bacillus circulans WL-12	Esteban et al., 1982	85	5.5-7.0	-	8	-
Bacillus Stearothermophilus T-6	Khasin et al., 1993	43	6.5	75	1.63	288
Streptomyces T-7	Keskar et al., 1997	20.643	4.5-5.5	60	10	7600
Thermotoga maritima	(Winterhalter and Liebl, 1995	-	6.5	85	-	-

2.3 Bioprocess Parameters in Enzyme Production

Any operation involving the transformation of some raw material (biological or non-biological) into some product by means of microorganisms, animal or plant cell cultures, or by materials derived from them (e.g. enzymes, organelles), may be termed as a "bioprocess" (Moses and Cape, 1991).

During the design of a bioprocess there are several parameters that should be taken into account. These parameters are the microorganism, medium composition, temperature, pH, oxygen transfer rate depending on air inlet rate (Q_0/V_R) and agitation rate (N). In order to obtain the maximum yield of the product, investigation of these parameters is an important step in bioprocess engineering.

2.3.1 Microorganism

The microorganism that is to be used should give adequate yields, be able to secrete large amounts of protein and should not produce toxins or any other undesired products. Potential hosts should be suitable for industrial fermentations and produce large cell mass per volume quickly on cheap media (Kirk and Othmer, 1994).

2.3.1.1 The Genus *Bacillus*

Bacillus species continue to be dominant bacterial workhorses in microbial fermentations. *Bacillus subtilis* (natto) is the key microbial participant in the ongoing production of the soya-based traditional natto fermentation, and some *Bacillus* species are on the Food and Drug Administration's GRAS (generally regarded as safe) list. The capacity of selected *Bacillus* strains to produce and secrete large quantities (20–25 g/L) of extracellular enzymes has placed them among the most important industrial enzyme producers. The ability of different species to ferment in the acid, neutral, and alkaline pH ranges, combined with the presence of thermophiles in the genus, has lead to the development of a variety of new commercial enzyme products with the desired temperature, pH activity, and stability properties to address a variety of specific applications (Schallmey et al., 2004).

The genus *Bacillus*, exhibits a wide diversity of physiological abilities; although the majority are mesophilic, there are also psychrophilic and thermophilic species (Table 2.8).

Table 2.8 Allocation of some *Bacillus* species to groups on the basis of phenotypic similarities (Sonenshein, 1993).

Species	Characteristics of group
Group I *B. alvei* *B. amylolyticus* *B. apiarius* *B. azotofixans* *B. circulans* *B. glucanolyticus* *B. larvae* *B. lautus* *B. lentimorbus* *B. macerans* *b. macquariensis* *B. pabuli* *B. polymyxa* *B. popilliae* *B. psychrosaccharolyticus* *B. pulvifaciens* *B. thiaminolyticus* *B. validus*	All species are facultative anaerobes and grow strongly in absence of oxygen. Acid is produced from variety of sugars. Endospores are ellipsoidal and swell the mother cell. They have fairly complex nutritional requirements, and none are prototrops.
Group II *B. alcolophilus* *B. amyloliquefaciens* *B. anthracis* *B. atrophaeus* *B. carotorum* *B. firmus* *B. flexus* *B. laterosporus* *B. lentus* *B. licheniformis* *B. niacini* *B. pantothenicus* *B. pumilus* *B. simplex* *B. suptilis* *B. thuringiensis*	All species produce acid from variety of sugars including glucose. Most are able to grow at least weakly in absence of oxygen, particularly if nitrate is present. Spores are ellipsoidal and do not swell the mother cell. These bacteria are therefore an intermediate stage between true facultative anaerobes and strict aerobes.
Group III *(B. alginolyticus)* *B. aneurinolyticus* *B. azotoformans* *B. badius* *B. brevis* *(B. chondroitinus)* *B. frudenreichii* *B. gordonae*	These strict aerobes do not produce acid from sugars; names in bracket are exceptions. They produce ellipsoidal spores that swell the mother cell. Most species ("*B. aneurinolyticus*", *B. badius*, *B. brevis* ve "*B. freudenreichii*") have an oxidative metabolism and produce an alkaline reactions in peptone media.

Table 2.8 –Continued

Species	Characteristics of group
Group IV *B. aminovorans* *B. fusiformis* *B. globisporus* *B. insolitus* *B. marinus* *B. pasteurii* *B. psychrophilus* *B. sphaericus*	All species produce spherical spores that may swell the mother cell and contain L-lysine or ornithine in cell wall. These bacteria are strickly oxidative and in most cases will not use sugars as a source of carbon or energy, preferring acetate or amino acids such as glutamate as carbon sources.
Group V ***B. coagulans*** ***B. flovothermus*** ***B. kaustophilus*** ***B. pallidus*** ***B. schlegelii*** ***B. smithii*** ***b. stearothermophilus*** ***B. thermocloacae*** ***B. thermodenitrificans*** ***B. thermoantarcticus*** ***B. thermoglucusidasius*** ***B. thermoleovrans*** ***B. thermoruber***	These thermophilic species all grow optimally at >50^{o}C. Physiologically and morphologically, they are heterogeneous, but most produce ovel spores that swell the mother cell.
Group VI *A. acidocaldarius* *A. acidoterrestiris* *A. sikloheptanius*	Thermophilic, acidophilic species with membraneous ω-alicyclic fatty acids.
Unassigned species *B. benzoervorans* *B. fastidiosus* *B. nagonoensis*	It is not clear how can these species be allocated with conviction to one of these groups.

2.3.1.2 Glucose Isomerase from *Bacillus*

Novo Industries has developed glucose isomerase from *B. coagulans* for commercial use. This immobilized enzyme process is used today in many plants in the United States, Europe, Japan, and Korea. Since this glucose isomerase is primarily a xylose isomerase, xylose must be added for induction of the enzyme with all wide strains. Xylose is quite expensive; however, and may be replaced by xylan or wheat bran, which also contain xylose. Constitutive mutants have also been isolated.

Catabolic repression regulates the production of glucose isomerase. Glucose acts as a repressor in both batch process and continuous fermentation. In batch process, *B. coagulans* does not form the enzyme during log phase. As soon as the glucose content in the nutrient approaches zero, growth ceases. In a typically diauxy, additional carbon sources present in the medium are then metabolized, and enzyme production begins. Maximal enzyme activity obtained after 24 hours incubation (Figure 2.5) (http://rcw.raiuniversity.edu/course-bio-msc-lec-phrbio&dign.html).

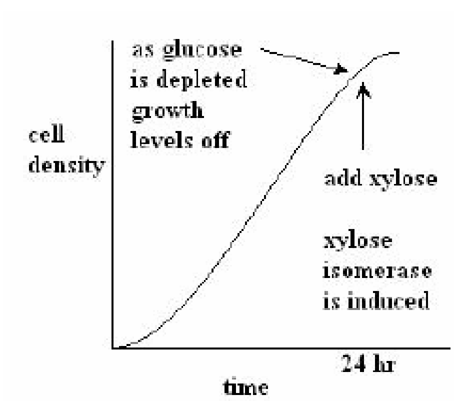

Figure 2.5 Diauxic growth curve

2.3.1.3 Thermophilic Microorganisms

Most organisms living at moderate optimum growth temperature of 24° to 40°C are mesophiles. Organisms living at the higher optimum growth temperature of 50° to 70°C are thermophiles and at an optimum growth temperature of greater than 80ºC are hyperthermophiles, including members of domains Archaea and Eubacteria (Trivedi et al, 2006).

The thermophilic bacteria are a ubiquitous group of microorganisms found in a wide variety of environmental conditions ranging from thermal pools and desert soils to arctic glaciers and freshly fallen snow (Singleton et al., 1976).

In order to survive at high temperatures, thermophilic prokaryotes (Archaea and Eubacteria) adopt different strategies. Among several important contributing factors for stability of proteins are GC-rich codons, the ratio of charged amino acids compared to uncharged amino acids, ionic interactions, amino acid preferences and their distribution, post-translational modifications, and solute accumulation (Trivedi et al., 2006).

Thermophiles are under constant destabilizing effects of high temperatures. To counteract ill effects of temperature on proteins, thermophiles adopt strategies that are characteristically different from mesophiles (Trivedi et al.,2006). Some of the strategies are replacement of Arg with Tyr, presence of sulfate ions (Kallwass et al., 1992), the type of metal ions as co-factors, thermamines, chaperones, substrate, flexibility and or rigidity of proteins (Vieille et al., 2001), protein mobility (Panasik et al., 2000) and pressure due to deep sea habitat (Konisky et al., 1995).

2.3.1.4 *Bacillus thermoantarcticus*

The novel thermophilic Gram-positive bacillus, *Bacillus thermoantarcticus*, is isolated from geothermal soil near the crater of Mount Melbourne. Besides glucose isomerase, it produces an exopolysaccharide, an exocellular xylanase, an intracellular alcohol dehydrogenase and exo-and endocellular α-glucosidase(s). The organism is oxidase-positive, catalase-negative and produces an exopolysaccharide, an exocellular xylanase, an intracellular alcohol dehydrogenase and exo- and endocellular a-glucosidase(s). The DNA base ratio is 53.7 mol% G+C. It grews aerobically between 37°C and 65°C with an optimum at 63°C, and in the pH range of 5.5-9.0 with an optimum at pH 6.0 (Nicolaus et.al., 1996).

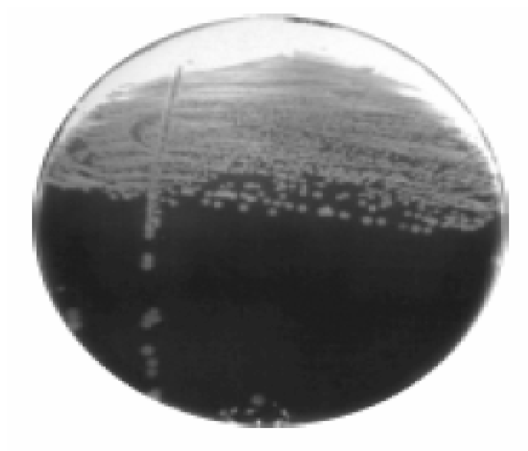

Figure 2.6 The cells form endospores and are Gram-positive regular rods, 0.5-2.0 μm in diameter and 3.0- to 5.0-μm long. On agar plates colonies appeared opaque, flat and circular with an entire margin (Nicolaus et al.,1996).

Bacillus thermoantarcticus belongs to the group V of the bacillus species on the basis of phenotypic similarities. Nazina et al. (2001) undertook a thorough, polyphasic examination of their group V isolates. Their results support the notion of a phylogenetically distinct, physiologically and morphologically consistent taxon, for which they have submitted the validly described genus name of *Geobacillus* (Table 2.9).

Table 2.9 Description of the *Geobacillus* genus, adapted from Nazina et al. (2001)

Morphology	
Vegetative cell	Rod-shaped cells, occurring either singly or in short chains and motile by means of peritrichous flagella. The cell wall structure is Gram-positive, but the Gram-stain reaction may vary between positive and negative.
Spore	One ellipsoidal or cylindrical endospore per cell, located terminally or subterminally in slightly swollen or non-swollen sporangia
Colony	Variable shape and size; pigments may be produced on certain media.
Metabolism	
Energy	Chemo-organotrophic
Oxygen	Aerobic or facultatively anaerobic. O2 is the electron acceptor, replaceable in some species by nitrate.
Temperature	Obligately thermophilic. The growth-temperature range is 37-75°C, with an optimum at 55-65°C.
pH	Growth occurs in a pH range of 6.0 to 8.5, with an optimum at pH 6.2-7.5.
Requirements	Growth factors, vitamins, NaCl and KCl are not required by most species.
Identification tests	
Carbonhydrates	Acid but no gas is produced from glucose, fructose, maltose, mannose and sucrose. Most species do not produce acid from lactose.
Enzymes	Most species form catalase. Phenylalanine is not deaminated, tyrosine is not degraded, indole is not produced, the Voges-Proskauer reaction is negative. Oxidase-positive or negative.
Biochemicals	The major cellular fatty acids are iso-15:0, iso-16:0 and iso-17:0, which make up more than 60% of the total. The main menaquinone type is MK-7.
DNA characterization	
G-C content	48.2-58 mol% (thermal denaturation method)
16S rDNA	Sequence identities higher than 96.5% among the members of this genus
Ecology	
Prelevance	Most species are widely distributed in nature.

2.3.2 Cell Growth, Kinetics and Yield Factors

For microbes, growth is their most essential response to their physicochemical environment. Growth is a result of both replication and change in cell size (Shuler and Kargi, 2002). Cellular growth is an autocatalytic process, as one cell is responsible for the synthesis of more cells, and at conditions with excess nutrients growth is therefore exponential with a certain doubling time for the number of cells. Besides the formation of new biomass (or new cells), the breakdown (or catabolism) of substrates leads to the formation of several by-products, with carbon dioxide being a typical by product formed, especially in respiring cells (Nielsen). The rate of microbial growth is characterized by the net specific growth rate, μ related to the rate of cell growth, which is,

$$r_X = \frac{dC_X}{dt} = \mu C_X \qquad (2.1)$$

Many biochemical processes involve batch growth of microorganisms. After seeding a liquid medium of appropriate composition with an inoculum of living cells, nothing (except possibly some gas) is added to the culture or removed from it as growth proceeds. Typically the number of living cells in the culture varies with time, as shown in Figure 2.7. After a lag phase, where no increase in cell numbers is evident, a period of rapid growth ensues, where the cell numbers increase exponentially with time. This stage of batch culture is called the logarithmic phase. Naturally in a closed vessel the cells cannot multiply indefinitely, and a stationary phase follows the period of exponential growth. At this point the population achieves its maximum size. Eventually a decline in cell numbers occurs during the death phase. Here an exponential decrease in the number of living individuals is often observed (Baily and Ollis, 1986).

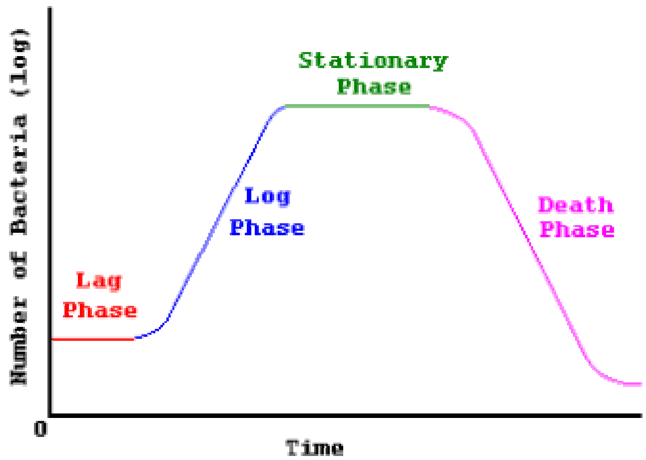

Figure 2.7 Typical growth curve for a bacterial population

Besides, the substrate consumption rate, r_S, and product formation rate, r_P, are described as follows:

$$r_P = \frac{dC_P}{dt} \tag{2.2}$$

$$r_S = \frac{dC_S}{dt} \tag{2.3}$$

To better describe growth kinetics, some stoichiometrically related parameters are defined. Yield coefficients are defined based on the amount of consumption of another material (Shuler and Kargi, 2002). A list of frequently used yield coefficients is given in Table 2.10. When yield for fermentation are reported, the time or time period to which they refer should be stated (Doran, 1995).

Table 2.10 Definition of yield coefficients

Symbol	Definition	Unit
$Y_{X/S}$	Mass of cells produced per unit mass of substrate consumed	kg cell kg^{-1} substrate
$Y_{X/O}$	Mass of cells produced per unit mass of oxygen consumed	kg cell kg^{-1} oxygen
$Y_{S/O}$	Mass of substrate produced per unit mass of oxygen consumed	kg substrate kg^{-1} oxygen
$Y_{P/X}$	Mass of product formed per unit mass of substrate consumed	kg product kg^{-1} cell
$Y_{P/S}$	Mass of product formed per unit mass of substrate consumed	kg product kg^{-1} substrate
$Y_{P/O}$	Mass of product formed per unit mass of oxygen consumed	kg product kg^{-1} oxygen

It is important to realize that the yield coefficients are not constant throughout the growth phase since they chance with growth rate due to the maintenance energy (m) requirement (Scragg, 1988). Cellular maintenance represents the energy expenditures to repair damaged cellular components, to transfer some nutrients and products in and out of the cell, for motility, and to adjust the osmolarity of the cells' interior volume (Shuler and Kargi, 2002). Microbial growth, product formation and substrate utilization rates are usually express in the form of specific rates:

$$q_P = \frac{1}{C_X}\frac{dC_P}{dt} \qquad (2.4)$$

$$q_S = \frac{1}{C_X}\frac{dC_S}{dt} \qquad (2.5)$$

The oxygen consumption for oxygen denoted by m_0 is the oxygen consumption for maintenance. In an aerobic process oxygen is consumed for cell growth, product formation and by-product formations, and maintenance. The oxygen consumption rate for cell growth can be defined as (Calik et al., 2006):

$$-r_{O1} = \frac{dCx/dt}{\bar{Y}_{X/O}} \qquad (2.6)$$

Oxygen consumption for by-product formation is defined as:

$$-r_{O2} = \frac{dC_{BP}/dt}{\bar{Y}_{BP/O}} \qquad (2.7)$$

Oxygen consumption for maintenance is defined as:

$$-r_{O3} = m_0 Cx \qquad (2.8)$$

The total oxygen consumption rate is as follows:

$$-r_O = (-r_{O1}) + (-r_{O2}) + (-r_{O3}) \qquad (2.9)$$

Equations (2.6)-(2.8) are substituted into (2.9) to obtain,

$$-\frac{dCo}{dt} = -r_O = \left(\frac{1}{\bar{Y}_{X/O}}\right)\frac{dCx}{dt} + \left(\frac{1}{\bar{Y}_{BP/O}}\right)\frac{dC_{BP}}{dt} + m_0 Cx \qquad (2.10)$$

Also,

$$q_{BP} = \frac{1}{Cx}\frac{dC_{BP}}{dt} \qquad (2.11)$$

and

$$\mu = \frac{1}{Cx}\frac{dCx}{dt} \tag{2.12}$$

Substituting (2.11) and (2.12) into (2.10),

$$-r_O = \left(\frac{1}{\bar{Y}_{X/O}}\right)(\mu Cx) + \left(\frac{1}{\bar{Y}_{BP/O}}\right)(q_{BP}Cx) + m_0 Cx \tag{2.13}$$

Dividing (2.13) by Cx

$$\frac{-r_O}{Cx} = \frac{\mu}{\bar{Y}_{X/O}} + \frac{q_{BP}}{\bar{Y}_{BP/O}} + m_0 \tag{2.14}$$

q_{BP} can be defined as follows, where α is the term for growth associated organic acid formation and β is the term for non-growth associated organic acid formation:

$$\frac{dC_{BP}}{dt} = \alpha\frac{dCx}{dt} + \beta Cx \tag{2.15}$$

Dividing (2.15) to Cx,

$$q_{BP} = \frac{1}{Cx}\frac{dC_{BP}}{dt} = \alpha\mu + \beta \tag{2.16}$$

Substituting (2.16) into (2.14),

$$\frac{-r_O}{Cx} = \frac{\mu}{\bar{Y}_{X/O}} + \frac{\alpha\mu + \beta}{\bar{Y}_{BP/O}} + m_0 \tag{2.17}$$

Dividing (2.17) by μ,

$$\frac{-r_O}{\mu C_X} = \frac{1}{\bar{Y}_{X/O}} + \frac{\alpha + (\beta/\mu)}{\bar{Y}_{BP/O}} + \frac{m_0}{\mu} \qquad (2.18)$$

Rearranging (2.18),

$$\frac{-r_O}{\mu C_X} = \left(\frac{\alpha}{\bar{Y}_{BP/O}} + \frac{1}{\bar{Y}_{X/O}}\right) + \left(\frac{1}{\mu}\right)\left(m_0 + \frac{\beta}{\bar{Y}_{BP/O}}\right) \qquad (2.19)$$

and,

$$\frac{1}{Y_{X/O}} = \left(\frac{\alpha}{\bar{Y}_{BP/O}} + \frac{1}{\bar{Y}_{X/O}}\right) + \left(\frac{1}{\mu}\right)\left(m_0 + \frac{\beta}{\bar{Y}_{BP/O}}\right) \qquad (2.20)$$

From the slope of the plot of $\frac{1}{Y_{X/O}}$ versus $\frac{1}{\mu}$, $\left(m_0 + \frac{\beta}{\bar{Y}_{BP/O}}\right)$(g oxygen g^{-1} dry cell)

weight h^{-1}) and from the intercept, $\left(\frac{\alpha}{\bar{Y}_{BP/O}} + \frac{1}{\bar{Y}_{X/O}}\right)$ could be determined, where Y represents the apparent and $\bar{Y}_{X/O}$ represents the true yield (Calik et al., 2006).

When by-product formation is neglected, equation (2.10) becomes:

$$-\frac{dC_O}{dt} = -r_O = \left(\frac{1}{\bar{Y}_{X/O}}\right)\frac{dC_X}{dt} + m_0 C_X \qquad (2.21)$$

If the above equation is reorganized:

$$\frac{-r_O}{dC_X/dt} = \frac{1}{Y_{X/O}} = \frac{1}{\bar{Y}_{X/O}} + \frac{m_0}{\mu} \qquad (2.22)$$

is obtained (Calik et al., 2004). From the slope of $\frac{1}{Y_{X/O}}$ versus $\frac{1}{\mu}$, oxygen consumption for maintenance is obtained.

2.3.3 Medium Design

An important aspect in design of a fermentation process is the choice of fermentation medium, which represents the raw material for the process. The fermentation medium should fulfill the following criteria:

- It should contain a carbon, nitrogen and energy source
- It should contain all essential minerals required for growth
- It should contain all necessary growth factors to ensure rapid growth and high yield of the desired product
- It should be of a consistent quality and be readily available throughout the year
- It causes a minimum of problems in the downstream processing
- It causes a minimum of problems in other aspects related to the fermentation process, i.e., it has no negative effect on the gas-liquid mass transfer (Nielsen, Villadsen, Liden, 2003).

Nutrient media can be simple mixtures of a few pure chemical compounds, or contain a large number of preformed monomers as growth factors. As long as well-characterized chemicals are used, the medium is defined. However, it is simpler to add amino acids, vitamins and other natural compounds as crude digests of animal or plant substances. Thus, this type of media is called complex, because its precise chemical composition is not known. Often, complex media is less expensive than defined media. However, the primary advantage of defined media is that the results are more reproducible and the operator has better control of the fermentation. Further, recovery and purification of a product is often easier and cheaper in defined media (Shuler and Kargi, 2002).

The advantages of applying complex media are that they often contain an organic nitrogen source, essential minerals and different growth factors. Thus, two frequently applied complex media, corn steep liquor and pharmamedia; both contain a very large variety of amino acids, many different minerals and many different vitamins. The disadvantages of complex media are that:

1) There may be a seasonal variation in the composition,

2) The composition changes with storage

3) There may be compounds present that are undesirable (Nielsen, Villadsen and Liden, 2003).

Optimization of the fermentation medium of glucose isomerase has been extensively studied with a view to development of an economically viable fermentation technology for the production of GI. Research efforts were directed mainly toward:

(i) Replacement of xylose by another, inexpensive inducer;
(ii) Evaluation of the effect of cheaper nitrogen sources on the yield of enzyme;
(iii) Optimization of pH and temperature for maximum enzyme production; and
(iv) Substitution of Co^{+2} ions by other divalent metal ions in the fermentation medium. There is no concrete composition of medium for the best production of the enzyme from different microorganisms. Each organism or strain requires its own special conditions for maximum enzyme production (Takasaki et al., 1966).

In literature glucose isomerase production was performed by the use of several mediums depending on type of microorganism, activity and the production capacity of the enzyme. Table 2.11 summarizes the mediums used for GI production by different microorganisms.

Table 2.11 Different growth mediums of glucose isomerase producing microorganisms

MICROORGANISM	MEDIUM	REFERENCE
Bacillus thermoantarcticus	5 kg m^{-3} carbon source: xylose, xylan, arabinose, galactose, fructose, maltose, glucose 1 kg m^{-3} yeast extract 3 kg m^{-3} NaCl	Lama et. al., 2001
Thermus aquaticus HB8	5 kg m^{-3} xylose 9.5 mM $(NH_4)Cl$	Lehmaher at al., 1989
Steptomyces fusant D3	5 kg m^{-3} xylose/xylan 3 kg m^{-3} yeast extract 3 kg m^{-3} $(NH_4)_2HPO_4$ 0.3 kg m^{-3} KCl 1 kg m^{-3} $MgSO_4$ 0.02 kg m^{-3} $FeSO_4$	Teeradakorn at al.,1997
Bacillus KX-6 *Bacillus TX-3*	5 kg m^{-3} xylose 5 kg m^{-3} yeast extract 5 kg m^{-3} polypeptone 1 kg m^{-3} K_2HPO_4 0.1 kg m^{-3} $MnCl_2$ 0.05 kg m^{-3} $CoSO_4.7H_2O$ 10 kg m^{-3} Na_2CO_3	Kwon et al., 1987 ; Kwon et al., 1989
Thermus thermophillus	0.5 kg m^{-3} xylose 4 kg m^{-3} yeast extract 8 kg m^{-3} polypeptone 2 kg m^{-3} NaCl	Ensari et al., 1995
Bacillus sp. *(NCIM 59)*	10 kg m^{-3} carbon source: xylose, xylan, glucose, sorbitol, wheat bran 5 kg m^{-3} yeast extract 5 kg m^{-3} peptone 1 kg m^{-3} K_2HPO_4 10 kg m^{-3} Na_2CO_3 0.1 kg m^{-3} $MnCl_2.4H_2O$ 0.05 kg m^{-3} $CoSO_4.7H_2O$	Chauthaiwale at al.,1994
Streptomyces flavogriseus	10 kg m^{-3} straw hemicellulose 24 kg m^{-3} corn steep liquor 1 kg m^{-3} $MgSO_4.7H_2O$	Chen et al., 1979
Thermotoga martima *(DSMZ 3109)*	ASW media supplemented with: 5 kg m^{-3} xylose 1 kg m^{-3} yeast extract 5 kg m^{-3} tryptone	Brown et al., 1993

Most of the GI-producing organisms have an obligate requirement for D-xylose to induce production of the enzyme. However, xylose is expensive and hence impractical for use on a commercial scale. Starch, glucose, sorbitol, or glycerol could be used in place of 75% of xylose (Drazic et al.,1980). Takasaki and Tanabe showed that *Streptomyces* strain YT-5 was able to grow on xylan or xylan-containing material such as corn cobs or wheat bran (Takasaki et al., 1966). This was the first landmark in selecting strains which could grow in a cheaper medium. Today, several strains are capable of producing GI-utilizing glucose instead of xylose. These include *Actinoplanes* strains, mutant strains of *Bacillus coagulans*, and *Streptomyces olivochromogenes*. Another approach to eliminate the requirement of xylose as an inducer is to generate mutants which can produce GI constitutively. One of the wild-type strains of *Actinoplanes missouriensis* is able to produce GI constitutively and is used for commercial production of the enzyme by Gist Brocades (Anheuser-Busch Inc., 1974.).Another approach to achieving constitutive enzyme production involves cloning the *xylA* gene in front of a strong *Streptomyces* promoter. The P1-*xylA* gene has been integrated into the chromosome by using the integrative vector pTS55. The resultant strain (CBS1) gave about sevenfold greater activity in the absence of xylose compared with the wild-type strain fully induced by xylose (Bejar et al., 1994).

Xylose is the main inducer that is used in the production of glucose isomerase. In most of the GI fermentations microorganisms, such as *Thermus aquaticus, Streptomyces fusant D3, Bacillus KX-6,Bacillus TX-3, Thermus thermophilus* …etc, GI is produced on the media containing 5 kgm-3 of xylose. Induction of glucose isomerase formation by xylose was studied in 1977 by Vaheri and Kauppinen and 5 kgm^{-3} concentration of xylose is found to be the optimum.

Even though several carbon sources can be used to optimize the production of glucose isomerase, the most important point in the optimization of a fermentation process is the economical point of view. As xylan is a cheaper media when compared to xylose, glucose isomerase production can be optimized by using xylan in the fermentation medium. Also glucose isomerase production from xylan depends greatly on the enzymatic degradation of xylan. As *Bacillus thermoantarcticus* synthesize xylanolytic enzymes xylanase and β-xylosidase, it is obvious to use xylan in the glucose isomerase production by *Bacillus thermoantarcticus.*

Teeradakorn et al. (1998) studied glucose isomerase production in medium containing either xylose or xylan in shake-flask cultures. Glucose isomerase activity was observed to reach the highest activity with the initial xylose concentration in the range of 5-6 kg m^{-3}. The glucose isomerase activity decreased when the initial xylose concentration was 10 kg m^{-3}. When xylose replaced with xylan, the highest glucose isomerase activity was obtained as 1100 UL^{-1} with initial xylan concentration of 8 kg m^{-3}.

Sapunova et al. (2004) studied screening of microorganisms producing glucose isomerase, namely *Arthrobacter sp, A. ureafaciens, A. citreus.* They investigated the carbon sources with a concentration of 10 kg m^{-3} on glucose isomerase activity. Medium containing xylan yielded higher GI activity than the GI activity obtained by the medium containing xylose.

The nitrogen source is a critical factor, which needs to be optimized for each source of glucose isomerase. Although complex nitrogen sources are usually used for GI production, the requirement for a specific nitrogen supplement differs from organism to organism. Peptone, yeast extract, or ammonium salts can be used with *Bacillus coagulans*, but urea and nitrate are not suitable (Yoshimura er al., 1966). Corn steep liquor was found to be a cheap and suitable source of nitrogen by some workers, but its use is limited by its seasonal and interbatch variability. Suitable nitrogen sources as substitutes for corn steep liquor are still being evaluated. Soy flour gives a 50% higher yield than corn steep liquor (Shieh et al., 1977). A constitutive mutant of *Streptomyces coelicolor* utilizes corn steep liquor better than yeast extract or yeast autolysate (Hafner et al., 1985). Moreover, Fogarty and Kelly (1990) reported the effect of organic nitrogen sources, corn steep liquor, casein hydrolysate, soy peptone, yeast extract and malt extract on glucose isomerase activity. While corn steep liquor was found as the best organic nitrogen source, casein hydrolysate, soy peptone, yeast extract and malt extract showed 73, 66, 58, 24% of the glucose isomerase activity obtained with the corn steep liquor containing medium respectively.

Peptone and beef extract are necessary organic nitrogen sources in the production of glucose isomerase. For example, the media optimization for glucose isomerase production by *Arthobacter sp.* is studied and the optimal medium was containing 10 kgm^{-3} of peptone besides 5 kg m^{-3} of yeast extract (Prabhakar et al., 2004). Also in the study done by Park et al., glucose isomerase production was performed in the media containing 2.5 kg m^{-3} of beef extract besides yeast extract and peptone (Park et al., 1976). However, in the present work, it is observed that GI activity did not differ so much when they were omitted from the medium. So experiments further carried out by the medium without peptone and beef extract.

Moreover, literature studies done with *Bacillus thermoantarcticus* supports the conclusion that yeast extract should be present in the production medium of *Bacillus thermoantarcticus.* As shown in Table 2.12 yeast extract was used in most of the fermentation mediums defined for the growth of *Bacillus thermoantarcticus*. Main function of yeast extract in the production of glucose isomerase is to support the growth of *Bacillus thermoantarcticus*. As glucose isomerase from *Bacillus thermoantarcticus* is an intracellular enzyme, its activity highly depends on the growth of the microorganism and hence, presence of yeast extract.

Table 2.12 Fermentation media used by *Bacillus thermoantarcticus*

MEDIUM	**PRODUCT/STUDY**	**REFERENCE**
1 kg m^{-3} yeast extract 3 kg m^{-3} NaCl 5 kg m^{-3} carbon source: xylose,xylan,arabinose,galactose, fructose,maltose,glucose	Glucose isomerase	Lama et al., 2001
1 kg m^{-3} yeast extract 3 kg m^{-3} NaCl 5 kg m^{-3} xylan	Xylanase	Lama et al., 2004
8 kg m^{-3} yeast extract 3 kg m^{-3} NaCl	Biochemical & physiological characteristics were determined	Nicolaus et al., 1996
8 kg m^{-3} starch 5 kg m^{-3} peptone 3 kg m^{-3} meat extract 3 kg m^{-3} KH_2PO_4		
6 kg m^{-3} yeast extract 3 kg m^{-3} glucose 3 kg m^{-3} NaCl		
6 kg m^{-3} yeast extract 3 kg m^{-3} NaCl 0.3 kg m^{-3} glutamic acid		
0.1 kg m^{-3} yeast extract 3 kg m^{-3} NaCl 6 kg m^{-3} glucose, mannose or sucrose	EPS (Exopolysaccharide) production	Manca et al., 1996

In this study, the medium offered by DSMZ used as the starting point of the medium design experiments. DSMZ offers the solid medium containing: 15 kgm^{-3} of agar, 10 kg m^{-3} of glycerol, 6 kg m^{-3} of yeast extract, 3 kg m^{-3} of NaCl, 2.5 kgm^{-3} of peptone and 1.5 kg m^{-3} of beef extract. Agar and soil extract were omitted from the medium and the remaining components were analyzed step by step. First step of the medium design was the evaluation of carbon sources. Xylan was found to be the most effective carbon source for glucose isomerase production. In order to support this result, structure and special digestion of xylan should be understand and related to the enzymatic degradation mechanism present in *Bacillus thermoantarcticus.*

2.3.3.1 Xylan, its structure and hydrolysis

In this study, besides xylose, glycerol, glucose, fructose and sucrose, effects of xylan on glucose isomerase production by *Bacillus thermoantarcticus* were investigated. The organism possesses extracellular xylanase and β-xylosidase that are able to degrade xylan (Lama et al., 2001).

Xylan, the major hemicellulose in most plants, represents one-third of the renewable biomass on the earth. The xylan percentages of the plant dry weight vary depending on plant source. That of hardwoods, softwoods, and annual plants is in the range of 15 to 30, 7 to 10 and up to 30, respectively (Bakir, 2005). Xylan a cheaper medium whose presence in the growth medium enhanced the expression of xylose isomerase about eightfold with respect to standart growth medium (Lama et al., 2001).

Plant cell walls are composed of several layers of polysaccharides and protein complexes (Figure 2.8). The outer layer, the primary wall, comprises 20% of cellulose and 50% of hemicellulose. Cellulose fibers are randomly oriented in loose array in the primary cell walls, which also contains hemicellulose, pectin, and proteins in its structure. The protein structure crosslinks the amorphous polysaccharides and forms a closely knit network. Moreover, the area between adjacent cell walls is composed predominantly of pectic materials.

The secondary cell wall, formed inside the primary wall, consists of mainly cellulose and hemicellulose in the range of 50-90% and 25%, respectively. The secondary wall also has distinct layers in which cellulose fibers are woven together in various ways (Yasinok, 2006).

β -1,4-Xylan, mainly found in the secondary walls of plants, does not have a homogenous chemical composition, except those present in esparto grass and tobacco. Xylan polymer largely consists of a β-D-1,4- linked 6 D-xylose backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains (Figure 2.9) (Yasinok,2006).

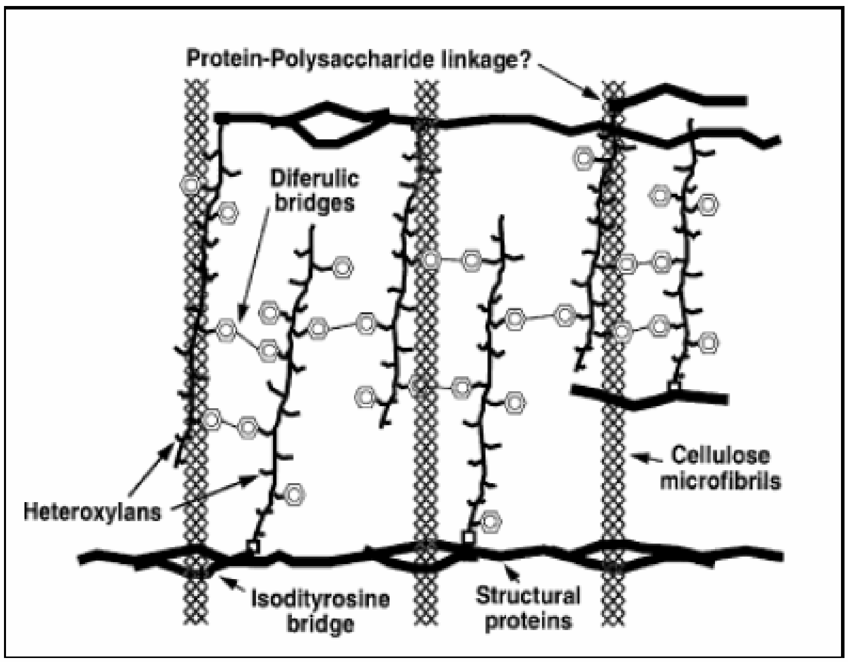

Figure 2.8 Maize bran cell walls model (Saha, 2000)

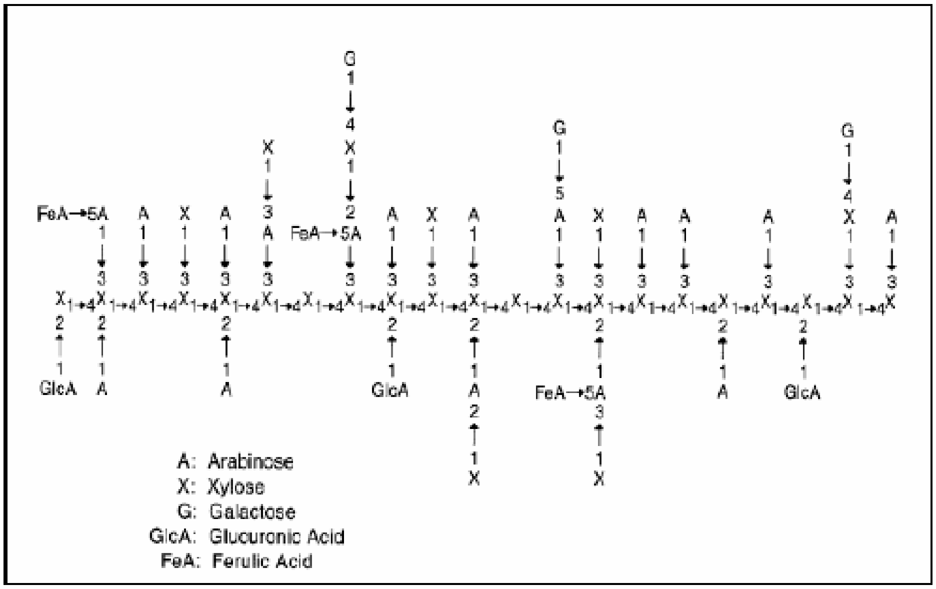

Figure 2.9 Schematic maize bran heteroxylan structure (Saha, 2000).

Several enzymes are required for complete hydrolysis and assimilation of xylan, including β-1,4-xylanase (1,4-β-D-xylan xylanohydrolase; E.C. 3.2.1.8) and β-xylosidase (1,4-β-D-xylan xylohydrolase; E.C. 3.2.1.37). The xylanases attack internal xylosidic linkages on the backbone and β-xylosidase releases xylosyl residues by endwise attack of xylooligosaccharides (Lama et al., 2004).

Lama et al. (2004) investigated purification and characterization of thermostable xylanase and β-xylosidase by the thermophilic bacterium *Bacillus thermoantarcticus*. Analysis of hydrolytic products revealed the action of these two enzymes. Initially, xylanase cleaved the substrate to liberate xylooligosaccharides and than the resulting oligosaccharides were cleaved to form xylose by β-xylosidase action.

A series of accessory debranching enzymes also take part in effective full degradation of xylan. α -L-arabinofuranosidases (EC 3.2.1.55, AF) and α-glucuronidase (EC 3.2.1.131) remove the arabinose and 4-O-methly glucuronic acid substituents, respectively, from the xylan backbone. Esterases hydrolyze the ester linkages between xylose units of the xylan and acetic acid (acetylxylan esterase, EC 3.1.1.72**.**6) or between arabinose side chain residues and phenolic acids, such as ferulic acid (ferulic acid esterase, EC 3.2.2.73) and p-coumaric acid (p-coumaric acid esterases, EC 3.1.1-) (Sørensen et al., 2005).

2.3.3.2 Response Surface Methodology (RSM)

Response Surface Methodology, RSM, is essentially a particular set of mathematical and statistical methods used by the researchers to aid in the solution of certain types of problems, which are pertinent to scientific or engineering processes. Its greatest application has been in industrial research, particularly in situations where a large number of variables in some system influence some feature of the system. This feature (e.g., reaction yield, cost of production, etc) is termed the *response*; it is normally measured on a continuous scale and is a variable which likely represents the most important function of the systems, though this does not rule out the possibility of a study involving more than one response. Also contained in the system are input variables or *independent variables*, which have an effect on the response and are subject to the control of the scientist or experimenter (Myers, 1971).

Many processes in the biological industries are studied using response surface methodology. The use of biological materials, however, means that run-to-run variation is typically much greater than that in many experiments in mechanical or chemical engineering and so the designs used require greater replication. The data analysis which is performed may involve some variable selection, as well as fitting polynomial response surface models. This implies that designs should allow the parameters of the model to be estimated nearly orthogonally. A class of three-level response surface designs is introduced which allows all except the quadratic parameters to be estimated orthogonally, as well as having a number of other useful properties (Gilmour, 2006).

The main three-level designs in the literature are central composite designs (CCDs) with the axial points at $\alpha = 1$, sometimes called face-centered cubes, and Box–Behnken designs (BBDs).

Central composite designs consist of

· 2^K or 2^{K-1} factorial points (also called cube points), where K is the number of factors

· axial points (also called star points)

· center points

A central composite design with three factors is shown below. Points on the diagrams represent the experimental runs that are performed:

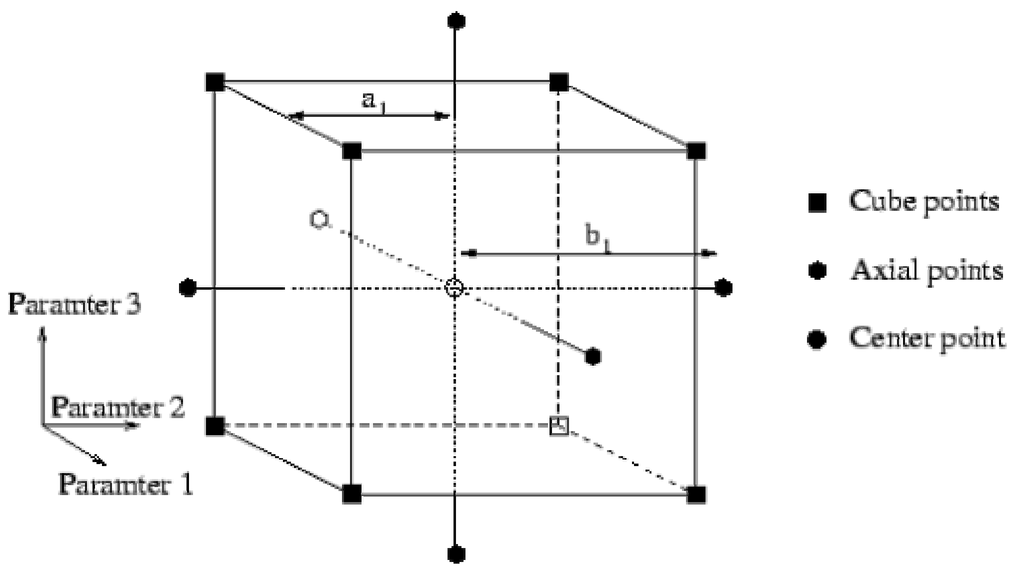

Figure 2.10 Central Composite Design

Central composite designs are often recommended when the design plan calls for sequential experimentation because these designs can incorporate information from a properly planned factorial experiment. The factorial and center points may serve as a preliminary stage where you can fit a first-order (linear) model, but still provide evidence regarding the importance of a second-order contribution or curvature.

The illustration below shows a three-factor Box-Behnken design. Points on the diagram represent the experimental runs that are performed:

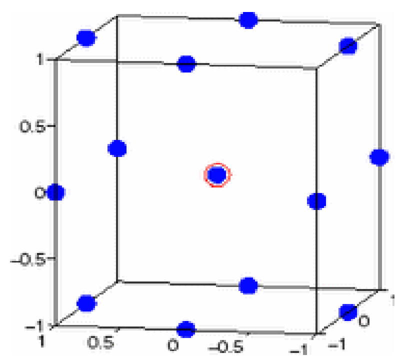

Figure 2.11 Box-Behnken Design

Box-Behnken designs are used when performing non-sequential experiments. That is, are only planned to perform the experiment once. These designs allow efficient estimation of the first- and second-order coefficients. Because Box-Behnken designs have fewer design points, they are less expensive to run than central composite designs with the same number of factors.

Box-Behnken designs can also prove useful if the safe operating zone for process is known. Central composite designs usually have axial points outside the "cube" (unless you specify an a that is less than or equal to one). These points may not be in the region of interest, or may be impossible to run because they are beyond safe operating limits. Box-Behnken designs do not have axial points, thus, all design points fall within safe operating zone. Box-Behnken designs also ensure that all factors are never set at their high levels simultaneously.

2.4 Bioreactor Operation Parameters

pH, temperature and oxygen transfer are the main environmental conditions that affect growth kinetics and product formation. Bioreactors are designed to control these parameters in order to achieve an effective bioprocess.

2.4.1 pH and Temperature

Temperature is an important factor affecting the performance of cells. As the temperature is increased toward optimal growth temperature, the growth rate approximately doubles for every 10°C increase in the temperature. Above the optimal temperature range, the growth rate decreases and thermal death may occur. Temperature also affects product formation. However, the temperature optimum for growth and product formation may be different. When temperature is increased above the optimum value, the maintenance requirements of cells increase. The yield coefficient is also affected by temperature (Shuler and Kargi, 2002).

pH (the hydrogen ion concentration) is an important factor influencing the microbial growth. The influence of pH on cellular activity is determined by the sensitivity of the individual enzymes to changes in the pH. Enzymes are normally active only within a certain pH interval, and the total enzyme activity of the cells is therefore a complex function of the environmental pH (Nielsen, Villadsen, Liden, 2003). There is a pH optimum for each microorganism at which growth is maximal. The pH optimum for many bacteria ranges from pH=3 to 8; for yeast, pH=3 to 6; for molds, pH=3 to 7 (Shuler and Kargi, 2002).

In most fermentation, pH can vary substantially. Often the nature of the nitrogen source can be important. If the ammonia is the sole nitrogen source, hydrogen ions are released into the medium as a result of the microbial utilization of ammonia, resulting in a decrease in pH. Also, the pH can change because of the production of organic acids, the utilization of acids (particularly amino acids), or the production of bases. The evolution or supply of CO_2 can alter pH greatly in some systems. Thus, pH control by means of a buffer or an active pH control system is important (Shuler and Kargi, 2002).

Most GI-producing fermentations are carried out between pH 7.0 and 8.0 without control of pH. *Streptomyces* spp., *Arthrobacter* sp., and *Actinoplanes missouriensis* are grown at around 30°C. Thermophilic *Bacillus* spp. are incubated at 50°C to 60°C (Bhosale et al., 1996). Table 2.13 shows the pH and temperature used for the fermentation medium of GI producing microorganisms.

Ensari et al. (1995) investigated the effect of temperature on GI producing microorganism namely, *Thermus thermophilus*. They studied GI production in batch fermentations at 4 different temperatures (60, 65, 70, 75°C). They observed that specific growth rate, μ, at various high temperatures of Thermus thermophilus can be correlated by a shallow Arrhenius curve.

Ward et al. (1972) performed a study on the thermophilic *Bacillus* namely, *Bacillus stearothermophilus* which is also one of the GI producing microorganisms. They investigated the optimum growth temperature as 64.5°C by relating the specific growth rate to temperature.

Table 2.13 Fermentation conditions for GI production

STRAIN	pH	TEMPERATURE	Reference
Bacillus thermoantarcticus	6.0	63°C	Lama et al.,2001
Thermotoga maritima	6.5	90°C	Brown et al., 1993
Thermotoga neapolitana	6.5	80°C	Hess at al., 1998
Thermoanaerobacter strain B6A	5.5	60°C	Lee and Zeikus, 1991
Bacillus TX-3	8.0-10.0	55°C	Kitada et al., 1989
Thermus thermophilus	7.0	65°C	Dekker et al., 1992
Bacillus sp. (NCIM 59)	10.0	50°C	Chauthaiwale et al., 1994
Bacillus KX-6	7.5-11.0	37°C	Kwon et al., 1983
Stretopmyces phaeochromogenes	7.2	28°C	Faloci et al., 1983
Streptomyces flavogriseus	7.0	30°C	Chen et al., 1979
Streptomyces albus	7.5	30°C	Sanchez et al., 1975
Arthrobacter sp.	6.9	30°C	Sapunova et al., 2004
Actinoplanes misouriensis	7.0	30°C	Quax et al., 1991

Bacillus thermoantarcticus is found to grow between 37°C and 65°C with an optimum at 63°C, and in the pH range of 5.5-9.0 with an optimum at pH 6.0 (Nicolaus et al., 1996).

2.4.2 Oxygen Transfer

Oxygen concentration into the bioreactor is one of the more decisive aspects of aerated bioprocess; the concentration of dissolved oxygen into the broths is very low, because of the low solubility of oxygen (Nielsen et al., 2003). The oxygen consumption by the microorganisms can provoke a depletion of this nutrient, limiting the cellular growth and metabolism, and even provoking cellular death. Microorganisms prevail in the liquid phases; consequently the oxygen must be continuously supplied and dispersed into the broths to satisfy the oxygen demand of the cells. Stirred tank bioreactors are frequently used to reach an adequate contact between different phases, because high-energy input can be achieved. Thus, both oxygen transport and uptake rates (OTR and OUR) must be known in order to design and simulate bioreactors. Oxygen transfer is a physical phenomena, therefore OTR can be predicted, while oxygen consumption involves biochemical reactions and OUR must be measured (Çalık et al., 1998; Çalık et al., 2000; Gomez et al., 2006).

The transfer of oxygen from gas to microorganism takes place in several steps (Figure 2.12), which are 1) transfer from the interior of the bubble to the gas-liquid interface; 2) movement across the gas-liquid interface; 3) Diffusion through the relatively stagnant liquid film surrounding the bubble; 4) Transport through the bulk liquid; 5) Diffusion through the relatively stagnant liquid film surrounding the cells; 6) Movement across the liquid-cell interface; 7) If the cells are in a floc, clump or solid particle, diffusion through the solid to the individual cell; 8) Transport through the cytoplasm to the site of reaction (Scragg, 1988 and Bailey, 1986).

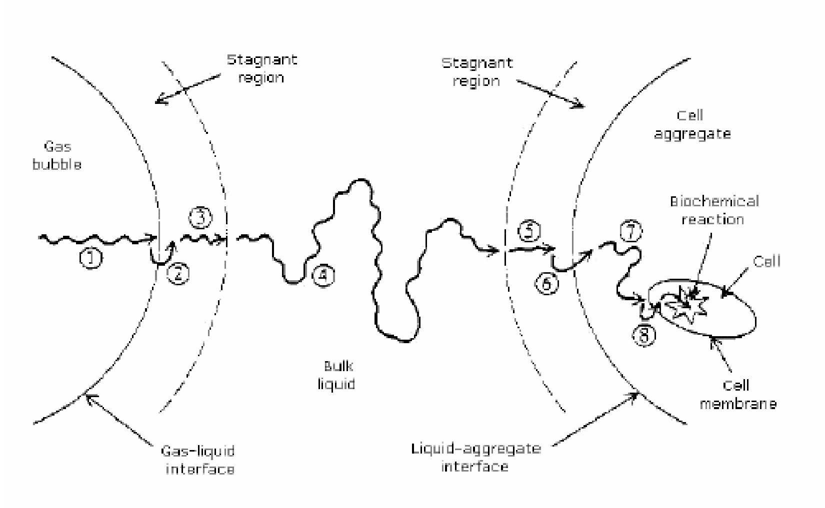

Figure 2.12 Schematic diagram of steps involved in transport of oxygen from a gas bubble to inside a cell (Bailey and Ollis, 1986)

The rate of oxygen transfer from the gas to liquid phase is given by

$$\text{OTR}=\ N_{O2}=k_L a(C_O^* - C_O) \qquad (2.23)$$

where k_L is the oxygen transfer coefficient (cm/h), a is the gas-liquid interfacial area(cm^2/cm^3), k_La is the volumetric oxygen transfer coefficient (h^{-1}), C* is saturated concentration(mg/L), C_L is the actual DO concentration in the broth (mg/L) and the N_{O2} is the rate of oxygen transfer (mg O_2/l.h) (Shuler and Kargi, 2002).

The rate of oxygen uptake is denoted as OUR (oxygen uptake rate) where q_{O2} is the specific rate of oxygen consumption (mgO_2/g dw cells .h) (Shuler and Kargi, 2002).

$$OUR = q_0 C_X \qquad (2.24)$$

2.4.2.1 Measurement of k_La: Dynamic Method

Dynamic method is a widely and simple method applied during the fermentation to determine the value of k_La experimentally. This method based on the unsteady-state mass balance for oxygen given by the following question:

$$\frac{dCo}{dt} = K_La(Co * -Co) - q_0 C_X \qquad (2.25)$$

A typical response curve of the dynamic method is given in Figure 2.13. As shown in Figure 2.13, at some time t_0, the broth is de-oxygenated by stopping the air flow. During this period, dissolved oxygen concentration, Co_0, drops, and since there is no oxygen transfer (region-II), equation reduces to:

$$\frac{dC_O}{dt} = r_0 \qquad (2.26)$$

Using equation in region-II of Figure 2.16, oxygen uptake rate, $-r_0$, can be determined.

Air inlet is then turned back on, and the increase in C_O is monitored as a function of time. In this period, region-III, equation is valid. Combining equations and and rearranging,

$$C_O = -\frac{1}{K_La}\left(\frac{dC_O}{dt} - r_0\right) + C_O^* \qquad (2.27)$$

From the slope of a plot of C_O versus (dC_O/dt – r_0), K_La can be determined (Figure 2.14).

The Dynamic Method can also be applied to conditions under which there is no reaction, i.e., r_0=0 (Nielsen and Villadsen, 1994). In this case, the broth is de-oxygenated by sparging nitrogen into the vessel. Air inlet is turned back on and again the increase in Co is monitored as a function of time (Figure 2.15). Modifying equation (2.27):

$$C_O = \frac{1}{K_L a}\frac{dC_O}{dt} + C_O^* \quad (2.28)$$

From the slope of a plot of C_O versus dC_O/dt, the physical mass transfer coefficient, K_La_0, can be determined.

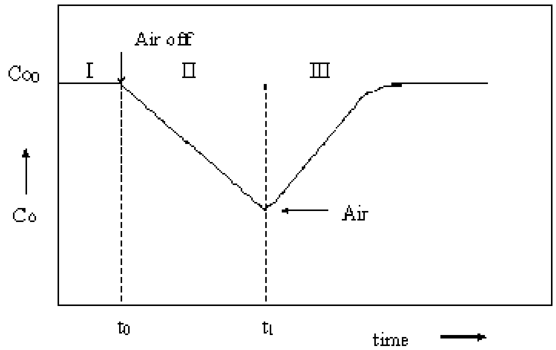

Figure 2.13 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La.

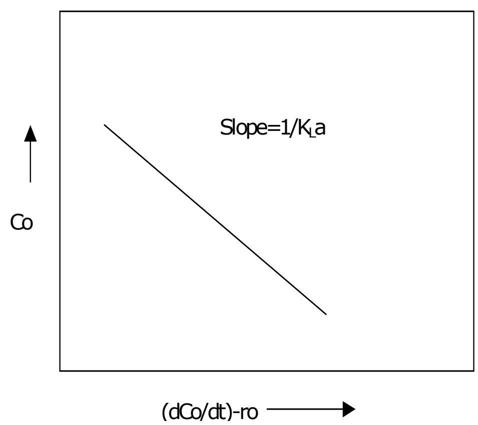

Figure 2.14 Evaluating K_La using the Dynamic Method

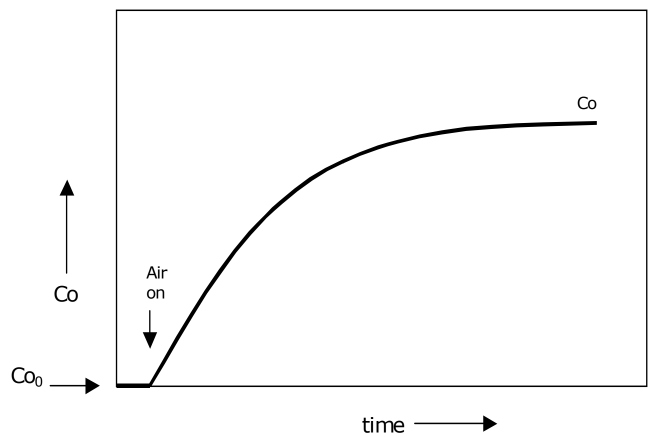

Figure 2.15 Variation of dissolved oxygen concentration with time in dynamic measurement of K_La_0 using the Dynamic Method

In the study Wang et al. (1998), production of glucose isomerase in a recombinant strain of *Streptomyces lividans* is performed. They investigated the effect of aeration on glucose isomerase production in a 250 ml double-jacket culture flask with a working volume of 150 ml in continuous operation mode. The culture was operated at 30°C and 200 rpm agitation rate. They studied the effect of oxygen transfer in the following aeration rates: 0, 0.45, 1.00, 1.60 L min^{-1}, being equal to the Q_o/V_R values: 0, 0.068, 0.15, 0.24 vvm respectively. In these operation conditions, cell density, dilution rate, and glucose isomerase activity were analyzed at a continous operation mode. The results are given below:

- The highest cell density was obtained at aeration rate of 1 L min^{-1}.
- When the level of sparged air was increased from 0.45 L min^{-1} to 1.60 L min^{-1}, there was no significant difference in the medium dilution rate necessary to stabilize the culture.
- The productivity of glucose isomerase increased, from 3.38 GIU/h to 12.13 GIU/h as the aeration rate increased from 0 to 1.60 L min^{-1}. The increased enzyme production was evident both in the activity in the cell lysates and in the amount of enzyme activity that was apparently released into the culture medium.

Glucose isomerase production is also performed in anaerobic conditions. Thermophilic anaerobe namely, *Thermoanaerobacterium saccharolyticum* was used as the producer of glucose isomerase in a bioreactor of 14 L with a working volume of 10 L. The fermentor cultures were incubated at 60°C, gassed continuously with N_2-$C0_2$ (95:5), and mixed at 100 rpm (Lee et al., 1990). Moreover, Brown et al. (1992) reported glucose isomerase production from a hyperthermophilic and anaerobic microorganism namely, *Thermotoga maritima.* Production of glucose isomerase was conducted in 400 ml of bioreactor under nitrogen sparge approximately 5 L min^{-1} at 250 rpm.

2.4.2.2 Oxygen Transfer Parameters

In order to compare the relative rates of maximum oxygen transfer and biochemical reactions and find the rate limiting step of the bioprocess, the maximum possible oxygen utilization rate (OD=oxygen demand) which is defined as (Çalık et al., 2004),

$$OD = \frac{\mu_{\max} C_X}{Y_{X/O}} \qquad (2.29)$$

and the maximum possible mass transfer rate is defined as,

$$OTR_{\max} = k_L a C_{DO}^{*} \qquad (2.30)$$

should be determined throughout the bioprocess.

In order to express the oxygen limitation in an aerobic process, the effectiveness factor, η (oxygen uptake rate per maximum possible oxygen utilization rate) and Damköhler number, Da (maximum possible oxygen utilization rate per maximum mass transfer rate) are defined according to the equations given below:

$$\eta = \frac{OUR}{OD} \qquad (2.31)$$

$$Da = \frac{OD}{OTR_{\max}} \qquad (2.32)$$

2.5 The scope of study

This study attempts the investigation of bioprocess parameters for glucose isomerase production from *Bacillus thermoantarcticus.* Investigation of the optimum operation conditions for glucose isomerase production is essential because of the commercial importance of glucose isomerase in the production of high-fructose syrups from starch. To achieve an efficient enzymatic conversion of glucose to fructose, optimum combinations of bioprocess parameters for maximizing glucose isomerase production should be investigated in terms of medium components, pH and oxygen transfer conditions.

Within the context of this work, the following steps were carried out:

- The novel bacterium namely, *Bacillus thermoantarcticus* was used as the potential producer of thermostable glucose isomerase. Thermostable protein synthesis of this novel bacterium was considered as an advantage that thermostable glucose isomerase can easily catalyze the conversion of glucose to fructose at high reaction temperatures. Because higher reaction temperatures enhance the fructose yield in the reversible conversion of glucose to fructose.
- A medium design was performed in terms of its carbon and nitrogen sources by Response Surface Methodology (RSM) which explored the relationship between the medium components (variables) and the GI volumetric activity (response). RSM offered an efficient way of finding the optimum combination of the medium components to maximize the GI volumetric activity.
- Glucose isomerase production was investigated in terms of bioreactor operation parameters namely, pH and oxygen transfer conditions by using the designed medium. The effect of pH on glucose isomerase production was analyzed in terms of pH control throughout the bioprocess. The effect of oxygen transfer parameters were investigated by varying agitation rate (N) of the pilot scale bioreactor at a constant air inlet rate (Q_0/V_R).

CHAPTER 3

MATERIALS AND METHODS

3.1 The Microorganism

Bacillus thermoantarcticus (DSMZ 9572) was used as the potential producer of glucose isomerase. The microorganisms, which were freeze dried when received, were kept at –50°C and brought to active state by incubating for 30 min at 30°C in a liquid medium, V=0.3 ml containing (kg m^{-3}): soytryptone,5 ;peptone,5 ;$MnSO_4.2H_2O$,0,010. Afterwards, the microorganisms were inoculated onto a solid medium and stored at 4°C.

3.2 The Solid Medium

The microorganisms, stored on agar slants at 4ºC, were inoculated onto the newly prepared agar slants under sterile conditions, and they were incubated at 55ºC for 24h. The composition of the solid medium for glucose isomerase production by *Bacillus thermoantarcticus* is given in Table3.1.

Table 3.1 The composition of the solid medium for *Bacillus thermoantarcticus*

Compound	Concentration, kg m^{-3}
Soytryptone	5.00
Peptone	5.00
$MnSO_4.2H_2O$	0.01
Agar	30.00

3.3 The Precultivation Medium

Microorganisms grown in the solid medium for 24 h, were inoculated into precultivation medium, and incubated at 55ºC and N=200 min^{-1}, for 10-12 h in laboratory scale bioreactor experiments. Microorganism growth was conducted in orbital shakers under agitation and heating rate control, using air-filtered Erlenmeyer flasks 150 ml in size that had working volume capacities of 33 ml. The composition of the precultivation medium for cell growth and glucose isomerase production is given in Table 3.2.

Table 3.2 The composition of the precultivation medium.

Macronutrients	Concentration, kg m^{-3}
Yeast extract	6.0
Peptone	2.5
Beef extract	1.5
NaCl	3.0
Xylan	10.0
Micronutrients (salt solution)	**Concentration, kg m^{-3}**
$MgSO_4.7H_2O$	0.25
$FeSO_4.7H_2O$	1.0×10^{-3}
$ZnSO_4.7H_2O$	1.0×10^{-3}
$MnSO_4.H_2O$	7.5×10^{-5}
$CuSO_4.5H_2O$	1.0×10^{-5}

3.4 The Production Medium

When the microorganism concentration in the precultivation medium reached to 0,53 kg m^{-3}, the microorganisms were inoculated to the production medium in the laboratory scale bioreactor (V=150 ml) with 1/10 inoculation ratio.

For laboratory scale experiments, air filtered Erlenmeyer flasks 150 ml in size, having working volume of 33 ml, were used as bioreactors. Agitation and heating rate controlled orbital shakers, at a cultivation temperature of 55ºC and an agitation rate of 200 min^{-1}, unless otherwise stated, were used as incubators.

The parameters investigated in the production medium for glucose isomerase production are given in Table 3.3. The composition of the reference medium is given in Table 3.4 (DSMZ). The medium given by DSMZ (reference medium) is used as the starting point of the medium design experiments.

Table 3.3 The investigated parameters for glucose isomerase production

Medium Components	Bioreactor Design Parameters
Glycerol	pH
Xylose	Oxygen Transfer Rate
Xylan	
Xylan types	
Glucose	
Fructose	
Sucrose	
$(NH_4)_2HPO_4$	
$(NH_4)_2SO_4$	
$(NH_4)Cl$	
Yeast extract	
Peptone	
Beef Extract	
NaCl	
H_3PO_4,HCl,H_2SO_4 (for pH adjustment)	

Table 3.4 The composition of the reference production medium.

Compound	Concentration, kg m^{-3}
Yeast extract	6.0
Peptone	2.5
Beef extract	1.5
NaCl	3.0
Glycerol	10.0

All the medium components except the salt solution were steam sterilized at 121°C for 20 min, carbon sources being sterilized separately. The micronutrients all together, referred to as the salt solution, was filter sterilized with a sterile filter of 0.2 μm pore size.

3.5 Analysis

Throughout the bioprocesses, samples were taken at characteristic cultivation times. After determining the cell concentration, the medium was centrifuged at 13200 min^{-1} for 10 min at 4°C to precipitate the cells. The cells were used for the determination of glucose isomerase activity. The supernatant was used for the determination of xylanase activity in laboratory scale bioreactor experiments. In pilot scale experiments, amino acid and organic acid concentrations, oxygen uptake rate and liquid phase mass transfer coefficient values were determined in bioreactor system experiments.

3.5.1 Cell Concentration

Cell concentrations based on dry weights were measured with a UV-Vis spectrophotometer (Thermo Spectronic, Heλios α) using a calibration curve (Appendix A) obtained at 600 nm.

3.5.2 Glucose Isomerase Activity

Glucose isomerase activity was determined by measuring the conversion of D-glucose into D-fructose. The steps concerning the measurement of glucose isomerase activity are given below:

1. Samples from the culture broth were taken in eppendors.

2. Cells were precipitated by centrifugation at 13,200 g for 10 min.

3. Supernatant was removed and crude cell extracts were dissolved in a 970 µl of 0.1 M potassium phosphate buffer.

4. Glass beads were put into the dissolved cell solutions to the 1.5 ml volume of the ependorf in order to disrupt the cell wall. Cell were lyzed at f=10 s^{-1} for 10 minutes at the agitator bead mill (Retsch, MM 200).

5. After distrupting cells, 50 µl of enzyme solution was taken from ependorf and added to 50 µl of a solution of fresh activity buffer which was already maintained at 80°C. Fresh activity buffer was prepared daily by dissolving 0.4 M D-Glucose, 10mM $MgSO_4$ and 1mM $CoCl_2$ in 0.02 M potassium phosphate buffer, pH 7.0.

6. 100 µl or reaction mixture consisting, 50 µl of enzyme solution and 50 µl of a solution of fresh activity buffer were incubated for 10 min at 80°C. At the end of the reaction, the concentration of D-Fructose was estimated by cysteine-carbazole-sulfuric acid method (Dische and Borenfreund, 1951). One unit of glucose isomerase activity was defined as the formation of 1 µmol of D-fructose per minute at 80°C (Lama et al., 2001). Calibration curve for fructose is given in Appendix B.

3.5.2.1 Cysteine-Carbazole-Sulfuric Acid Method

The formation of D-fructose from D-Glucose was measured using the colorimetric assay of Dische and Borenfreund (1951), known as cysteine-carbazole-sulfuric acid method, in which 50 µl of enzyme solution was added to the 50 µl of a solution of 0.4 M D-Glucose, 10mM $MgSO_4$ and 1mM $CoCl_2$ in 0.02 M potassium phosphate buffer, pH 7.0, and incubated for 10 min at 80°C.Then 60 µl of reaction mixture was putted into 540 µl of 0.1 M HCl to terminate the reaction. 60 µl of freshly prepared 1.5%(w/v) cysteine-HCl, 60 µl of 0.12%(w/v) carbazole in 95% ethanol, and 1.8 ml of 70% sulfuric acid were added to the reaction mixture and the absorbance at 560 nm was measured after a 30 minute incubation at room temperature. The absorbance was compared to a linear standard curve prepared by using D-fructose in the reaction mix (Appendix B).

3.5.3 Xylanase Activity

One unit of xylanase activity is expressed as 1 µmol of reducing sugars (xylose equivalent) released in 1 min under the conditions described below (Lama et al., 2004). The amount of reducing sugars was determined by 3,5-dinitrosalicyclic acid method (DNS method) as reported by Miller (1959).

Xylanase activity was assayed by using 1% birchwood xylan as the substrate, which was preliminarily suspended homogeneously in 0,05 M Potassium phosphate buffer (pH 5.6). The mixture, containing 0.5 ml of an appropriate dilution of the enzyme and 5 ml of the substrate, was incubated at 80°C for 1 min (Lama et al., 2004). During the enzymatic reaction, 1ml of samples were taken from the reaction mixture and mixed with 1.5 ml of DNSA reagent in tubes at 15 second intervals up to 1 min. Then, the tubes were boiled for five minutes and cooled immediately under tap water. Finally, tubes were equilibrated to room temperature and read against a buffer blank at 540 nm**.** The absorbance related to the enzymatic activity was compared to a linear standard curve with a slope of m_1, which was prepared by using xylose (Appendix C).

Xylanase activity (Uml^{-1}) was calculated according to the formula given below:

$$Xylanase\ Activity\ (Uml^{-1}) = \frac{m_2}{m_1} \times \frac{60\sec}{1\min} \times \frac{1\mu\text{mol of xylose}}{150\mu\text{g xylose}} \times \text{Dilution Ratio}$$

m1: Slope of the calibration curve for reduced sugar (xylose) concentration
m2 : Slope of the curve plotted by the absorbance of the reaction mixture samples treated with DNS against time (sec)

3.5.3.1 Reduced Sugar Concentration

Reduced sugar, xylose, concentration was determined by the DNS (dinitrosalisylic acid) method (Miller, 1959) at 540 nm with a UV spectrophotometer. The reaction mixtures contained 0.5 ml of properly diluted enzyme and 5 ml of substrate solution. The reaction mixtures were incubated at suggested temperature and the 1ml of samples were mixed with 1.5 ml of DNSA reagent at 15 second intervals up to 1 min.

3.5.4 Organic Acid Concentration

Organic acid concentrations were measured with an organic acid analysis system (Waters, HPLC, Alliance 2695). The method is based on reversed phase HPLC, in which organic acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard organic acids solution. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and loaded to the analysis system with a mobile phase of 3.12% (w/v) NaH_2PO_4 and $0.62x10^{-3}$% (v/v) H_3PO_4. Needle and seal wash were conducted with water. The analysis was performed under the conditions specified below (İleri, 2005):

Column: Capital Optimal ODS, 5µm
Column dimensions: 4.6x250 mm
System: Reversed phase chromatography
Mobile phase flow rate :0.8 ml/min
Column temperature: 30 °C
Detector and wavelength: Waters 2487 Dual absorbance detector, 210 nm
Injection volume: 5 µl
Analysis period: 15 min

3.5.5 Amino Acids Concentrations

Amino acid concentrations were measured with an amino acid analysis system (Waters, HPLC), using the Pico Tag method (Cohen, 1983). The method is based on reversed phase HPLC, using a pre-column derivation technique with a gradient program developed for amino acids. The amino acid concentrations were calculated from the chromatogram, based on the chromatogram of the standard amino acids solution. Samples were filtered with 45 µm filters (ACRODISC CR PTFE) and loaded to the analysis system with a mobile phase of 6.0% (v/v) acetonitrile, 1.79% (w/v) NaAc from solvent A and 66.6% (v/v) acetonitrile from solvent B. The analysis was performed under the conditions specified below:

Column: Amino acid analysis column (Nova-Pak C18, Millipore)
Column dimensions: 3.9 mm x 30 cm
System:Reversed phase chromatography
Mobile phase flow rate: 1 ml/min
Column temperature: 38 °C
Detector and wavelength: UV/VIS, 254 nm
Injection volume: 4 µl
Analysis period: 18 min

3.5.6 Liquid Phase Mass Transfer Coefficient and Oxygen Uptake Rate

In order to determine the liquid phase mass transfer coefficient and oxygen uptake rate in the glucose isomerase production process, the Dynamic Method (Bandyopadhyay and Humprey, 1967) was used. Prior to inoculation of the microorganism to the production medium in the bioreactor, the physical mass transfer coefficient (KLa_0) was determined. After inoculation of the microorganism to the bioreactor, the dynamic oxygen transfer experiments were carried out at certain cultivation times for a short period of time, so that the biological activities of the microorganisms are unaffected. During this period, the air inlet was totally ceased, the agitation rate was lowered to N=150 min^{-1} in order to lower the effect of surface aeration.

3.5.7 Saturation oxygen concentration determination

In order to calculate the dissolved oxygen concentration in the fementation during the bioprocesses, saturation oxygen concentrations, C_{O2}^{*}, were determined. For the saturation oxygen concentration measurements, approximately 1000 ml of fermentation broth was prepared according to the medium composition used at the beginning of the bioprocess and 1000 ml of fermentation broth was collected at the end of each experiment performed in pilot scale bioreactor. Fermentation broth sample was set to 55°C and stirred with a magnetic stirrer. Air was compressed to the fermentation broth and dissolved oxygen concentration was measured by an oxygen probe. After a short period, dissolved oxygen concentration attained a constant value, namely the saturation oxygen concentration, C_{O2}^{*}.

3.5.8 Response Surface Methodology

In order to determine the optimum compositions of medium components that maximize the glucose isomerase activity, the statistical approach, Response Surface Methodology (RSM) was used. For the medium design experiments performed by RSM, statistical software MINITAB ® Release 14 was employed.

The steps followed during the creation of a response surface design are given below. There are 3 main commands used when carrying out response surface designs. Each command in MINITAB ® Release 14 is given at the beginning of the steps and explained in details.

1. Stat > DOE > Response Surface > Create Response Surface Design: This command is used to create the response surface design.

- The appropriate response surface design is choosen by concerning the experimental region of interest, number of runs that can be performed and other considerations such as cost and time. The Minitab provides two response surface designs namely, Central Composite and Box-Behnken designs (Section 2.3.3.2).
- The number of factors are specified.
- The names, the lowest and highest values of factors are identified.
- The value of α (alpha) is determined in which the α value less than 1 places the axial points inside the cube; values greater than 1 places them outside the cube. Placing α=1 indicates a face-centered design.
- The blocking of the central composite design is specified. When the number of runs is too large to be completed, it is necessary to be concerned with introducing blocks to the experiment. Running an experiment in blocks allows you to work separately in which block effects (different experimental conditions) are concerned by the program. When the experimental runs can be carried out at the same time, there is no need to introduce blocking to the experiment.
- After carrying out the steps above one by one, a response surface design with definite combinations of runs is created. A table displaying the combinations of experimental runs is generated by Minitab.

2. Stat > DOE > Response Surface > Analyze Response Surface Design: After performing the experiment, the responses were placed into the design table, and the results were analyzed by the command given above.

- Coding of the analysis is performed in which coding places −1 for the low level, +1 for the high level, and 0 for the center points. Minitab displays the output based in this coded form. However, uncoding performs the analysis using the values that is assigned in the factors subdialog box. Minitab displays the output using the assigned values.
- After carrying out the analysis of the design with Minitab, a session window output was created and the results are interpreted in terms of the lack fit of the model (p), R^2 and the estimated regression coefficients. Lack of fit and R^2 is important for checking the adequacy of the fitted model as an incorrect or under-specified model can result in misleading conclusions. The estimated regression coefficients are used to determine the second order polynomial defined by the relation between variables and the response.

3. Stat > DOE > Response Surface > Analyze Response Surface Design > Graphs: This command is used to create graphs that define the relation between the response and the variables. Generally, the experimenters performing response surface designs use two types graphs namely, contour and surface plots. A contour plot provides a two-dimensional view where all points that have the same response are connected to produce contour lines of constant responses. A surface plot provides a three-dimensional view that may provide a clearer picture of the response surface.

CHAPTER 4

RESULTS AND DISCUSSION

This study focuses on investigation of bioprocess parameters for glucose isomerase production. For this purpose this study was carried out in two parts. Firstly, a medium design was carried out by Response Surface Methodology for finding the optimum glucose isomerase production conditions. Secondly using the optimum conditions pH and oxygen transfer effects were investigated in pilot scale bioreactor.

4.1 Determination of effects of various medium components on glucose isomerase activity

A defined medium was designed to optimize glucose isomerase production. For this purpose, the medium given by DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) was taken as the reference medium. After omitting the soil extract from this medium, the medium containing: 10 kg m^{-3} of glycerol, 6 kgm^{-3} of yeast extract, 3 kgm^{-3} of NaCl, 2.5 kgm^{-3} of peptone and 1.5 kgm^{-3} of beef extract was considered as the starting point of the medium design experiments.

In this context, the effects of carbon sources, i.e.,glycerol, xylose and xylan; inorganic nitrogen sources, i.e., $(NH_4)_2HPO_4$,NH_4Cl and $(NH_4)_2SO_4$; the organic nitrogen sources, i.e., yeast extract, peptone and beef extract and their concerted effects, NaCl and salt solution were investigated in agitation (N=200 min^{-1}) and heating rate (T=55°C) controlled laboratory scale bioreactors.

4.1.1 Effect of various carbon sources and salt solution

The variations in glucose isomerase activity with the cultivation time utilizing different carbon sources were investigated, in media containing 10 kg m^{-3} carbon source, 6 kg m^{-3} of yeast extract, 3 kg m^{-3} of NaCl, 2.5 kg m^{-3} of peptone and 1.5 kg m^{-3} of beef extract. The effects of various carbon sources: glycerol, xylose and xylan with or without salt solution on glucose isomerase activity were investigated between t=0-9 h of the bioprocess and the highest glucose isomerase activity and cell concentration for each medium were obtained at t=9 h of the bioprocess. So the results obtained at t=9 h of the bioprocesses were given to compare the effects of various carbon sources and salt solution on glucose isomerase activity. As it is seen in Figure 4.1 medium containing xylan with salt solution was founded as the most effective medium for glucose isomerase activity (A_{max}=720 UL^{-1} and Cx=0.61 kgm^{-3} at t=9 h).

In the study performed by Lama et al (2001), glucose isomerase activity was measured in terms of specific activity, namely the GI activity per mg protein (U/mg) produced by *Bacillus thermoantarcticus.* They investigated the effects of various carbon sources, xylose, xylan, arabinose, galactose, fructose, maltose, and glucose, having concentrations of 5 kg m^{-3} and found that the highest GI activity was obtained with xylose as 0.18 U mg^{-1} .The following highest activity was obtained with the use of xylan as 0.009 U mg^{-1} which is % 5 of the GI activity obtained by xylose. However, in the present work, carbon sources were used in the concentration of 10 kg m^{-3} and xylan gave the highest activity (volumetric activity; GI activity per unit volume of sample taken from the fermentation broth) as 720 UL^{-1} which is about 4.1 times higher than that obtained by xylose containing medium at t=9 h.

As explained in section 2.3.3.1, xylose is the common carbon source used for glucose isomerase production. However, xylan or xylan containing materials could be used instead of purified xylose in the fermentation medium. When the production of glucose isomerase on a commercial scale is considered, a cheaper carbon source provides the advantage of reducing the production cost. Also, *Bacillus thermoantarcticus* possesses xylanase activity, which enables the metabolization of xylan during the fermentation. When these results are taken into consideration, an economically feasible medium design by using xylan as the sole carbon source for glucose isomerase production is reasonable.

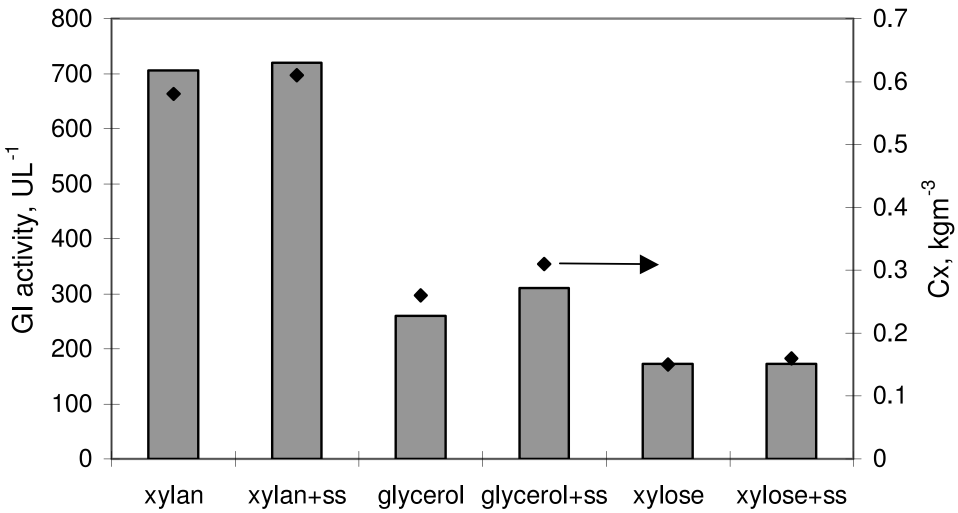

Figure 4.1 The variations in glucose isomerase activity and cell concentration with different carbon sources and salt solution at t=9 h of the cultivation, V=33 cm^3, T=55°C, pH_0 =6.0, N=200 min^{-1}.

4.1.2 Effect of organic nitrogen sources

After obtaining the medium containing 10 kg m^{-3} of xylan, 6 kg m^{-3} of yeast extract, 3 kg m^{-3} of NaCl, 2.5 kg m^{-3} of peptone, 1.5 kg m^{-3} of beef extract with 33 μl salt solution, analysis proceeded by investigating the effect of peptone and beef extract on glucose isomerase activity. This effect was investigated between t=12-24 h of the cultivation and the highest glucose isomerase activity in both medium was attained at t=24 h of the cultivation. So the results obtained at t=24 h of the bioprocesss were given in order to determine the effect of peptone and beef extract on glucose isomerase activity.

As it seen from Figure 4.2 there is a slight difference reflected to the glucose isomerase activity. The medium, which does not contain beef extract and peptone, showed 1330 U L^{-1} GI activity which is 1.1 times higher than the medium containing beef extract and peptone (1224 U L^{-1}).

As explained in section 2.3.3, glucose isomerase production has been carried out with different organic nitrogen sources. Although the use of peptone and beef extract were given in literature studies done by Prabhakar et al., (2004) and Park et al., (1976), their effects on GI activity were not investigated. However in this study, their effects were investigated in order to continue the medium design more simply. Presence of peptone and beef extract did not change GI activity significantly.

Chen et al. (1979) investigated the effect of nitrogen sources on GI activity. They used *Streptomyces flavogriseus* for the production of glucose isomerase and observed that 10 kgm-3 of yeast extract showed a GI activity of 2.00 Uml^{-1} after 48 hours of cultivation. The mediums containing 10 kg m^{-3} peptone and 10 kgm-3 beef extract were having GI activities of 1.80 U ml^{-1} and 0.78 U ml^{-1}, respectively.

Main function of yeast extract in the production of glucose isomerase is to support the growth of *Bacillus thermoantarcticus* (Nicolaus et al., 1996). As glucose isomerase from *Bacillus thermoantarcticus* is an intracellular enzyme, its activity highly depends on the growth of the microorganism and hence, presence of yeast extract in the fermentation broth. Also, as shown in Figure 2.5, yeast extract was used in most of the fermentation mediums of *Bacillus thermoantarcticus*. As a result, yeast extract was used as the sole organic nitrogen source in the medium design experiments.

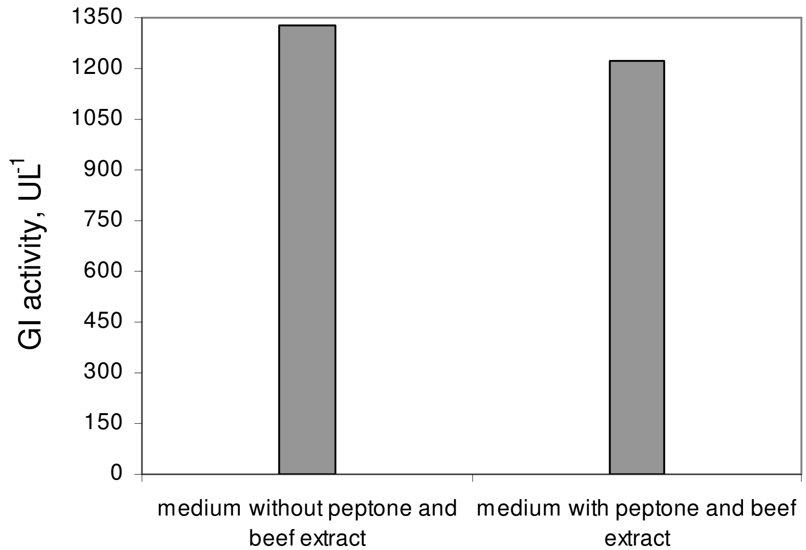

Figure 4.2 The variations in glucose isomerase activity with the organic nitrogen sources: peptone and beef extract at t=24 h of the cultivation, V=33 cm^3, T=55°C, pH_0 =6.0, N=200 min^{-1}.

4.1.3 Effect of inorganic nitrogen sources and yeast extract

Effects of $(NH_4)_2HPO_4$,NH_4Cl, $(NH_4)_2SO_4$ and yeast extract with concentrations of 6 kg m^{-3} on glucose isomerase activity and cell concentration were investigated in the medium containing 10 kg m^{-3} of xylan, 3 kg m^{-3} of NaCl and 33 μl salt solution between t=0-13 h of the cultivation and the highest glucose isomerase activity and cell concentration were obtained at t=13 h of the cultivation. So the results obtained at t=13 h of the bioprocesses were given in order to compare the effect of 6 kg m^{-3} of inorganic nitrogen sources and yeast extract on glucose isomerase activity.

In Figure 4.3 it is seen that $(NH_4)_2SO_4$ was founded as the best inorganic nitrogen source due to the sulphate metabolism among thermophiles (A_{max}=932 UL^{-1} and Cx=0.81 kg m^{-3} at t=13 h). $(NH_4)_2HPO_4$ and NH_4Cl showed GI activities of 876 U L^{-1} and 830 U L^{-1} respectively being 94% and 89% of the GI activity obtained with the medium containing $(NH_4)_2SO_4$. When the effects of $(NH_4)_2SO_4$

and yeast extract on glucose isomerase activity and cell concentration were compared, $(NH_4)_2SO_4$ was found as a more effective nitrogen source for glucose isomerase production. The medium containing yeast extract attained a GI activity of 850 U L^{-1} which is 91% of the glucose isomerase activity obtained with the medium containing $(NH_4)_2SO_4$.

The study performed about sulfate metabolism among thermophiles explains the result obtained by the use of ammonium sulfate in the fermentation medium. Sulfate reduction rates of thermophiles in natural aquatic systems studied by Ruychoudhury (2004). Thermophiles and hyperthermophiles can utilize a multitude of electron acceptors and donors for metabolic purposes. Sulphur isotope studies have provided the earliest evidence of a specific metabolic pathway – sulphate reduction. This focuses on the effect of temperature on sulfate reduction rate of thermophilic microorganisms present in hydrothermal springs. Within individual springs sulfate reduction rate is observed to increase with increasing temperature (Ruychoudhury, 2004).

Chen et. al. (1979) investigated the effect of inorganic nitrogen sources, $(NH_4)_2HPO_4$, NH_4Cl and $(NH_4)_2SO_4$ having concentrations of 5 kgm^{-3}, on GI activity after 48 hours of cultivation of *Streptomyces flavogriseus*. The medium containing $(NH_4)_2HPO_4$ showed a GI activity of 0.69 Uml^{-1}. However the mediums containing NH_4Cl and $(NH_4)_2SO_4$ showed no activity.

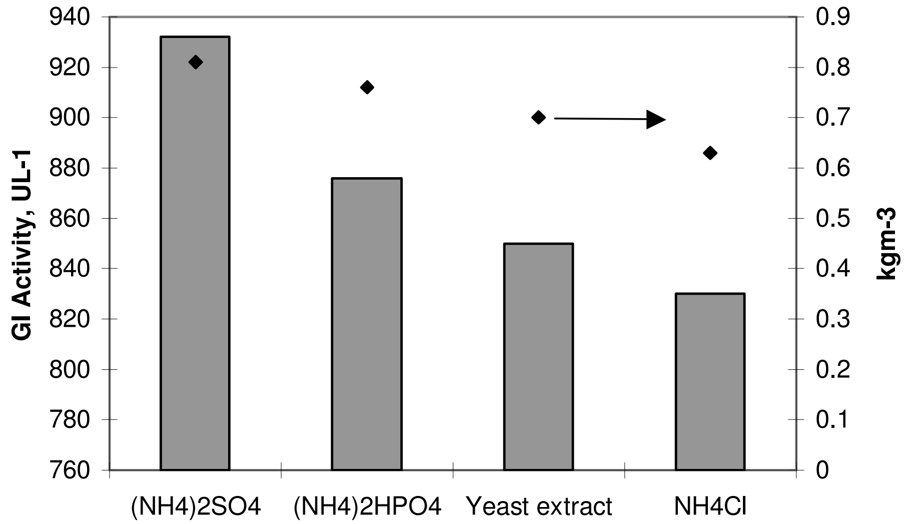

Figure 4.3 The variations in glucose isomerase activity and cell concentration with nitrogen sources :$(NH_4)_2HPO_4$,NH_4Cl, $(NH_4)_2SO_4$ and yeast extract at t=13 h of the cultivation , V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

4.1.4 Effect of NaCl

Effect of NaCl (3 kg m^{-3}) on GI activity and cell concentration was investigated between t=0-13 h of the cultivation in the medium containing 10 kg m^{-3} of xylan, 6 kg m^{-3} of yeast extract and 33 μl of salt solution. The highest glucose isomerase activity and cell concentration were obtained at t=13 h of the cultivation. So the results obtained at t= 13 h of the bioprocesses were given in order to investigate the effect of NaCl on glucose isomerase.

As it seen in Figure 4.4, the medium containing NaCl showed a lower glucose isomerase activity due to the inhibitory effect of NaCl on the enzyme. Presence of NaCl depressed the activity of the enzyme (A_{max}=1032 U L^{-1} and Cx=0.93 kg m^{-3} at t=13 h). The medium containing NaCl showed a GI activity of 850 U L^{-1} which is 82% of the glucose isomerase activity obtained in the medium that does not contain NaCl. As shown in Table 2.13, growth media for *Bacillus antarcticus* all contain 5 kg m^{-3} of NaCl. However, omitting NaCl from the medium increased the glucose isomerase activity.

Ward et al. (1972) investigated the properties of thermophilic *Bacillus stearohermophilus.* Several tests have been performed and no growth is observed by *Bacillus stearothermophilus* in the media containing 3 kg m^{-3} of NaCl. *Bacillus stearothermophilus* belongs to the group V which also comprises *Bacillus thermoantarcticus*. Group V is a phenotypically and phylogenetically coherent group of thermophilic bacilli displaying very high similarity among their 16S rRNA sequences (98±5±99±2%) (Nazina et al., 2001).

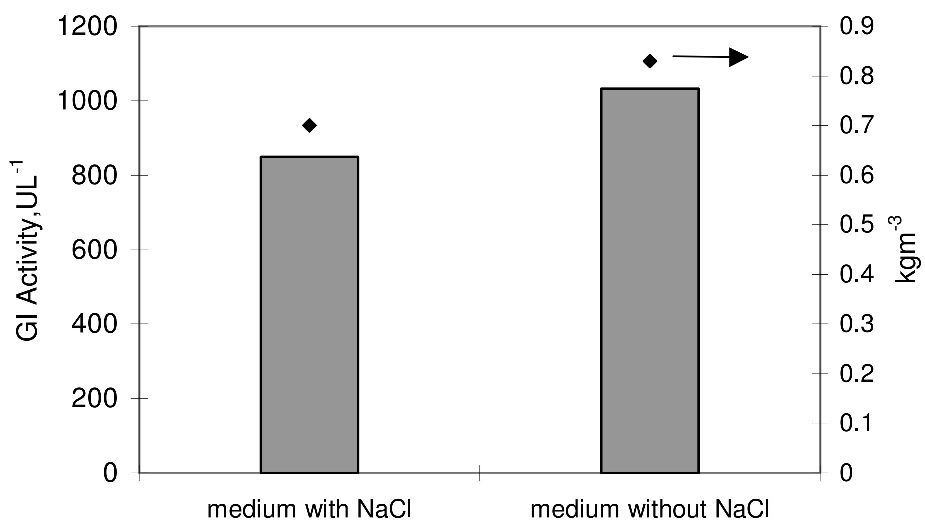

Figure 4.4 The variations in glucose isomerase activity and cell concentration with the NaCl at t=13 h of the cultivation respectively, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

4.1.5 Variation of Glucose Isomerase Activity with time

Before performing the medium design experiments, variation of glucose isomerase activity according to the period of fermentation should be evaluated. To carry out the medium design experiments at the point where glucose isomerase reaches its maximum activity, *Bacillus thermoantarcticus* was examined for 30 hours of cultivation and the response of glucose isomerase activity during this period of bioprocess was investigated.

As seen in Figure 4.5 the maximum activity of glucose isomerase was obtained as 1618 U L^{-1} at 24^{th} hour of cultivation in the medium containing: 10 kg m^{-3} of xylan, 5 kg m^{-3} of yeast extract and 5 kg m^{-3} of ammonium sulfate. As a result, results of the following experiments were all analyzed at the 24^{th} hour of the glucose isomerase fermentation.

The result obtained by Lama et al (2001), who first studied the characterization of glucose isomerase by *Bacillus thermoantarcticus*, shows a similar fashion with the result obtained in the present work. Lama et al. investigated the change of glucose isomerase activity for 48 hours of cultivation and found that the maximum glucose isomerase activity was expressed at approximately 24^{th} hour of fermentation.

Also, intracellular glucose isomerase produced by thermophilic *Bacillus sp. (NCIM 59)* showed its maximum activity at the 24^{th} hour of the fermentation (Chauthaiwale et al., 1994).

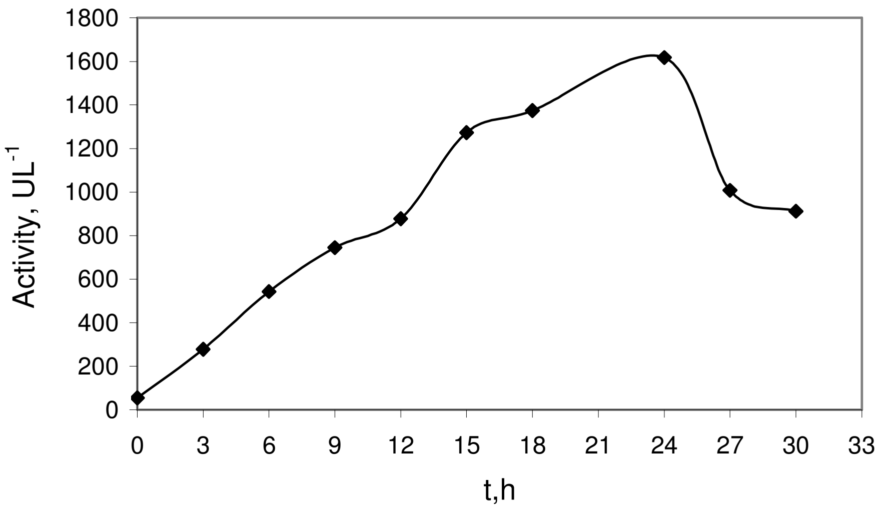

Figure 4.5 The variations in glucose isomerase activity with the cultivation time in the medium containing 10 kgm^{-3} of xylan, 5 kgm^{-3} of yeast extract, 5 kgm^{-3} of ammonium sulfate and 33 μl of salt solution, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

4.2 Medium Design with Response Surface Methodology (RSM)

Previous experiments showed that the medium optimization with RSM should comprise three parameters, namely: xylan, yeast extract and $(NH_4)_2SO_4$. But before RSM was applied, approximate ranges of these three factors were determined by varying one factor at a time while keeping the others constant. An appropriate range for each factor was determined for RSM. The investigated range of independent variables were 0 and 15 kg m^{-3} of xylan concentration, 0 and 8 kg m^{-3} of yeast extract concentration and 0 and 8 kg m^{-3} of $(NH_4)_2SO_4$ concentration. The individual effect of these independent variables on glucose isomerase activity and the RSM experiment were both carried out in laboratory scale bioreactors at conditions: V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

RSM methodology was applied after determination of the appropriate range for each factor. A central composite circumscribed (CCCD) and face-centred design (α=1) with 3 factors was used as the starting point and extra points were added to the experiment plan. There were 20 combinations with 6 central points determined by the program. By omitting 3 central points, 8 more points were added and as a result, 25 total numbers of runs having 22 combinations with 3 replications of the central point were adapted in this study. The independent variables and their levels are presented in Table 4.1. The highest and lowest levels of the interested range for each variable were coded as plus and minus one, respectively, and the center point of the range was coded as zero.

Table 4.1 The production medium variables for three factor three level second order RSM experiments

		Coded Variables		
Independent Variables	*Symbols*	*-1*	*0*	*+1*
Xylan (kg m^{-3})	C_{XYLAN}	7	10	13
Yeast Extract (kg m^{-3})	C_{YE}	3	5	7
$(NH_4)_2SO_4$ (kg m^{-3})	$C_{(NH4)2SO4}$	4	6	8

The second order polynomial model being predicted for glucose isomerase activity, was:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + e$$

Where β_0, β_1. β_2. β_3, β_{12}, β_{13}, β_{23} , β_{11} , β_{22} and β_{23} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively. x_1, x_2, x_3 are independent variables. The coefficients in the above equation were determined by using an algorithm written by MINITAB ® Release 14.

4.2.1 Preliminary experiments (Selection of factor levels)

The individual effect of each parameter on glucose isomerase activity was investigated before an experimental design with RSM was applied to the system. The experiments were performed in laboratory scale bioreactors at conditions: $V=33\ cm^3$, $T=55°C$, $pH_0=6.0$, $N=200\ min^{-1}$.

While the effect of xylan concentration on glucose isomerase activity was investigated in the concentration range $C_{XYLAN}=0\text{-}15\ kg\ m^{-3}$, the yeast extract and $(NH_4)_2SO_4$ concentrations were kept at $C_{YE}=5\ kg\ m^{-3}$ and $C_{(NH4)2SO4}= 5\ kg\ m^{-3}$.As it is shown in Figure 4.6 the maximum GI activity was attained at a xylan concentration of $C_{XYLAN}=12\ kg\ m^{-3}$ ($A_{max}=1578\ U\ L^{-1}$), while GI activity was 1426 and 912 $U\ L^{-1}$ at $C_{XYLAN}=9$ and 15 $kg\ m^{-3}$ respectively. Therefore, 7,10 and 13 kgm^{-3} of xylan were chosen as the lower, middle and upper points respectively for RSM analysis.

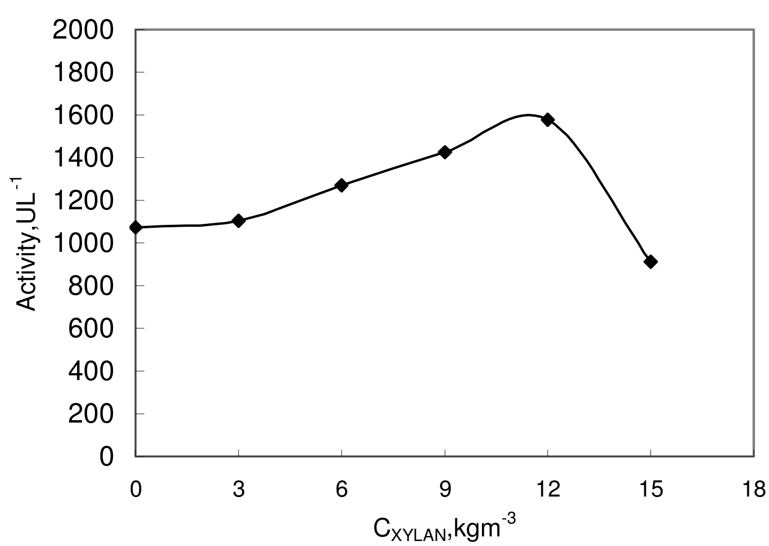

Figure 4.6 The effect of xylan concentration on glucose isomerase activity, $V=33\ cm^3$, $T=55°C$, $pH_0=6.0$, $N=200\ min^{-1}$.

Effect of yeast extract was investigated in the concentration range of C_{YE}=0-8 kg m^{-3}, while the xylan and ammonium sulfate concentrations were kept at C_{XYLAN} = 9 and $C_{(NH4)2SO4}$= 5 kg m^{-3}, respectively. As seen in Figure 4.7 glucose isomerase activity is maximized at the yeast extract concentration of 5 kg m^3 (A_{max}=1425 U L^{-1}) while GI activity was attained as 1335 and 1116 U L^{-1} at C_{YE}=4 and 6 kg m^{-3} respectively. Therefore, 3, 5 and 7 kgm^{-3} of yeast extract concentrations were chosen as the lower, middle and upper points respectively for RSM analysis.

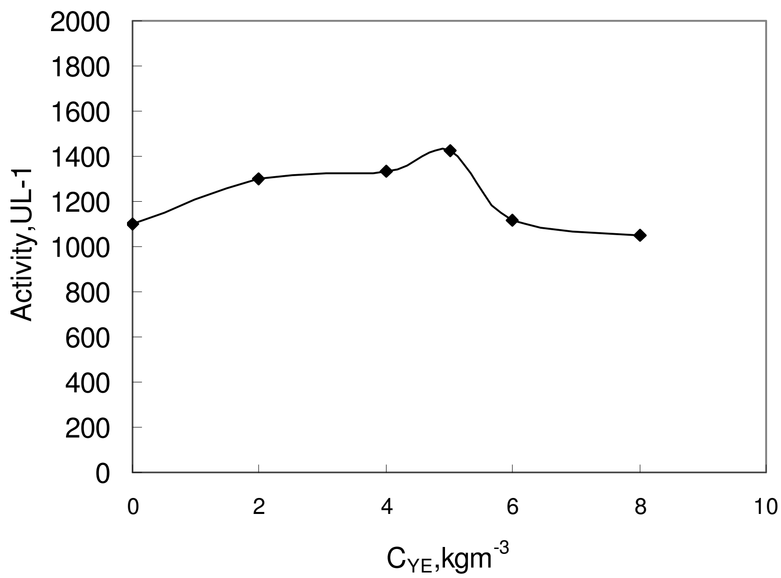

Figure 4.7 The effect of yeast extract concentration on glucose isomerase activity, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

Ammonium sulfate, being the last component of the medium designed, affected glucose isomerase activity as shown in Figure 4.8. During this analysis, the concentrations for xylan and yeast extract were kept at 9 and 5 kg m^{-3} respectively. The highest glucose isomerase activity was obtained at ammonium sulfate concentration of $C_{(NH4)2SO4}$=6 kg m^{-3} (A_{max}=1610 U L^{-1}). The glucose isomerase activities at $C_{(NH4)2SO4}$=4 and 8 kg m^{-3} were obtained as 1490 and

1585 U L^{-1} respectively. Factor levels for ammonium sulfate concentrations were selected as 4, 6 and 8 kg m^{-3}, being the lower, middle and upper limits respectively.

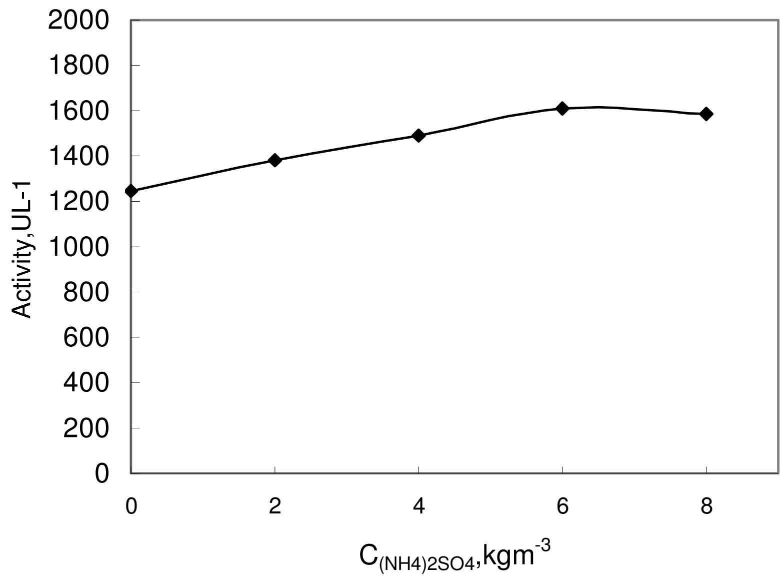

Figure 4.8 The effect of ammonium sulfate concentration on glucose isomerase activity, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

4.2.2 Response Surface Analysis

As the factor levels for each component of the medium design were selected, a face-centered, central composite circumscribed design with three factors was employed (Table 4.2). Three replicates were taken at the design center, (10,5,6) and with addition of 8 points the total number of observations was 25 as mentioned previously. The main advantage of the design is that it enables to study three variables simultaneously in a single experiment.

Table 4.2 Face centered cube design arrangement and responses

RUNS	**INDEPEPENDENT VARIABLES**			**RESPONSE, GI Activity, U L^{-1}**
	C_{XYLAN} kg m^{-3}	C_{YE} kg m^{-3}	$C_{(NH4)2SO4}$ kg m^{-3}	
1	7	3	4	1145.56
2	13	3	4	1393.85
3	7	7	4	1381.56
4	13	7	4	1457.77
5	7	3	8	1101.31
6	13	3	8	1347.14
7	7	7	8	1320.10
8	13	7	8	1425.81
9	7	5	6	1450.39
10	13	5	6	1482.35
11	10	3	6	1460.22
12	10	7	6	1543.81
13	10	5	4	1482.35
14	10	5	8	1570.85
15	8.5	4	5	1452.85
16	11.5	4	5	1526.60
17	8.5	6	5	1543.81
18	11.5	6	5	1629.85
19	8.5	4	7	1506.93
20	11.5	4	7	1548.72
21	8.5	6	7	1573.31
22	11.5	6	7	1600.00
23	10	5	6	1610.18
24	10	5	6	1600.00
25	10	5	6	1625.00

The data obtained for glucose isomerase activity from the 25 experimental points were used for statistical analysis to optimize the medium components. The design matrix and the fitness of the each term were analyzed by means of ANOVA (Table 4.3). The regression coefficients for each term were given in Table 4.4. The second-order polynomial equation for the glucose isomerase activity was found to be:

$$\text{GI Activity}(U\ L^{-1}) = -1727.80 + 339.14C_{XYLAN} + 332.81C_{YE} + 205.52C_{(NH4)2SO4} - 14.31\ C_{XYLAN}2 - 23.28C_{YE}2 - 17.13\ C_{(NH4)2SO4}2 - 6{,}14\ C_{XYLAN}\ C_{YE} + 0.08\ C_{XYLAN}\ C_{(NH4)2SO4} - 0.64C_{YE}\ C_{(NH4)2SO4}$$

The fitness of the model (R^2) was calculated to be 0.974, meaning the variability of the model response could be explained by the proposed model. The adjusted R^2 value was determined as 0.915 and the lack of fitness was determined as 0.084 ($p < 0.05$), so the lack of the model was insignificant.

Table 4.3 Analysis of Variance (ANOVA) for the selected quadratic model

Model	**Degrees of Freedom**	**Sum of Squares**	**Mean Sum of Squares**	**F**	**p < 0.05**
Regression	9	417490	46387.8	29.54	0.000
Linear	3	195812	65270.8	41.56	0.000
Square	3	290947	96982.3	61.76	0.000
Interaction	3	11576	3858.6	2.46	0.103
Residual Error	13	23556	1570.4		
Lack of Fit	15	23240	1787.7	11.31	0.084
Pure Error	2	316	158.0		
Total	24				

Table 4.4 Regression coefficients of predicted polynomial model for response (Y)

Term	**Coefficient**	**t**	**$p < 0.05$**
Constant	-1727.8	-5.880	0.000
Linear			
C_{XYLAN}(kg m^{-3})	339.14	6.028	0.000
C_{YE}(kg m^{-3})	332.81	4.932	0.000
$C_{(NH4)2SO4}$(kg m^{-3})	205.52	2.652	0.018
Quadratic			
C_{XYLAN} (kgm^{-3})* C_{XYLAN} (kg m^{-3})	-14.31	-5.371	0.000
C_{YE} (kg m^{-3})* C_{YE} (kg m^{-3})	-23.28	-3.884	0.001
$C_{(NH4)2SO4}$ (kg m^{-3})* $C_{(NH4)2SO4}$ (kg m^{-3})	-17.13	-2.859	0.012
Interactions			
C_{XYLAN} (kg m^{-3})* C_{YE} (kg m^{-3})	-6.14	-2.708	0.016
C_{XYLAN} (kg m^{-3})* $C_{(NH4)2SO4}$ (kg m^{-3})	0.08	0.036	0.971
C_{YE} (kg m^{-3})* $C_{(NH4)2SO4}$ (kg m^{-3})	-0.64	-0.187	0.854

When the interaction terms are considered (Table 4.4), the interaction of $(NH_4)_2SO_4$ with xylan and yeast extract seems insignificant. However, the utilization of $(NH_4)_2SO_4$ in the fermentation medium increased glucose isomerase activity remarkably depending on the sulfate metabolism of thermophiles. So, it can be concluded the interaction terms calculated by the software Minitab contradicts with the experimental data obtained in the previous analysis.

The plot of experimental values of GI Activity versus those calculated from the equation (4.1) indicated a very good fit for GI Activity with a correlation coefficient of 0.947.

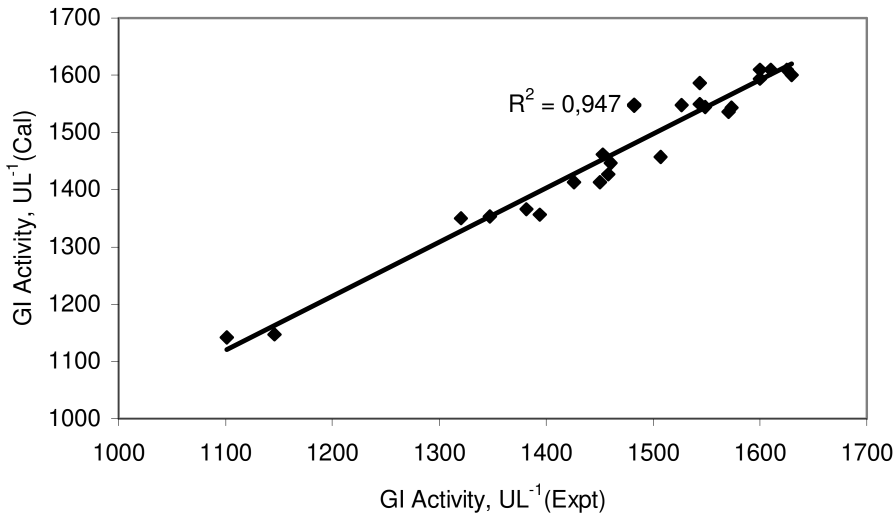

Figure 4.9 Correlation of calculated versus experimental GI Activity

The effects of the independent variables on glucose isomerase activity were represented via response surface plots (Figure 4.10, 4.11 and 4.12).

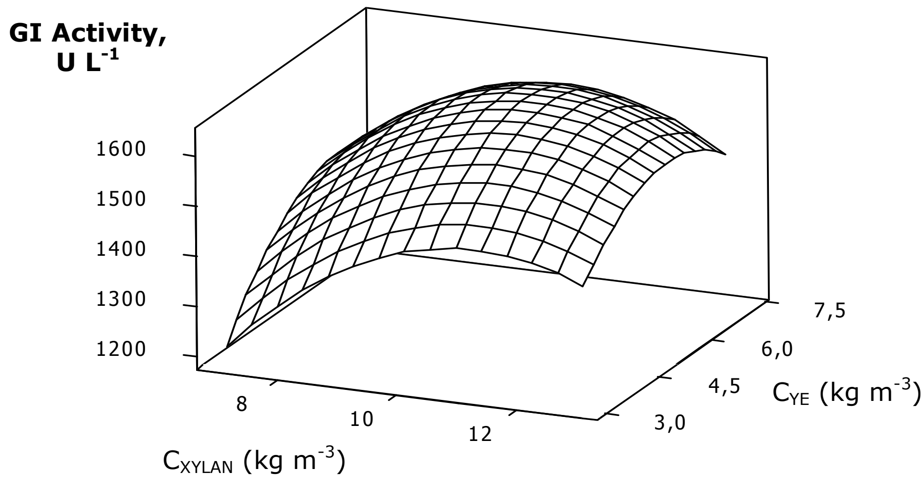

Figure 4.10 Response surface plot for xylan and yeast extract interaction at ammonium sulfate concentration of 6 kg m^{-3}, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

It has been verified that the enzyme has similar affinity for the three variables, xylan, yeast extract and ammonium sulfate. The optimized glucose isomerase activity was found by taking the derivation of the second-order polynomial equation with respect to xylan, yeast extract and ammonium sulfate one by one and equating each equation to zero. After carrying out the calculations, optimized glucose isomerase activity was found as 1630 UL^{-1} at concentrations: 10,64, 5,66 and 5,92 kgm^{-3} of xylan, yeast extract and ammonium sulfate respectively.

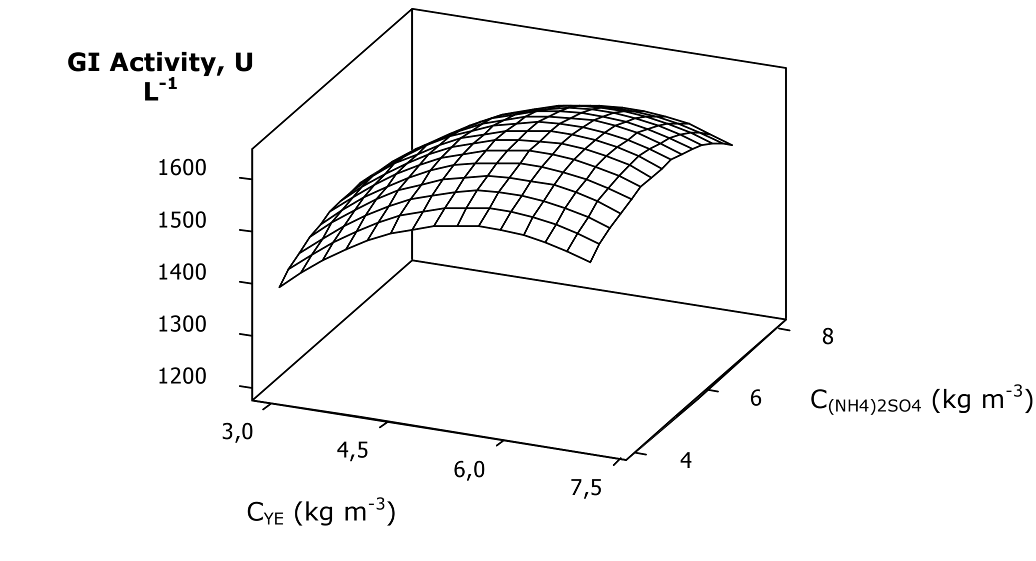

Figure 4.11 Response surface plot for yeast extract and ammonium sulfate interaction at xylan concentration of 10 kg m^{-3}, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

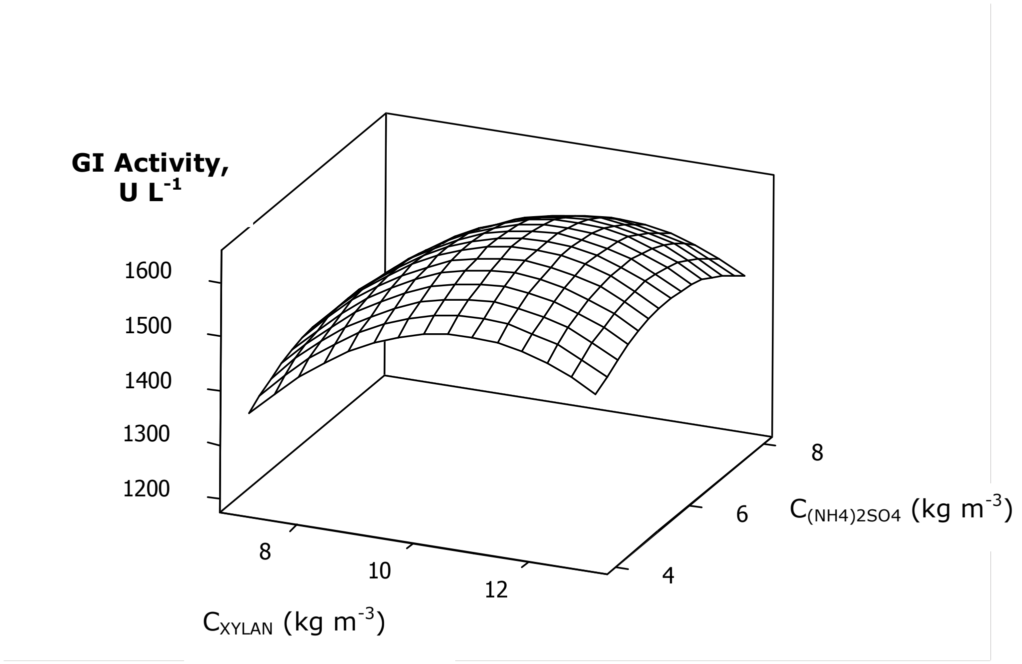

Figure 4.12 Response surface plot for xylan and ammonium sulfate interaction at yeast extract concentration of 5 kg m^{-3}, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

The interaction of xylan, yeast extract and ammonium sulfate can be also observed from the contour plots (Figures 4.13, 4.14, 4.15). In a contour plot, each contour represents a specific value for the height of the surface.

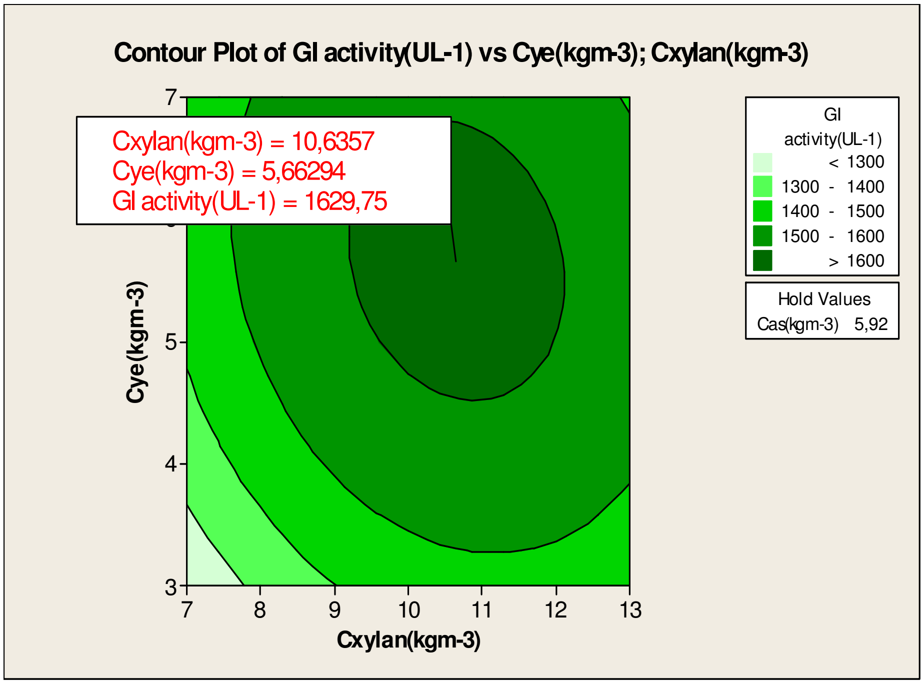

Figure 4.13 Contour plot for xylan and yeast extract interaction at ammonium sulfate concentration of 5.92 kg m^{-3}, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

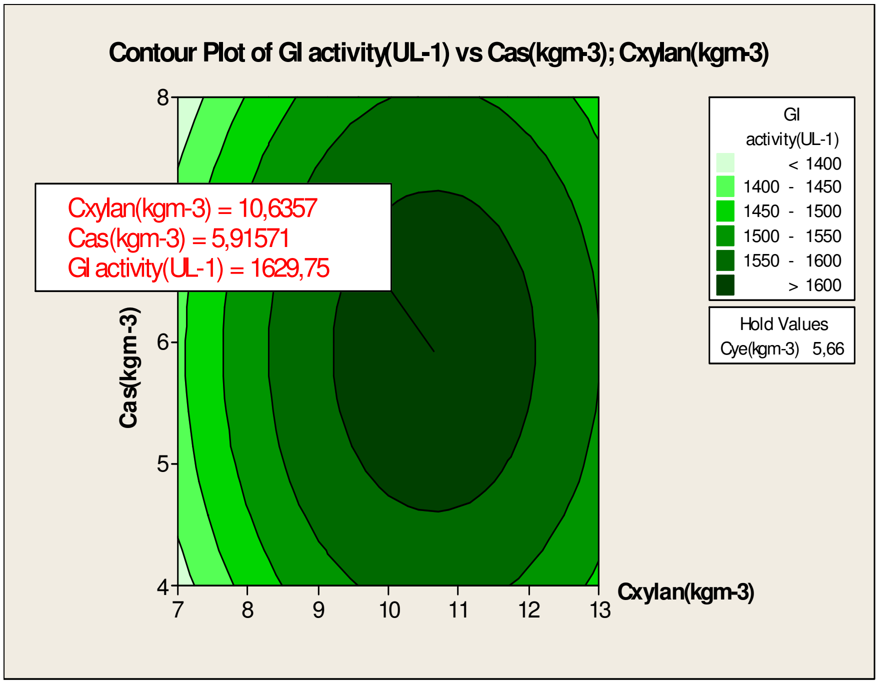

Figure 4.14 Response surface plot for xylan and ammonium sulfate interaction at yeast extract concentration of 5.66 kg m^{-3}, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

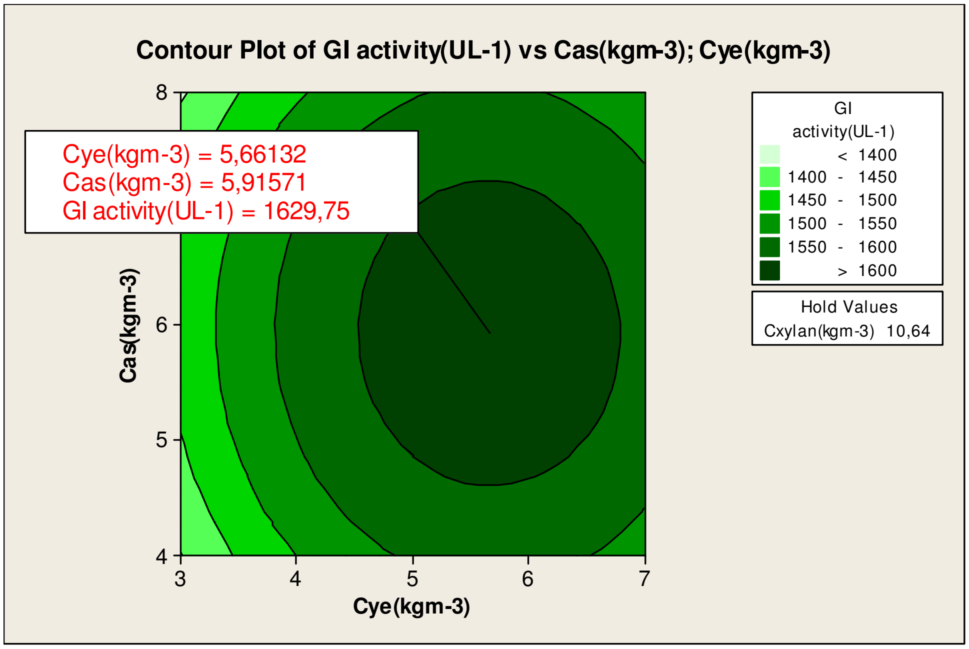

Figure 4.15 Contour plot for yeast extract and ammonium sulfate interaction at xylan concentration of 5.66 kg m^{-3}, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

4.3 Optimized medium

As a consequence, among the investigated media, the highest glucose isomerase activity was obtained as 1630 UL^{-1}, in the medium containing 10,64 kg m^{-3} birchwood xylan, 5,66 kg m^{-3} yeast extract, 5,92 kg m^{-3} $(NH_4)_2SO_4$, and 0.25 kg m^{-3} MgSO4.7H2O, 0.001 kg m^{-3} FeSO4.7H2O, 0.001 kg m^{-3} ZnSO4.7H2O , 0.000075 kg m^{-3} MnSO4. H2O, 0.00001 kg m^{-3} CuSO4.5H2O. The activity obtained in the optimized medium at the 24th hour of the fermentation process was 1.6 fold higher than the activity obtained in the reference production medium (A_{ref}=1020 UL^{-1}). Before investigating the oxygen transfer parameters on the pilot scale bioreactor, the effect of initial pH, xylan types and base used to adjust the pH of the fermentation medium on glucose isomerase activity were evaluated. Also effects of various carbon sources: glucose, fructose and sucrose on glucose isomerase and xylanase activity were determined by using the designed medium.

4.3.1 Effect of initial pH on Glucose Isomerase Activity

In this part, the effect of initial pH of the fermentation on glucose isomerase activity and cell concentration was investigated. The initial pH of the fermentation medium was adjusted to pH=6 with 5 M NaOH in the previous experiments carried out in laboratory scale bioreactor. Using the designed medium containing, 10,64 kg m^{-3} birchwood xylan, 5,66 kg m^{-3} yeast extract, 5,92 kg m^{-3} $(NH_4)_2SO_4$, 0.25 kg m^{-3} $MgSO_4.7H_2O$, 0.001 kg m^{-3} $FeSO_4.7H_2O$, 0.001 kg m^{-3} $ZnSO_4.7H_2O$, 0.000075 kg m^{-3} $MnSO_4.H_2O$, 0.00001 kg m^{-3} $CuSO_4.5H_2O$ at conditions: V=33 cm^3, T=55°C, N=200 min^{-1}, the effect of initial pH was investigated between pH=5-9 at the 24th hour of the cultivation.

The pH of the media was adjusted by NaOH. As seen in Figure 4.16, the optimum initial pH for glucose isomerase activity was found as pH=6. The glucose isomerase activity was found as 1630 U L^{-1} (Figure 4.16) at pH_0=6. Also the highest cell concentration at the 9th hour of the cultivation was attained as 0.75 kg m^{-3} at pH_0=6 (Figure 4.17).

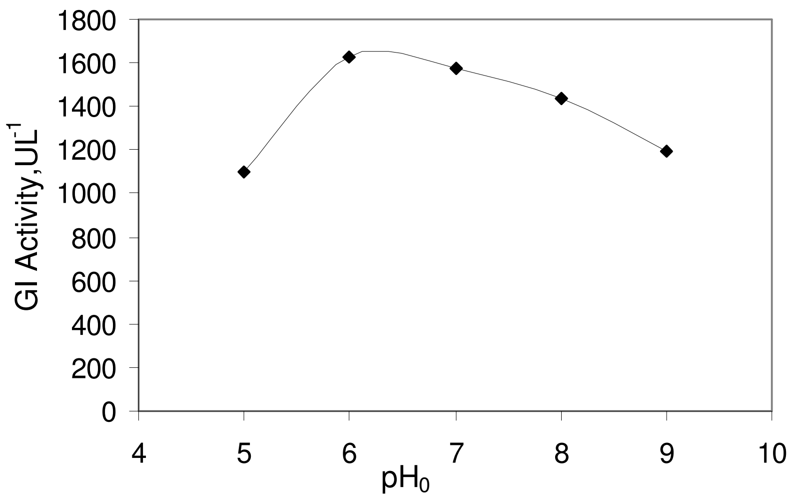

Figure 4.16 The variation of glucose isomerase activity with the initial pH of the medium at the 24th hour of the cultivation in the medium containing 10,64 kg m^{-3} of xylan, 5,66 kg m^{-3} of yeast extract, 5,92 kg m^{-3} of ammonium sulfate, V=33 cm^3, T=55°C, N=200 min^{-1}.

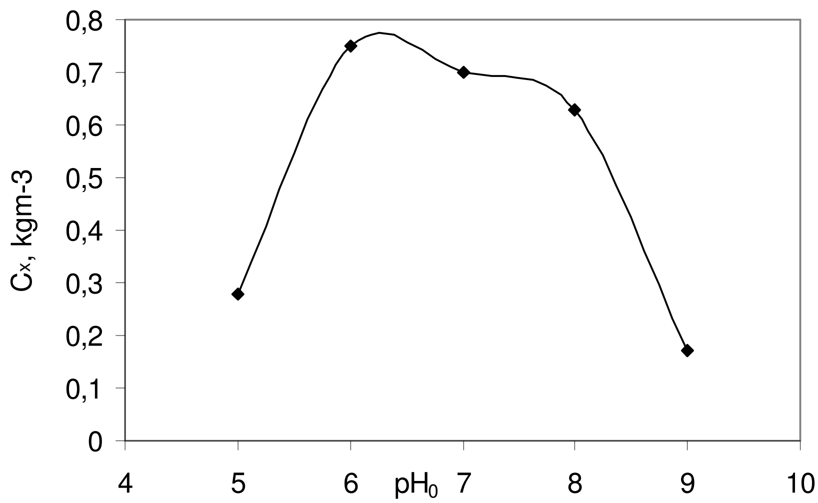

Figure 4.17 The variation of cell concentration with the initial pH of the medium at the 9th hour of the cultivation in the medium containing 10,64 kg m^{-3} of xylan, 5,66 kg m^{-3} of yeast extract, 5,92 kg m^{-3} of ammonium sulfate, V=33 cm^3, T=55°C, N=200 min^{-1}.

The result is in accordance with that found by Nicolaus et al (1996). They evaluated the physicochemical properties of *Bacillus thermoantarcticus* and found the optimal pH for microbial growth is 6.0.

Chen et al. (1979) investigated the effect of initial pH of the fermentation medium on glucose isomerase production by *Streptomyces flavogriseus.* After 48 hours of cultivation the maximum GI activity was attained at pH=6.0 to 8.5. So pH=7.0 was selected as the optimal initial pH for enzyme production.

4.3.2 Effect of xylan types on Glucose Isomerase Activity

In this part, the effects of xylan types namely, i.e., birchwood, beechwood, oat spelt xylan on glucose isomerase activity were investigated. In the previous experiments, birchwood xylan was used as the sole carbon source for glucose isomerase production from *Bacillus thermoantarcticus*.

As seen in Figure 4.18, the medium containing birchwood xylan attained 1630 UL^{-1} of glucose isomerase activity. However, the medium containing oat spelt and beechwood xylan attained glucose isomerase activities of 1372 and 1308 U L^{-1} which are 84% and 80% of the glucose isomerase activity obtained in the medium containing birchwood xylan as a carbon source.

Effects of birchwood xylan and oat spelt xylan were investigated by *Streptomyces sp. EC 10.* Glucose isomerase activity in the medium containing birchwood xylan and oat spelt xylan were 8,78 and 6.65 U mg^{-1} protein respectively (Belfaquih et al., 2000). Use of birchwood xylan increased the GI activity 1.3 fold. In the present analysis, use of birchwood xylan increased GI activity 1.2 fold which is close to the value found in the literature study.

4.3.3 Effect of the base used to adjust initial pH of the medium

In order to adjust the pH of the medium, effects of NaOH and KOH were investigated at t=24 h of the cultivation. In the previous experiments, the pH of the fermentation medium was adjusted to pH=6 by NaOH.

The highest GI activity was obtained with NaOH as, 1630 U L^{-1} (Table 4.5). Therefore for the pH adjustment of the bioreactor experiments, NaOH was selected as it has been used in the previous experiments.

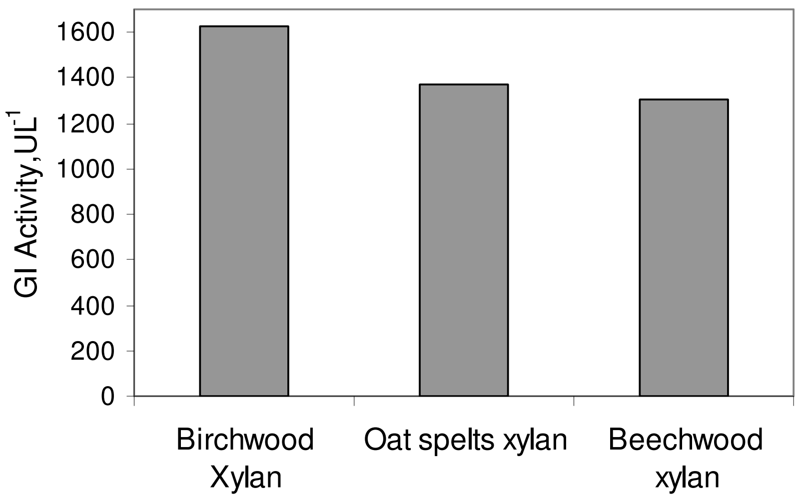

Figure 4.18 The variation of glucose isomerase activity with xylan types, T=55°C, pH_0 =6.0, N=200 min^{-1}: medium containing 10,64 kg m^{-3} of birchwood, beechwood and oat spelts xylan, 5,66 kg m^{-3} of yeast extract, 5,92 kg m^{-3} of ammonium sulfate, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

Table 4.5 Effect of base used to adjust initial pH of the production medium containing 10,64 kg m^{-3} of birchwood xylan, 5,66 kg m^{-3} of yeast extract, 5,92 kg m^{-3} of ammonium sulfate, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

Base	**Glucose Isomerase Activity**
NaOH	1630 U L^{-1}
KOH	1276 U L^{-1}

4.3.4 Effects of glucose, fructose and sucrose on glucose isomerase and xylanase activity

The aim of this analysis is to compare the glucose isomerase and xylanase activities of mediums containing glucose, fructose, sucrose and xylan. For this reason, again the designed medium was used and 10.64 kg m^{-3} of glucose, sucrose and fructose were replaced instead of xylan. Figure 4.19 shows the effect of carbon sources on glucose isomerase activity at the 24^{th} hour of cultivation respectively. Figure 4.20 shows the variation of cell concentration with the effect of different carbon sources that was attained at the 9^{th} hour of the cultivation.

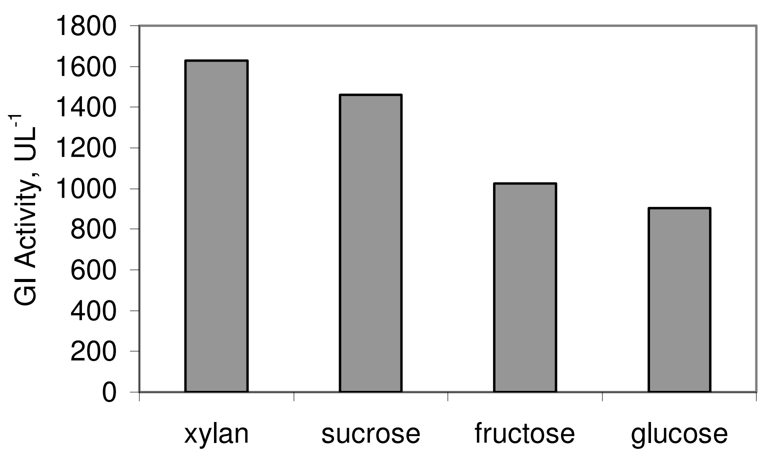

Figure 4.19 Effects of carbon sources on the production of glucose isomerase by *Bacillus thermoantarcticus* at the 24^{th} hour of cultivation, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

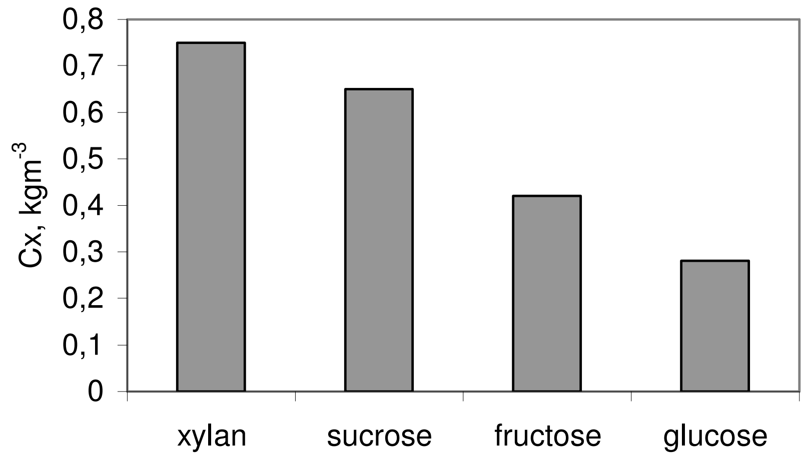

Figure 4.20 Effects of carbon sources on cell concentration at the 9th hour of cultivation, V=33 cm^3, T=55°C, pH_0=6.0, N=200 min^{-1}.

As seen in Figure 4.19, when sucrose was replaced with xylan, GI activity was obtained as 1460 U L^{-1}. This value is close to the GI activity obtained in the medium containing xylan (1660 U L^{-1}). This indicates that sucrose, which is again a cheaper inducer for glucose isomerase, can be used instead of xylan for the production of glucose isomerase from *Bacillus thermoantarcticus*. Also, medium containing xylan attained a cell concentration as 0.75 kg m^{-3}, medium containing sucrose showed 0.65 kg m^{-3} of cell concentration (Figure 4.20).

However, when xylanase activities were considered, it is more beneficial to use xylan as the sole carbon source. Because xylan containing medium showed a higher xylanase activity when compared to that of sucrose containing medium (Figure 4.21). Production of both glucose isomerase and xylanase from *Bacillus thermoantarcticus* is an advantage when the collaborative mechanism of these two enzymes is considered.

The increase in the production of xylanase results in the efficient digestion of xylan. Efficient digestion of xylanase increases the cell concentration and consequently increases glucose isomerase production. Xylanase also gains its importance in the digestion of xylan from agricultural by-products. Use of cheaper carbon sources provides the suitability of the fermentation conditions for commercial enzyme production. For example, production xylan from agricultural by-products such as cotton waste is beneficial for commercial glucose isomerase production. In the cotton industry, the ginning process generates a considerable amount of waste. About 18-65 kg of cotton gin waste is produced per 100 kg of cotton and xylan content of cotton gin waste is 9.41% (Thomasson, 1990 and Jeoh et al., 2001). As a result, utilization of cheap xylan sources and efficient xylanase production makes glucose isomerase production from *Bacillus antarcticus* a feasible bioprocess for industrial applications.

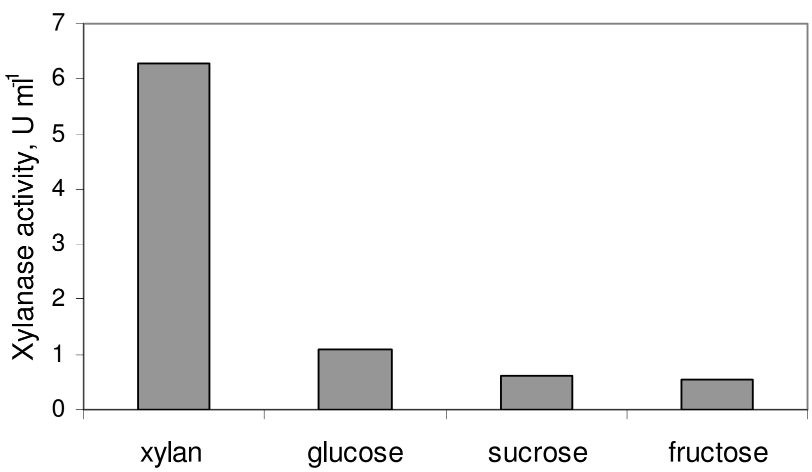

Figure 4.21 Effects of carbon sources on the production of xylanase by *Bacillus thermoantarcticus* at the 24th hours of cultivation, V=33 cm^3, T=55°C, pH$_0$=6.0, N=200 min^{-1}.

As shown in Figure 4.21, xylan containing medium possesses a xylanase activity of 6.30 U ml^{-1}. This is approximately 10 fold higher than that obtained by the medium containing sucrose as carbon source (0.62 U ml^{-1}). These results are in accordance with the results obtained by Lama et al., (2004) in which characterization of thermostable xylanase by *Bacilus thermoantarcticus* was investigated. They evaluated the effect of various carbon sources on xylanase activity and found that the highest xylanase activity was found in the medium containing 5 kg m^{-3} of xylan as 2.1 U mg $protein^{-1}$. Using sucrose instead of xylan as the carbon source resulted a xylanase activity of 0.17 U mg $protein^{-1}$ which is about 8% of the xylanase activity obtained in the medium containing xylan.

4.4 Bioreactor Experiments

Using the optimized medium with *Bacillus thermoantarcticus* DSMZ 9572, the fermentation and oxygen transfer characteristics of the bioprocess were investigated in the 3 dm^3 pilot scale batch bioreactor (Braun CT2-2) equipped with temperature, pH, foam, air inlet and stirring rate controls with V_R=1.65 dm^3 working volumes. Throughout the process cell, glucose isomerase activity, amino acid and organic acid concentrations, dissolved oxygen, pH, oxygen uptake rate, overall oxygen transfer coefficient, maintenance coefficient for oxygen, specific cell growth rate, yield coefficients were determined. In the pilot scale bioreactor, first of all the effects of uncontrolled and controlled pH on glucose isomerase production were evaluated and then the effects of oxygen transfer were carried out at three different conditions: air inlet rates of Q_0/V_R=0.5 vvm, and the agitation rates of N=300, 500, 750 min^{-1}.

The effect of pH on glucose isomerase activity was evaluated at pH=6 uncontrolled and controlled operations at air inlet rate of Q_0/V_R=0.5 vvm and agitation rate of N=500 min^{-1} as optimum initial pH for cell growth was found to be 6 at shake-bioreactor. Controlled pH experiments were carried out with 5M NaOH and 5M H_3PO_4. The abbreviation of the bioreactor operation conditions were given in Table 4.6.

Table 4.6 Oxygen transfer conditions and pH conditions and their abbreviations

Oxygen Transfer Conditions		**pH strategy**	**Abbreviation**
N, min^{-1}	Q_0/V_R, vvm		
300	0.5	pH_c=6.0	LOT- pH_c
500	0.5	pH_{uc}=6.0	MOT- pH_{uc}
500	0.5	pH_c=6.0	MOT- pH_c
750	0.5	pH_c=6.0	HOT- pH_c

The oxygen transfer condition Q_0/V_R = 0.5 vvm and N=300 min^{-1}, was named as 'low oxygen transfer' condition (LOT); Q_0/V_R = 0.5 vvm and N=500 min^{-1} was named as 'medium oxygen transfer' condition (MOT); and Q_0/V_R = 0.5 vvm, N=750 min^{-1} was named as 'high oxygen transfer' condition (HOT).

4.4.1 The Effect of pH on Glucose Isomerase Production

This section focuses on the effect of pH control on glucose isomerase production in laboratory scale bioreactor. pH control was evaluated at oxygen transfer condition: Q_0/V_R = 0.5 vvm and N=500 min^{-1}. As the highest GI activity of *Bacillus thermoantarcticus* was obtained at the initial pH of 6, pH control was carried at pH 6.0 throughout the process. The influence of pH_C=6.0 and pH_{UC}=6.0 operations on cell growth, glucose isomerase activity, amino acid and organic acid concentrations, dissolved oxygen, pH, oxygen uptake rate, overall mass transfer coefficient, maintenance coefficient for oxygen, specific growth rate, yield coefficients were investigated.

4.4.1.1 The variation of pH throughout the bioprocess

The variations in pH with cultivation time are given in Figure 4.22. Under the influence of the operational conditions pH and oxygen transfer including the designed medium, the cell pumps H+ ions in or out through the membrane. Whereupon, the pH of the environment has a natural tendency to change along with the bioprocess despite the fact that intracellular pH is kept constant (Çalık et al., 2003). As it is the case in the operation MOT-pHuc, pH was monitored frequently and observed to vary substantially. In the first 4 hours of the bioprocess pH increased to pH=7.3 (t=3.5 h) and between t=3.5-7 h a decrease in pH is observed. This decrease could outcome from consumption of the ammonium sulfate present in the designed medium of *Bacillus thermoantarcticus.* As ammonia was utilized, hydrogen ions were released into the medium, resulting a decrease in pH of the medium (Schuler and Kargı, 2002). Also, amino acid production in the first 6 hours of the bioprocess MOT-pHuc could explain this decrease. Because first 6 hours of the process is the period where the highest amino acid synthesis is observed (Table 4.12). After the 7th hour of the fermentation, pH increased and stayed constant at around pH=7.5.

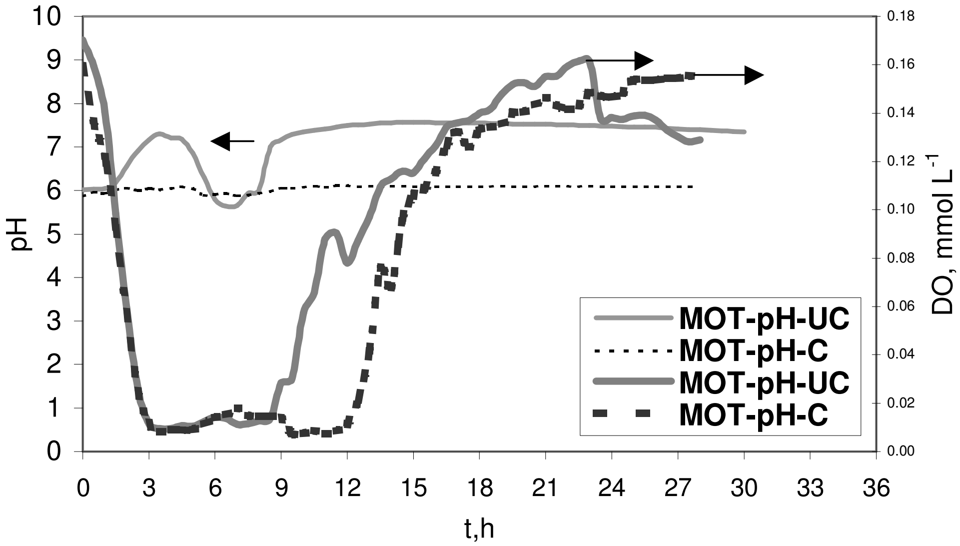

Figure 4.22 The variation of pH and dissolved oxygen concentration with the cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

4.4.1.2 Dissolved Oxygen Profiles

The variations in dissolved oxygen concentration with cultivation time, and pH strategy are given in Figure 4.22. Solubility of oxygen, C_{O2}^*, at the beginning and at the end of each experiment at 55°C were measured. They were found as 0,18 and 0,11 mmol L^{-1} respectively.

C_{DO} depends on the extent of the oxygen transfer rate to the media and the oxygen uptake rate of the cells. It varied with the cultivation time depending on the growth status of the cells (Calik et al.,2000). The relation between the dissolved oxygen concentration in the fermentation broth and the cell growth is discussed in more details in Section 4.4.1.3.

Dissolved oxygen concentrations of MOT-pHuc and MOT-pHc decreased to 0.011 and 0.012 mol m^{-3} at t=3 h of the bioprocess. This sharp decrease arises from rapid uptake of dissolved oxygen by the microorganism. As seen in Figure 4.22, dissolved oxygen profiles of MOT-pHuc and MOT-pHc were alike. However, uncontrolled-pH operation generally resulted with lower dissolved oxygen concentrations. Also, a significant difference of dissolved oxygen profiles is observed after t=9 h of the bioprocesses. While at MOT-pHuc, dissolved oxygen concentration increased after t=9 h, it increased after t=12 h at MOT-pHc.

4.4.1.3 Cell Growth Profiles

The variations in the cell concentration with cultivation time and pH strategy are given in Figure 4.23.

As seen in Figure 4.23, between t=0-6 h of the cultivation, cell profiles for uncontrolled-pH and controlled-pH were alike. However, when the variation of cell concentration throughout the whole bioprocess uncontrolled-pH operation is slightly favorable for cell formation. Cells generally reached to stationary phase after t=12 h. The highest cell concentration for operations MOT-pHuc and MOT-pHc were obtained as 2.26 and 2.05 kg m^{-3} at t=15 h and 16 h of the bioprocesses respectively.

The variation of cell concentration with the cultivation time should be related to the dissolved oxygen concentration of the fermentation broth. In a typical batch culture, the dissolved oxygen concentration decreases in the media along with the growth time, until the microorganism reaches the stationary phase and then the dissolved oxygen concentration increases. This evolution can be observed in many cultures of bacteria, yeast, and fungi.

Between t=0-9 h and 0-12 h of the uncontrolled and controlled-pH operations, cell concentration increased from to C_x=2.21 kg m^{-3} and C_x=2.03 kg m^{-3} respectively. Between t=0-9 h and 0-12 h dissolved oxygen concentration decreased from 0.17 to 0.028 and 0.159 to 0.011 mmol L^{-1} at uncontrolled and controlled-pH operations respectively due to the cell growth during the bioprocesses.

However after cell growth reached stationary phase, dissolved oxygen concentration increased that cells did not consume oxygen as much as they consumed in the growth phase. In the uncontrolled-pH operation cell growth reached its stationary phase at t=9 h (C_x=2.21 kg m^{-3}), meaning that its oxygen demand decreased resulting an increase in dissolved oxygen concentration from 0.028 to 0.115 mmol L^{-1} between t=9-15 h of the bioprocess. In the pH controlled operation, cells reached stationary phase at t=12 h of the bioprocess (C_x=2.03 kgm^{-3}) which explains the decrease in oxygen demand and hence the increase in dissolved oxygen in the fermentation broth from 0.011 to 0.115 mmol L^{-1} between t=9-16 h of the bioprocess.

As a result, when dissolved oxygen and cell concentration profiles were compared, results were in accordance.

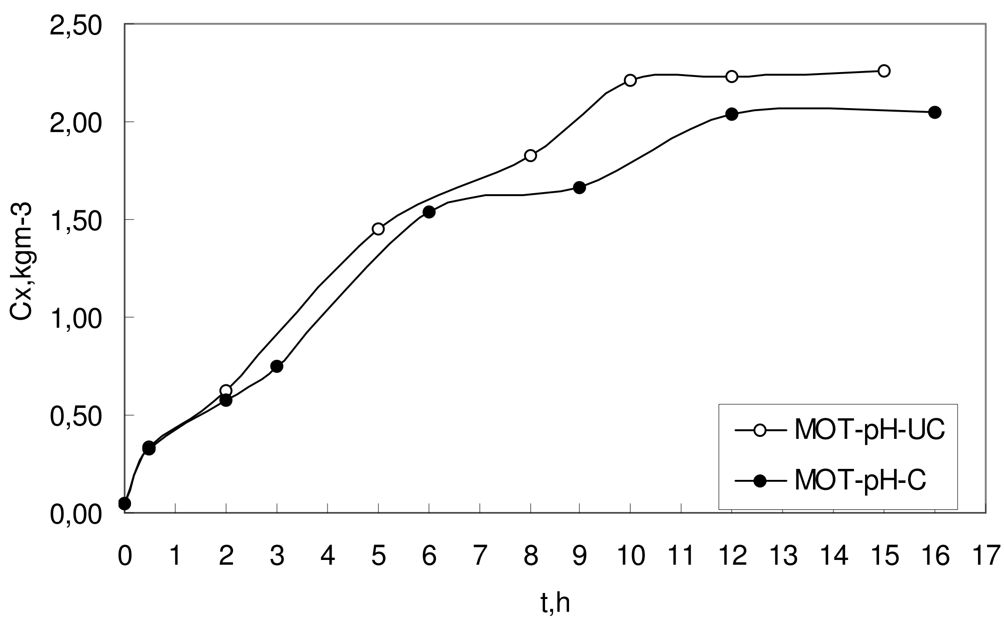

Figure 4.23 The variations in the cell concentration with the cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

4.4.1.4 Glucose Isomerase Activity Profiles

The variations of glucose isomerase activity with the cultivation time and pH strategy are given in Figure 4.24.

In this study, previous analyses were carried out by using the volumetric activity defined for glucose isomerase. Volumetric activity for glucose isomerase was defined as one unit of glucose isomerase activity per liters (U L^{-1}) of sample taken from the fermentation broth during the operation. Glucose isomerase volumetric activity increased with the cultivation time reaching the highest value for both operations at t=24 h. The highest volumetric activity was achieved as 1838 U L^{-1} at t=24 h of MOT-pHuc in which the highest cell concentration was attained (Cx=2.26 kg m^{-3}). MOT-pHc exhibited slightly lower volumetric GI activity profile and attained a glucose isomerase volumetric activity of 1814 U L^{-1} at t=24 h (Cx=2.05 kg m^{-3}) in which a lower cell concentration profile was obtained. On the other hand, when specific glucose isomerase activities which was defined as one unit of glucose isomerase activity per gram of microorganism (U g^{-1}) were compared, MOT-pHc possessed a higher specific glucose isomerase activity profile than the specific glucose isomerase activity profile obtained at MOT-pHuc. Although, GI volumetric activity profile was higher in uncontrolled pH operation, specific GI activity profile was higher in controlled pH operation. The highest specific GI activities were obtained as 885 U g^{-1} DW at t=24 h of MOT-pHc and 814 U g^{-1} at t=24 h of MOT-pHuc.

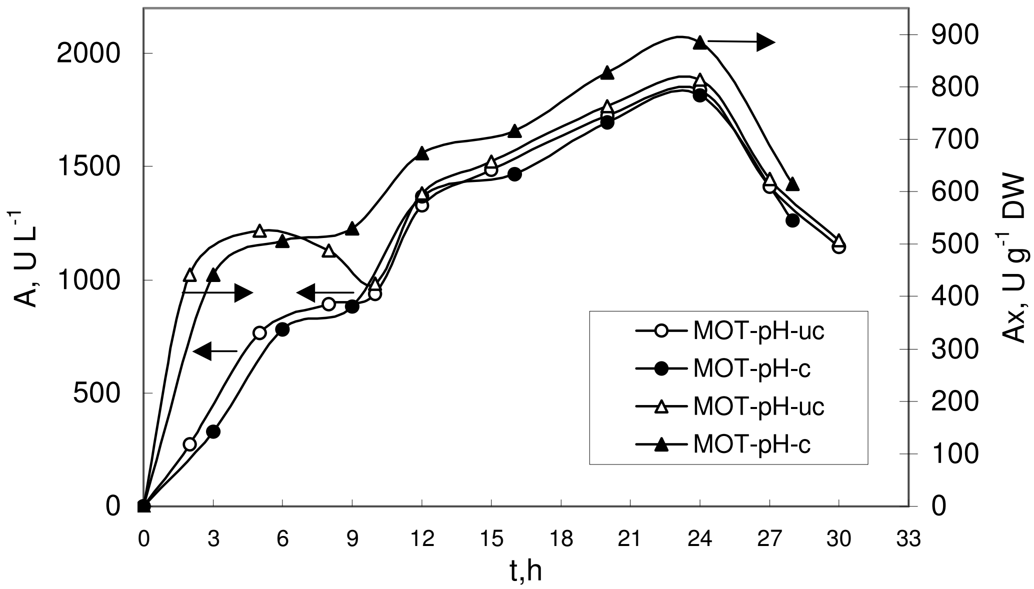

Figure 4.24 The variations in the glucose isomerase volumetric (UL^{-1}) and specific activity ($U\ g^{-1}$ DW) with the cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

4.4.1.5 Xylanase Activity Profiles

The variations of xylanase activity with the cultivation time and pH strategy are given in Figure 4.25.

In the section, materials and method, xylanase volumetric activity (U ml^{-1}) is defined as 1 μmol of reducing sugars (xylose equivalent) released in 1 minute under the reaction conditions defined by Lama et al., (2004). As seen in Figure 4.25, uncontrolled pH operation attained a higher volumetric xylanase activity profile and the highest xylanase activity was obtained as 9.3 U ml^{-1} at t=24 h of the uncontrolled pH operation in which the highest glucose isomerase volumetric activity was achieved at t=24 h (1838 U L^{-1}). The highest xylanase activity attained at controlled pH operation was 8.6 U ml^{-1} at t=24 h. While the highest specific xylanase acitivity was attained as 4.2 U mg-1 DW at t=24 h of controlled pH operation, it was attained as 4.1 U mg-1 at t=24 h of uncontrolled pH operation.

Xylanase production from *Bacillus thermoantarcticus* was first and only studied by Lama et al., (2004). Although Lama et al. investigated the physicochemical properties of xylanase from *Bacillus thermoantarcticus*, they did not evaluate the variation of the enzyme activity with cultivation time. Some literature studies concerning the variation of xylanase with cultivation time are given below. The common point in these literature studies is that the authors analyzed the production of both glucose isomerase and xylanase and they achieved the highest glucose isomerase and xylanase activities at the same period of cultivation as it is obtained in the present study.

Teeradakorn et al., (1998) investigated the variation of xylanase activity from *Streptomyces fusant D3* for 50 hours of cultivation and obtained the highest xylanase activity in a medium containing 29 kgm^{-3} of birchwood xylan at t=36 h of the cultivation as 1.6 U ml^{-1}. At the same time, they obtained the highest glucose isomerase activity as 1100 U L^{-1} at t=36 h of the bioprocess. In the present work, higher volumetric glucose isomerase and xylanase activities were attained as 1838 U L^{-1} and 9.3 U ml^{-1} at t=24 h. This difference obviously outcomes from the microorganism, *Bacillus thermoantarcticus,* and also the difference of medium compositions. Medium composition of GI-producing *Bacillus thermoantarcticus* is much richer that yeast extract, ammonium sulfate and salt solution are present in the medium. In addition, Chauthaiwale et al., (1994) investigated the change of xylanase activity from thermophilic *Bacillus sp*. (NCIM 59) with cultivation time and the highest xylanase activity was attained as 2.0 U mg^{-1} protein at t=24 h of the cultivation. They also investigated the production of glucose isomerase production and attained glucose isomerase activity of 0.035 U mg^{-1} protein at t=24 h of the bioprocess.

When the function of xylanase is considered the results obtained in the mentioned literature studies and the present study are reasonable. Because xylanase, which attacks internal xylosidic linkages on the backbone of xylan, has an important role in the digestion of xylan. Higher xylanase activity indicates the efficient digestion of xylan in the fermentation broth; in other words, efficient substrate consumption. As xylan is digested efficiently, cell concentration increases and hence glucose isomerase production increases.

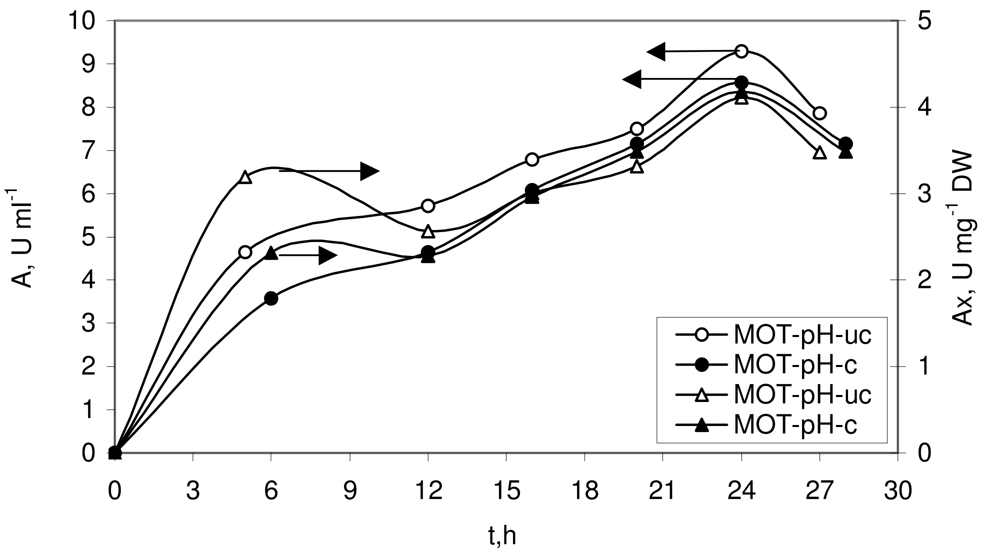

Figure 4.25 The variations in the xylanase volumetric (UL^{-1}) and specific activity (U g^{-1} DW) with the cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

4.4.1.6 Oxygen Transfer Characteristics

The Dynamic Method was applied to find the oxygen transfer parameters, i.e., oxygen uptake rate (OUR), r_0, and oxygen transfer coefficient, K_La. At t<0 h, the physical oxygen transfer coefficient K_La_o was measured in the medium in the absence of the microorganism. The variations in K_La, oxygen uptake rate, oxygen transfer rate, the enhancement factor E (=K_La/K_La_o), oxygen uptake rate (OUR), oxygen transfer rate (OTR), maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor (η) throughout the bioprocesses are given in Table 4.7.

Volumetric mass transfer coefficient, K_La, a physical parameter, depends on operational conditions such as geometrical parameters of the bioreactor, medium properties, and the presence of microorganism. Figure 4.26 shows the change of K_La with cultivation time for both processes. As shown in Figure 4.26, K_La values for operations MOT-pHuc and MOT-pHc are alike. K_La values for MOT-pHuc and MOT-pHc operations at t=0.5, 2, 15-16 and 24 h of the bioprocesses were 0.014 and 0.0145, 0.16 and 0.017, 0.014 and 0.015, 0.013 and 0.012 s^{-1} respectively. K_La is a physical parameter, which is a strong function of the oxygen transfer rate during an aerobic bioprocess. As the same stirring rates and air inlet rates were applied for the uncontrolled-pH and controlled-pH operations, results mentioned above are reasonable. When K_La values were evaluated in terms of their change with respect to time, the highest K_La was obtained at the t=2 h of MOT-pHc condition as 0.017 s^{-1}. For MOT-pHuc, the highest K_La was obtained at t=2 h of the bioprocess as 0.016 s^{-1}. E, the biological enhancement factor has been calculated by the ratio of experimental oxygen mass transfer coefficient in the presence of microorganisms and that measured in the absence of microorganisms. E values (Figure 4.27) varied between 1.25-2.00 and observed to increase between hours t=0.5-16 h. K_La and consequently the biological enhancement factor, E were higher in the growth phase of the bioprocess than in glucose isomerase production phase.

OUR was affected from the growth phases of the microorganism. Figure 4.28 and 4.29 show the variation of OUR with cultivation time and cell concentration respectively. OUR increased during the exponential growth phase of the microorganism, took the maximum value for a cell concentration of 0.62 and

0.58 kg m^{-3} of MOT-pHuc and MOT-pHc conditions respectively and afterwards decreased slowly as the cells reached the stationary phase of the growth. The highest OUR was observed as 0.00198 and 0.00215 mol m^{-3} s^{-1} at t=2.0 of MOT-pHuc and MOT-pHc conditions respectively. After microorganism has reached stationary phase at t=12, OUR began to decrease taking 0.0008 mol $m^{-3}s^{-1}$ for a cell concentration of 2.26 kg m^{-3} of MOT-pHuc and 0.0009 mol $m^{-3}s^{-1}$ for a cell concentration of 2.05 kg m^{-3} of MOT-pHc. The variation of oxygen transfer rate (OTR) with cultivation time is given in Figure 4.30. OTR, which is proportional to the difference between the equilibrium concentration, C_{DO}^* and the dynamic dissolved oxygen concentration in the medium, C_{DO} took the highest value at the beginning of the bioprocess (0.0012 mol m^{-3} s^{-1}) and decreased to an extent depending on the oxygen uptake rate.

To express the oxygen limitation in an aerobic process, Damköhler (Da) number is defined. Da is a dimensionless number, which relates maximum oxygen uptake and maximum transfer rates. At the beginning of the bioprocesses, biochemical reaction limitations were effective (Da<1); however after t=0.5 h of the bioprocesses, mass transfer resistances became more efficient (Da>>1). Da versus time profiles for both processes were similar to each other (Figure 4.31). However, at MOT-pHc condition Da values were slightly higher when compared to the uncontrolled bioprocess indicating that mass transfer resistances were more effective at MOT-pHc condition. This can be also observed in Figure 4.32 showing the change of Da with cell concentration.

Also the change of effectiveness factor along the bioprocess should be taken into consideration (Figure 4.33 and 4.34). It can be observed that the effectiveness factor, η, took values which were close to 1 at the beginning of the bioprocess, indicating that the cells are consuming oxygen with a high rate, maximum oxygen uptake rate (OD) is approached at both of the condition. For both conditions, η decreased with the increase of Da. This concludes the reverse proportionality between effectiveness factor and Damköhler number in a bioprocess. This approach can be easily seen in Figure 4.36.

The change of maximum oxygen uptake rate, oxygen demand (OD), with cultivation time is given in Figure 4.35. As seen, both MOT-pHuc and MOT-pHc show a similar profile for microbial oxygen demand along the bioprocesses. As seen in Figure 4.35, OD increased with the increase in cell concentration which indicates that maximum possible oxygen utilization rate (OD) depends on the microbial growth.

Table 4.7 The variations in oxygen transfer parameters with cultivation time and pH strategy

Condition	t	k_La (s^{-1})	E k_La/k_La_0	OTRx10^3 (mol $m^{-3}s^{-1}$)	OTR_{max}x10^3* (mol $m^{-3}s^{-1}$)	(OTR_{max}x10^3)′* (mol $m^{-3}s^{-1}$)	OURx10^3 (mol $m^{-3}s^{-1}$)	ODx10^3 (mol $m^{-3}s^{-1}$)	Da OD/OTR_{mx}	η OUR/OD
MOT-pH-UC	0.5	0.014	1.75	0.28	2.52	2.44	1.20	1.2	0.48	1.00
	2	0.016	2.00	1.92	2.88	2.71	1.98	7.38	2.56	0.27
	15	0.014	1.75	0.90	2.52	1.97	0.80	306.8	121.43	0.003
	24	0.013	1.63	0.58	2.34	1.56	0.60	_	_	_
	27	0.010	1.25	0.50	1.80	1.13	0.50	_	_	_
MOT-pH-C	0.5	0.015	1.61	0.62	2.61	2.52	1.30	1.36	0.52	1.00
	2	0.017	1.89	2.07	3.06	2.89	2.15	8.05	2.63	0.30
	16	0.015	1.67	0.98	2.70	2.07	0.90	706.7	281.85	0.001
	24	0.012	1.33	0.51	2.16	1.44	0.50	_	_	_

*OTR_{max} values are calculated in two ways. OTR_{max} is equal to the product of K_La and C_{DO}. However, $(OTR_{max})'$ is calculated by the product of K_La and C_{DO} which is measured by assuming the solubility of oxygen, $C_{DO}*$ in the fermentation broth changes in linear with time.

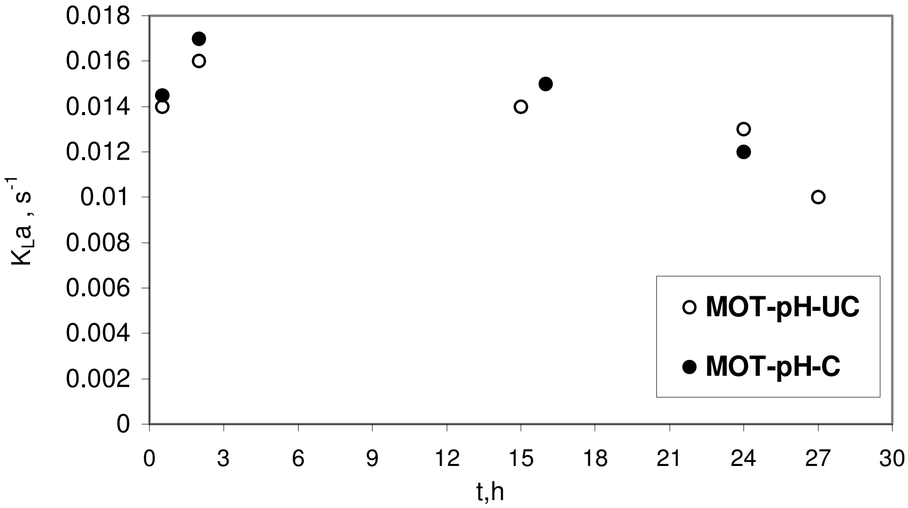

Figure 4.26 The variation of K_La with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

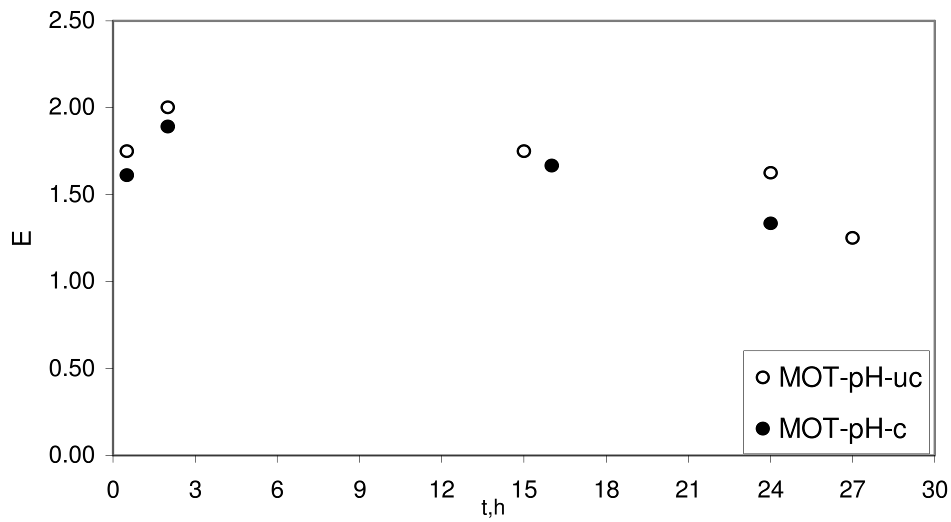

Figure 4.27 The variation of E with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}

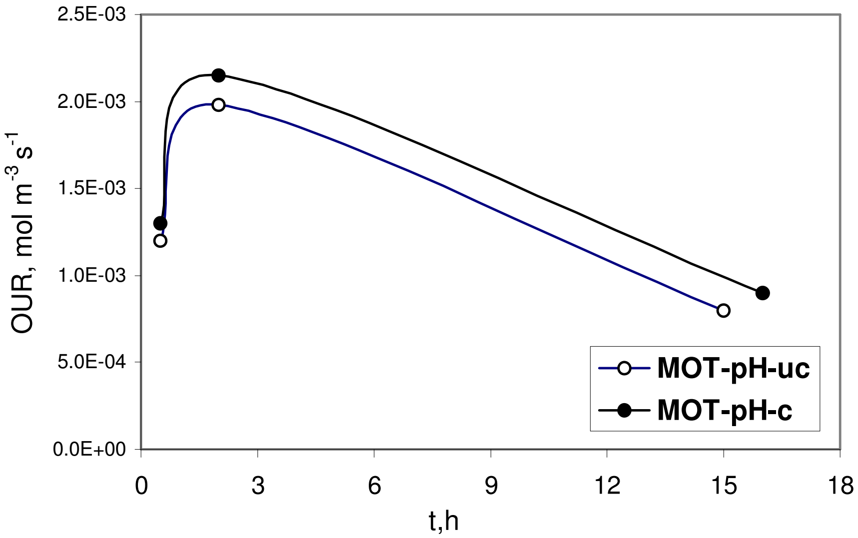

Figure 4.28 The variation of OUR with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

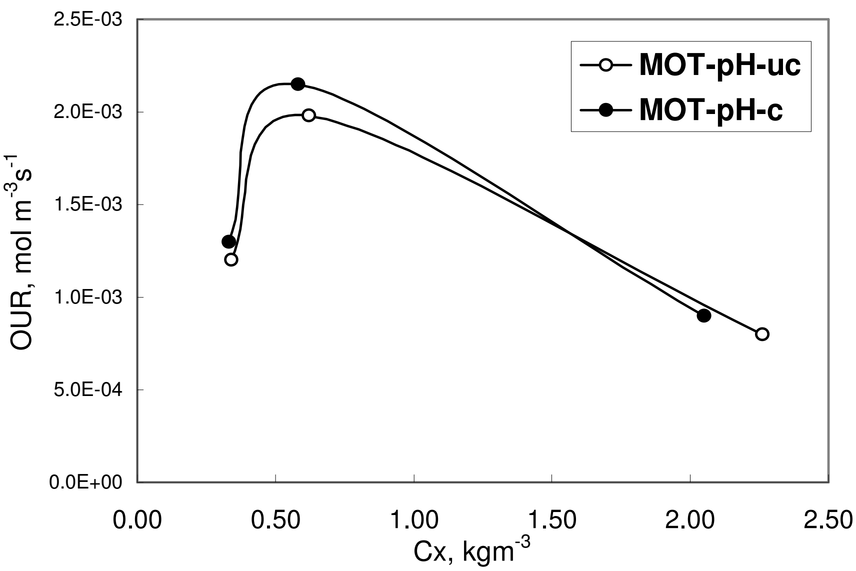

Figure 4.29 The variation of OUR with cell concentration and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min

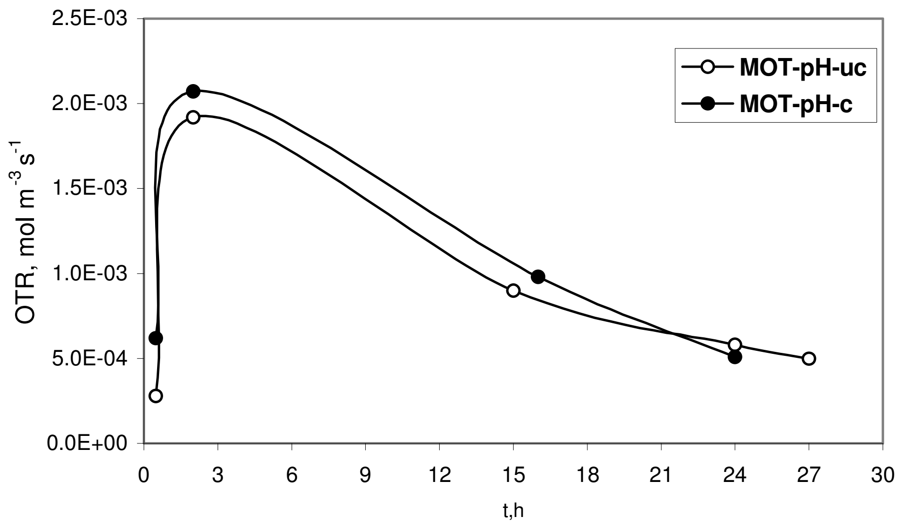

Figure 4.30 The variation of OTR with time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

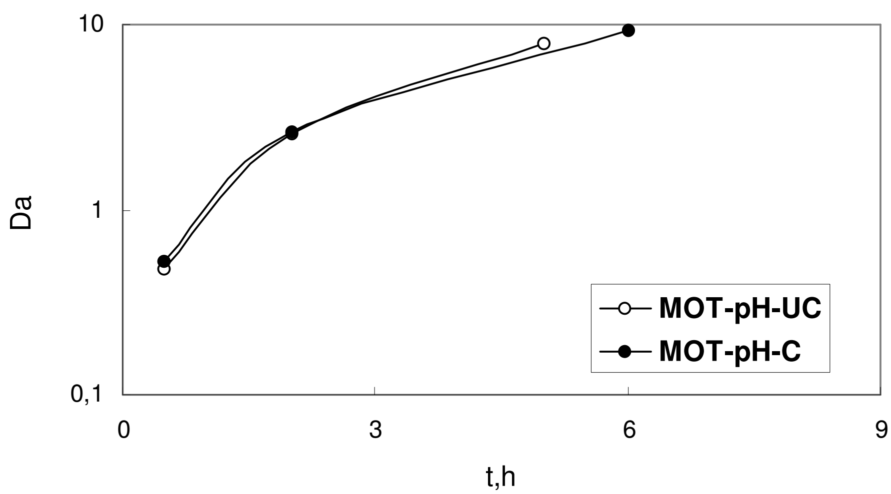

Figure 4.31 The variation of Da (OD/OTR_{max}) with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

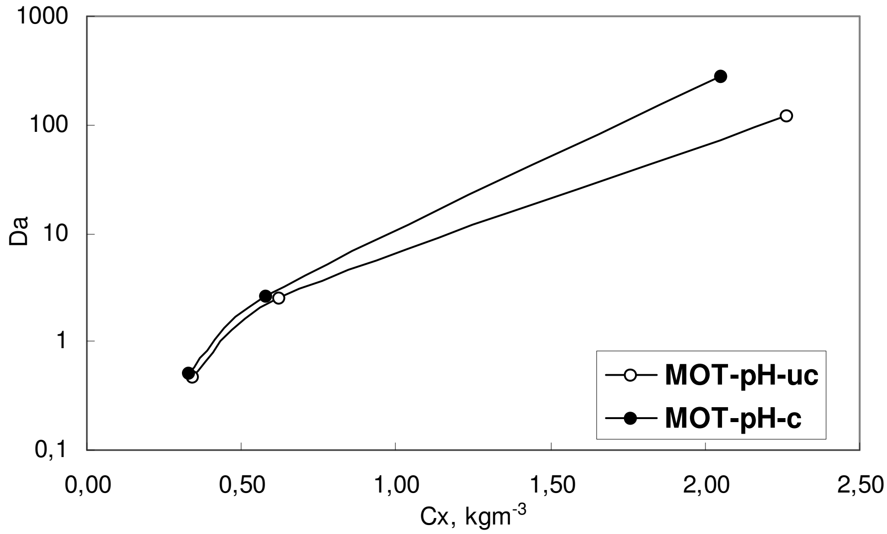

Figure 4.32 The variation of Da (OD/OTR_{max}) with cell concentration and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

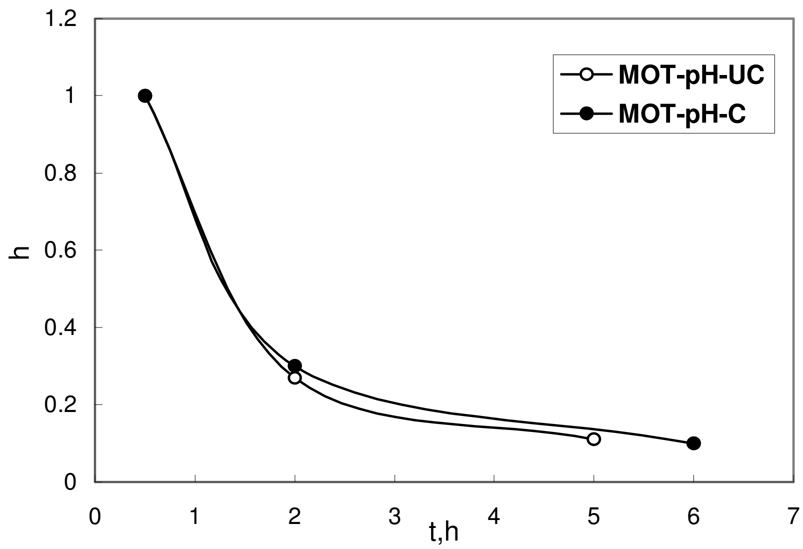

Figure 4.33 The variation of η (OUR/OD) with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

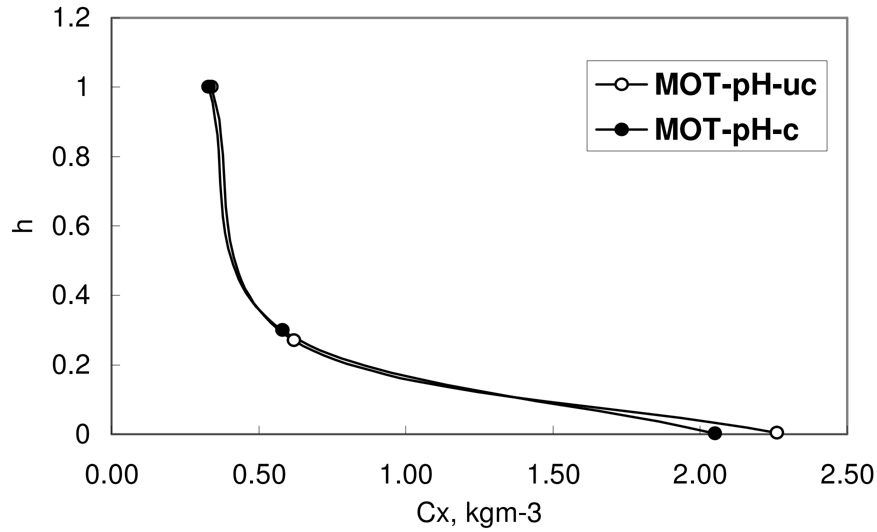

Figure 4.34 The variation of η (OUR/OD) with cell concentration and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

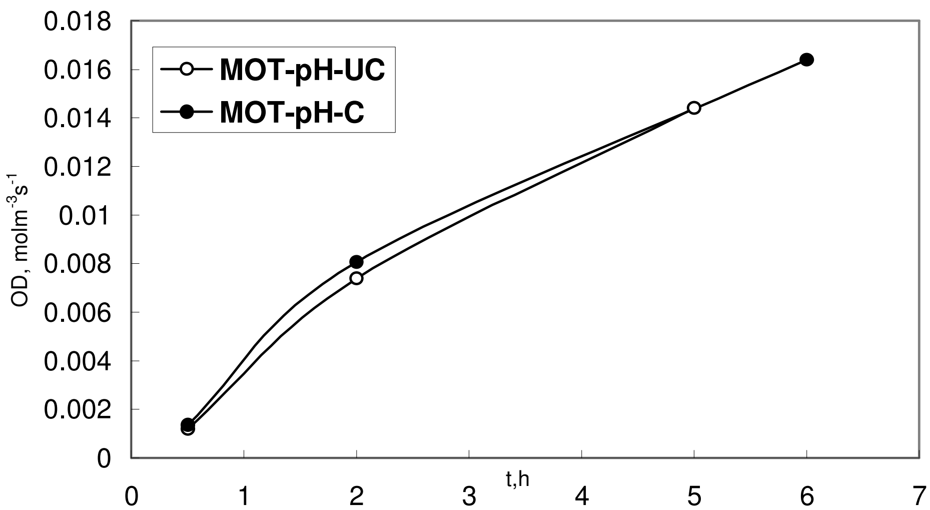

Figure 4.35 The variation of OD with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

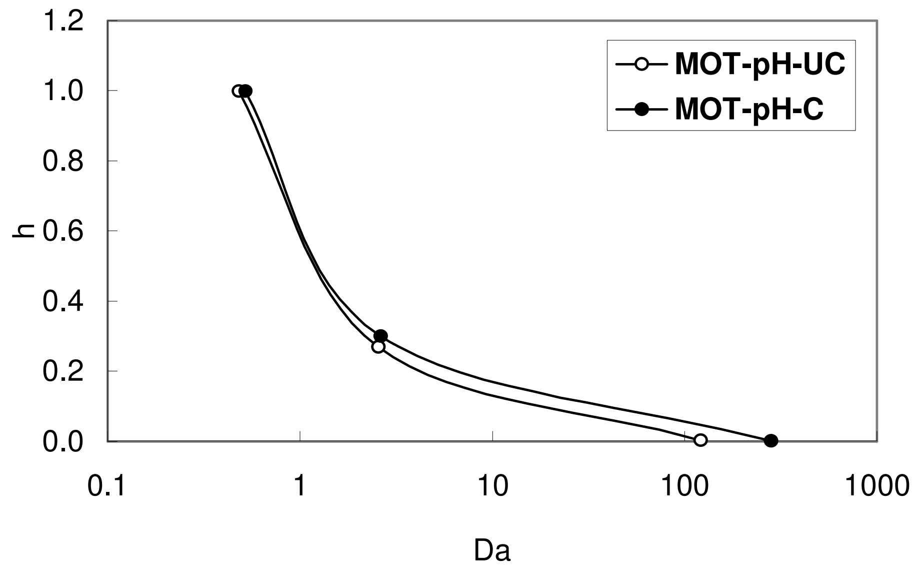

Figure 4.36 The relationship between Da and η at different pH conditions at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

4.4.1.7 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in the specific growth rate, μ, the specific oxygen uptake rate, q_0, maintenance coefficients for oxygen, m_0 and m_0' and the yield coefficients with the cultivation time are given in Table 4.8.

In both conditions, the specific growth rate (μ) decreased with cultivation time. As seen in Figure 4.37, the maximum value of μ was attained as 1.72 h^{-1} and 1.71 h^{-1} at t=0.5 h of MOT-pHuc and MOT-pHc in which the highest cell concentrations were obtained as 2.26 and 2.05 kg m^{-3}. At the beginning of the bioprocesses, a sharp decrease in specific growth rate was observed. After t=8 h, cell concentration did not change remarkably and specific growth rate attained almost μ=0 h^{-1} between t=8-16 h.

The variation of specific oxygen uptake rate q_0 with cultivation time and pH strategy are given in Figure 4.38. Specific oxygen uptake rate was observed to decrease with the cultivation time at both conditions. While the highest value q_0 was obtained as 0.46 kg kg^{-1} h^{-1} at t=0.5 h of MOT-pHc condition; it is found as 0.41 kg kg^{-1} h^{-1} at t=0.5 h of MOT-pHuc condition. MOT-pHc had a higher q_0 profile when compared to the operation MOT-pHuc. This difference resulted from higher oxygen uptake rate of the controlled-pH bioprocess. As seen in Figure 4.39, MOT-pHuc showed a higher trend of $Y_{X/O}$ indicating that higher cell yield on oxygen consumption was obtained. The highest $Y_{X/O}$ was obtained as 4.18 kg cell/kg O_2 at t=0.5 h of MOT-pHuc. $Y_{X/O}$ was observed to decrease with cultivation time. In general, at the beginning of the bioprocesses (t=2 or 3 h), a sharp decrease in $Y_{X/O}$ was observed due to the increase of OUR as seen in Figure 4.39. However after t= 2 or 3 h, OUR decreased with cultivation time and this resulted in a gentle decrease in $Y_{X/O}$.

The change of r_p (UL^{-1} h^{-1}) with cultivation time is seen in Figure 4.40. r_p is calculated in terms of glucose isomerase volumetric activity. As seen in Figure 4.40, MOT-pHuc showed a higher profile for r_p which also showed a higher profile for volumetric glucose isomerase activity. The specific glucose isomerase production rate, q_p was calculated in terms of GI volumetric activity namely, U L^{-1}. As a result q_p is termed as U kg $cell^{-1}$ h^{-1}. The variations of q_p with cultivation time, and pH strategy are given in Figure 4.41. As seen in Figure 4.41, the highest value of q_p was obtained as $2.21*10^5$ U kg cell^{-1} h^{-1}for MOT-pHuc and $1.47*10^5$ U kg cell^{-1} h^{-1}for MOT-pHc at the t=2h and t=3h of the bioprocesses respectively.

For all operation conditions, $Y_{P/X}$ values were calculated in terms of GI volumetric activity namely, U L^{-1}; $Y_{P/X}$ is termed as U kg cell^{-1}. The variations of $Y_{P/X}$ with cultivation time and pH strategy are given in Figure 4.42. MOT-pHc showed a higher profile of $Y_{P/X}$ and the maximum value was found as $6*10^6$ U kg cell^{-1} at t=16 h of the bioprocess, in which higher profile glucose isomerase specific activity (A_x, U mg^{-1} DW cell) was obtained. For MOT-pHuc the highest $Y_{P/X}$ was obtained as $3.7*10^6$ U kg cell^{-1}.

From the slope of the plot of $1/Y_{X/O}$ versus $1/\mu$, the rate of oxygen consumption for maintenance was obtained, and the variation of m_0 and m_0' with pH strategy is given in Table 4.8. m_0 is the oxygen consumption for maintenance calculated by neglecting the oxygen consumption for by-product formation and m_0' is the oxygen consumption for maintenance calculated by considering the oxygen consumption for by-product formation. The highest values of m_0 and m_0' were obtained at MOT-pH-c operation as 0.272 and 0.201 kg kg^{-1} h^{-1} respectively. For the operation MOT-pHuc, m_0 and m_0' were obtained as 0.266 and 0.195 kg kg^{-1} h^{-1} respectively. The calculation procedure of maintenance coefficients, m_0 and m_0' were explained in section 2.4.2.2; the Leudeking-Piret constants calculated for the bioprocesses are given in Table 4.9.

Table 4.8 The variations in specific growth rate and yield coefficients

Condition	t	μ h^{-1}	r_o kg O_2 $m^{-3}h^{-1}$	q_o kg O_2 kg^{-1} h^{-1}	r_p $UL^{-1}h^{-1}$	q_p U kg $cell^{-1}$ h^{-1}	$Y_{x/o}$ kg cell kg O_2^{-1}	$Y_{p/o}$ U kg O_2^{-1}	$Y_{p/x}$ U kg $cell^{-1}$	m_0 kg kg^{-1} h^{-1}	m_0' kg kg^{-1} h^{-1}
MOT-pH-uc	0.5	1.72	0.14	0.41	-	-	4.18	-	-	0.266	0.195
	2	0.46	0.23	0.37	137.1	$2.2*10^5$	1.26	$6*10^5$	$4.78*10^5$		
	15	0.0047	0.09	0.04	39.1	$0.17*10^5$	0.11	$4.2*10^5$	$3.7*10^6$		
	24	-	0.07	_	57.2	-	-	$8.3*10^5$	-		
	27	-	0.06	_	-	-	-	-	-		
MOT-pH-c	0.5	1.71	0.15	0.46	-	-	3.71	-	-	0.272	0.201
	2	0.46	0.25	0.43	-	-	1.07	_	-		
	16	0.0012	0.10	0.05	24.5	$0.12*10^5$	0.04	$2.4*10^5$	$6*10^6$		
	24	_	0.06	_	29.5	-	-	$5.1*10^5$	-		

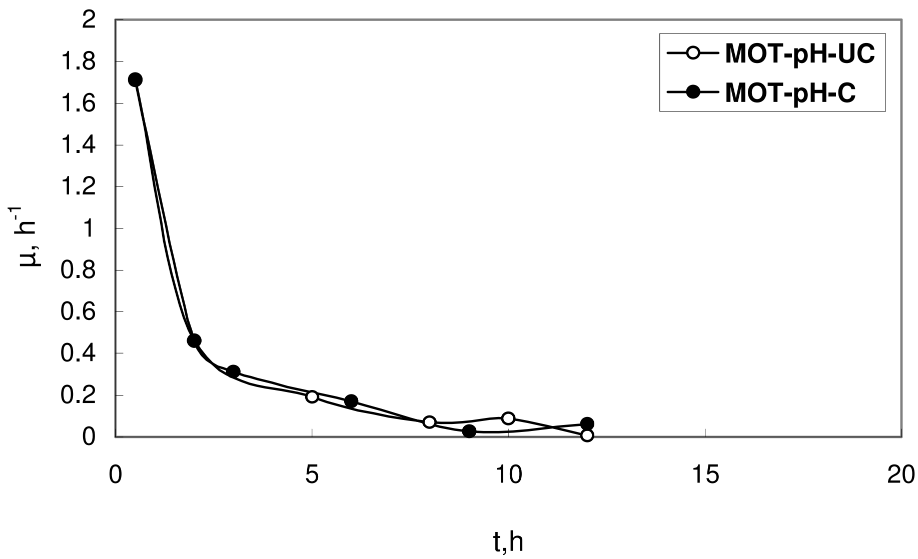

Figure 4.37 The variations in the specific growth rates with the cultivation time, agitation, air inlet rates and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

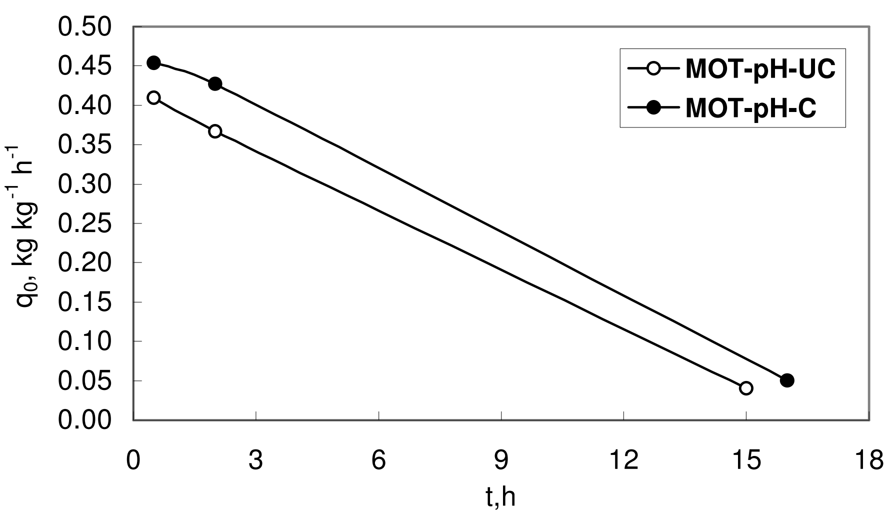

Figure 4.38 The variations in the specific oxygen uptake rates with the cultivation time, agitation, air inlet rates and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

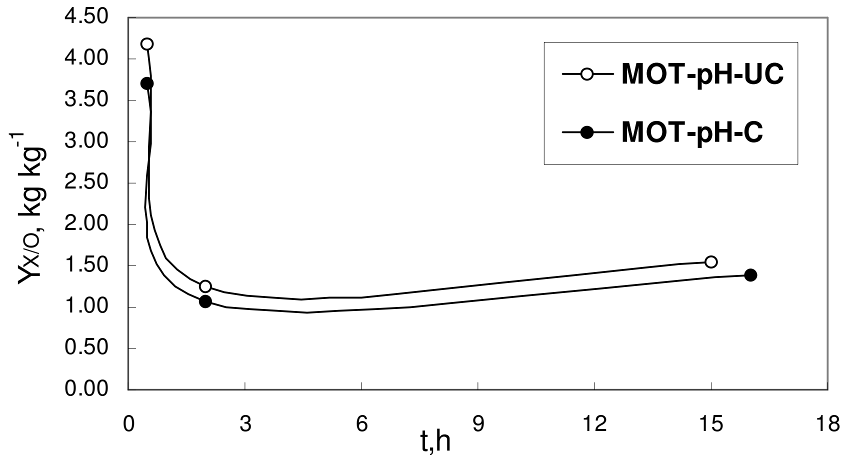

Figure 4.39 The variations in the yield coefficient, $Y_{X/O}$ with the cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

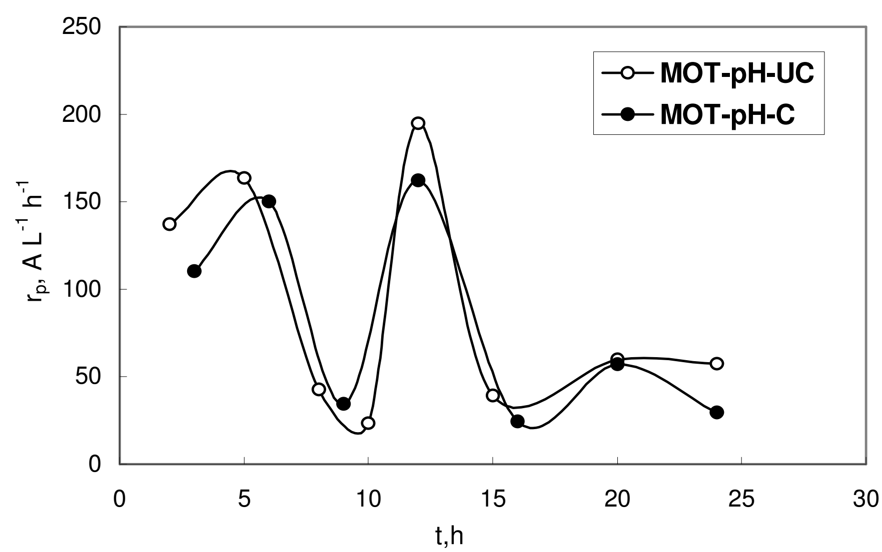

Figure 4.40 The variations in the r_p with the cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

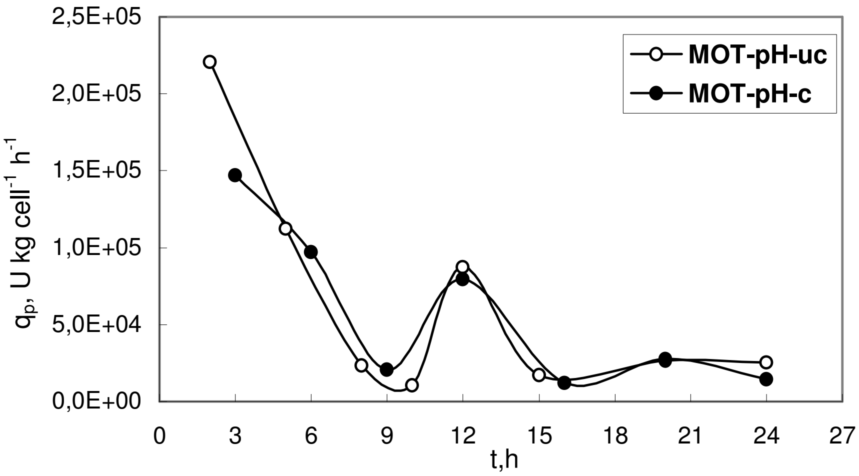

Figure 4.41 The variations in the specific glucose isomerase production rates with the cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

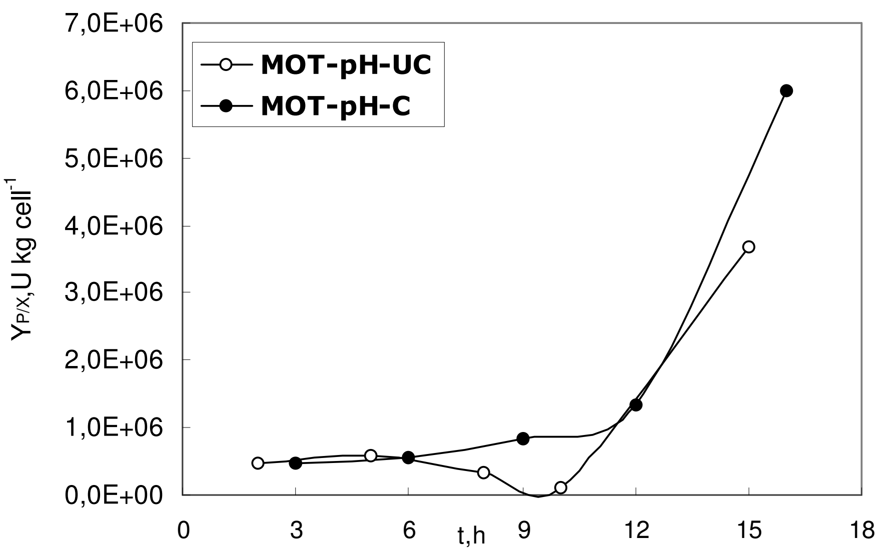

Figure 4.42 The variations in the yield coefficients, $Y_{P/X}$ with the cultivation time, and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

Table 4.9 The variations in Leudeking-Piret constants

Condition	**α**	**β**
MOT-pHuc	0.097	0.027
MOT-pHc	0.122	0.031

4.4.1.8 Organic and Amino Acid Concentration Profiles

The variations in the organic acids detected in the fermentation broth with cultivation time and pH strategy are given in Tables 4.10 and 4.11. As seen in Table 4.10 and 4.11, acetic acid and formic acid are the major organic acids excreted to the fermentation medium for both operations. As seen in Figure 4.43, higher organic acid concentration profile was attained at controlled pH operation. The highest total organic acid concentration was obtained as 0.7 kg m^{-3} at t=24 h of MOT-pHc. In addition to acetic and formic acids, at both operations moderate concentrations of malic and oxalic acids were excreted during bioprocess. At the pH controlled operation, citric acid was excreted into the fermentation medium after t=12 h of the bioprocess.

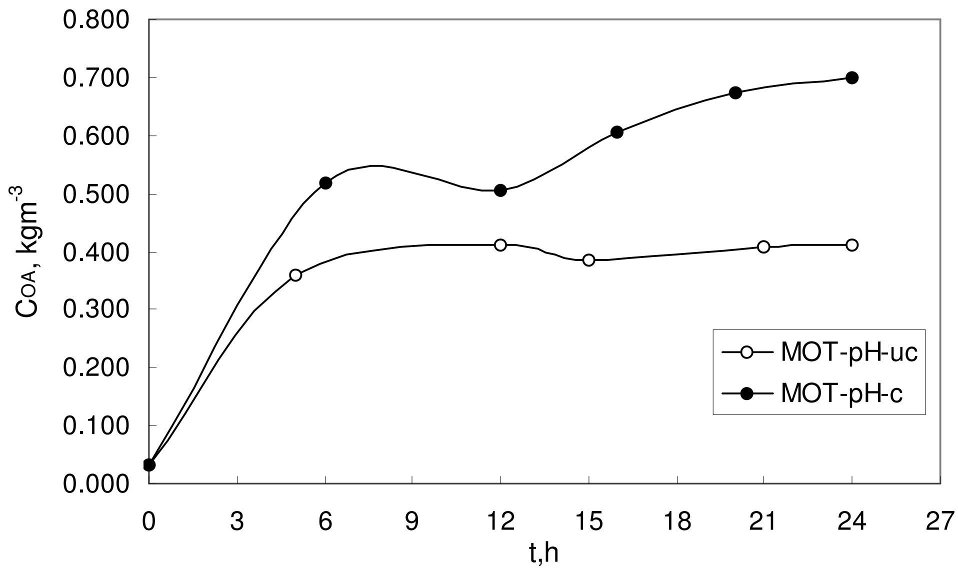

Figure 4.43 The variation of total organic acid concentration with cultivation time, air inlet rate, agitation rate and pH strategy at conditions, T=55°C, V_R= $1.65\text{x}10^{-3}$ m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

The variations in the amino acids detected in the fermentation broth with cultivation time and pH strategy were given in Table 4.12. At t=0 h for both operations, the amino acids coming from the precultivation medium were Asp, Glu, Arg, Ala, Tyr, Phe, Trp. When total aminoacid production capacities for perations MOT-pHuc and MOT-pHc were compared, it is observed that the total amino acid production was increased with pH control. The highest ΣC_{AA} amino acid concentration was attained as 0.228 kg m^{-3} at t=12 h of MOT-pHc. While tryptophane is the major amino acid secreted into the fermentation at MOT-pHc, phenylalanine is the major aminoacid secreted into the fermentation at MOT-pHuc. The highest tryptophane concentration was attained as 0.07 kg m^{-3} at t=24 h of MOT-pHc and the highest phenyalanine concentration was obtained as 0.043 kg m^{-3} at t=12 h of MOT-pHuc. Other aminoacids produced significantly during the fermentation were aspartic acid, glutamic acid, arginine, alanine and tyrosine.

Table 4.10 The variations in organic acid concentrations with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

MOT-pH-uc														
Time (h)	Ac	Cit	Form	Fum	Gluc	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc	ΣC_{OA}, kgm^{-3}
0	0.01	-	0.0068	0.0003	-	-	0.0084	0.0039	-	0.0048	-	-	-	0.034
5	-	-	0.106	0.0033	-	-	0.133	0.029	0.041	0.049	-	-	-	0.361
12	0.121	-	0.164	0.0032	-	-	-	0.017	0.059	0.048	-	-	-	0.412
15	0.077	-	0.171	0,0031	-	-	-	0.016	0.062	0.055	-	-	-	0.384
21	0.081	-	0.188	0.0030	-	-	-	0.015	0.066	0.053	-	-	-	0.408
24	0.196	-	0.130	0.0029	-	-	-	0.011	0.018	0.053	-	-	-	0.410

Table 4.11 The variations in organic acid concentrations with cultivation time and pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

MOT-pH-c														
Time (h)	Ac	Cit	Form	Fum	Gluc	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc	ΣC_{OA}, kgm^{-3}
0	0.01	-	0.0068	0.0003	-	-	0.0084	0.0039	-	0.0048	-	-	-	0.034
6	0.131	-	0.085	0.0035	-		0.134	0,011	-	0.055	-	-	-	0.519
12	0.163	0.06	0.162	0.0040	-	0.014	-	-	0.057	0.047	-	-	-	0.505
16	0.136	0.16	0.195	0.0042	-	0.015	-	-	0.049	0.053	-	-	-	0.606
20	0.163	0.17	0.215	0.0049	-	0.018	-	-	0.058	0.046	-	-	-	0.675
24	0.175	0.19	0.215	0.0054	-	0.020	-	-	0.061	0.035	-	-	-	0.700

Table 4.12 The variations in amino acid concentrations with cultivation time, pH strategy at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=500 min^{-1}.

MOT-pH-uc												
Time (h)	Asp	Glu	His	Arg	Thr	Ala	Tyr	Val	Met	Phe	Trp	ΣC_{AA}, kgm^{-3}
0	0.00058	0.0007		0.0004		0.0035	0.0069			0.0028	0.0059	0.021
5	0.012	0.022									0.05	0.084
12	0.01						0.0018			0.043		0.055
15	0.011						0.0018			0.021	0.017	0.051
21	0.012	0.021								0.016	0.016	0.064
24	0.018	0.017								0.025	0.013	0.073
MOT-pH-c												
Time (h)	Asp	Glu	His	Arg	Thr	Ala	Tyr	Val	Met	Phe	Trp	ΣC_{AA}, kgm^{-3}
0	0.00058	0.0007		0.0004		0.0035	0.0069			0.0028	0.0059	0.021
6	0.0071	0.021		0.044		0.028	0.037			0.031	0.057	0.225
12	0.0095	0.041	0.011	0.017		0.023	0,054		0,0039	0.01	0.059	0.228
16	0.018	0.017	0.017	0.0063		0.019		0.03	0.0043	0.0037	0.063	0.178
20	0.015	0.016	0.018								0.065	0.114
24	0.012	0.015	0.019			0.021				0.0046	0.070	0.142

4.4.2 Effect of Oxygen Transfer Rate (OTR) on Glucose Isomerase Production

In the previous section, effect of pH strategy on glucose isomerase production was discussed. After evaluating the effect of pH strategy on glucose isomerase activity throughout the process, following experiments were decided to perform with pH control. This decision was made according to the specific glucose isomerase activities found for controlled and uncontrolled operations. As higher specific GI activity was obtained for the operation with controlled pH, the effect of oxygen transfer rate on GI activity was examined by controlling the pH throughout the process. When volumetric glucose isomerase activities were compared, the bioprocess with uncontrolled pH showed slightly higher volumetric glucose isomerase activity (1838 U L^{-1}) than that obtained with controlled pH (1814 U L^{-1}). Applying an optimal pH control strategy provided the control of ambient pH of the cells present in the fermentation broth and increased the specific glucose isomerase activity 1.1 fold (from 814 to 885 U g^{-1} DW at t=24 h of the bioprocesses) and this increase was taken into account when deciding for the pH strategy for the following experiments.

As previously mentioned, oxygen transfer conditions applied are:

- Low oxygen transfer condition (LOT) as Q_0/V_R = 0.5 vvm, N=300 min^{-1}
- Medium oxygen transfer condition (MOT) as Q_0/V_R = 0.5 vvm, N=500 min^{-1}
- High oxygen transfer condition (HOT) as Q_0/V_R = 0.5 vvm, N=750 min^{-1}

4.4.2.1 Dissolved Oxygen Profiles

The influence of oxygen transfer rate on *Bacillus thermoantarcticus* was studied under three oxygen transfer conditions. The variations in dissolved oxygen concentration with cultivation time, air inlet rate and agitation rate are given in Figure 4.44.

As seen in Figure 4.44, at MOT and LOT conditions dissolved oxygen concentrations decreased below the concentration, 0.02 mol m^{-3} and remained almost constant for different periods of cultivation depending on the amount of oxygen transferred to the medium and amount of oxygen demanded by the microorganism. At LOT condition, the dissolved oxygen concentration decreased dramatically in 3 hours, approached 0.009 mol m^{-3}, and did not increase even when the microorganism reached the stationary growth phase. Whereas, at HOT condition, dissolved oxygen concentration decreased throughout the process at a lower rate when compared to the other two oxygen transfer conditions. Although oxygen concentration reached its minimum at t=8.5 h, it did not decrease below 0.02 mol m^{-3} due to the effective oxygen transfer mechanism at the stirring rate, 750 min^{-1}. The lowest dissolved oxygen concentration was obtained as 0.07506 $molm^{-3}$ at t=8.5 h of HOT operation.

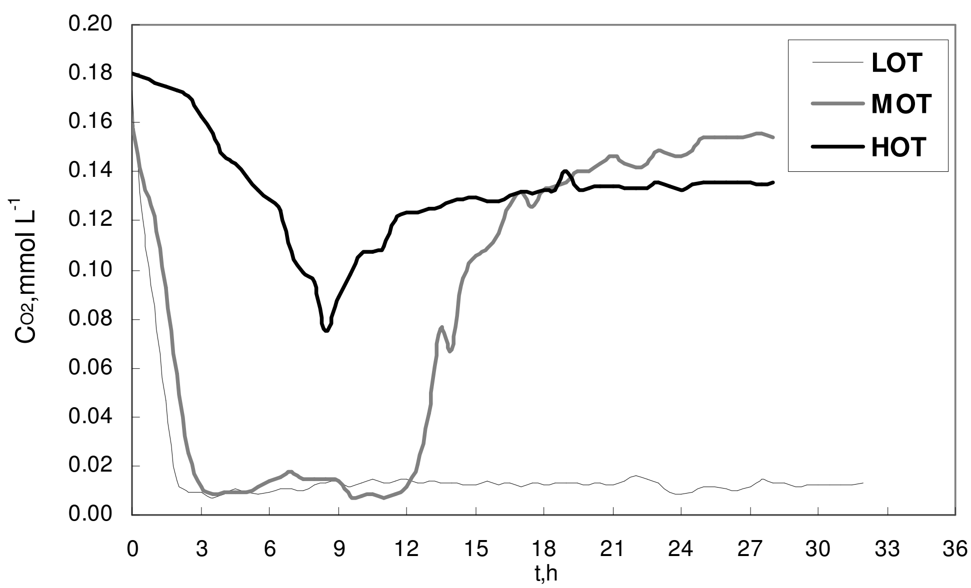

Figure 4.44 The variations in the dissolved oxygen concentration with the cultivation time, agitation and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

4.4.2.2 Cell Growth Profiles

As in many aerobic bioprocesses microbial growth is influenced by the oxygen supply. The variations in the cell concentration with cultivation time agitation and air inlet rates are given in Figure 4.45.

Until t=3 h, cell formation changed slightly with respect to the oxygen transfer conditions applied. On the other hand, after t=3 h cell profiles altered considerably throughout the bioprocess. In general, cell growth at all conditions reached to stationary phase after t=12 h. The highest cell concentration with a value of C_X=2.05 kg m^{-3} was obtained at MOT condition and lowest cell concentration was found as C_X=1.0 kg m^{-3} at LOT condition. Under the LOT condition, since oxygen has low solubility in the fermentation broth, oxygen supply that seems to be insufficient for maintenance of oxidative metabolism resulted in a lower cell growth. For HOT condition, the highest cell concentration was attained as C_X=1.31 kg m^{-3} indicating that a higher oxygen transfer rate inhibited the cell growth.

4.4.2.3 Glucose Isomerase Activity Profiles

The variations of volumetric and specific glucose isomerase activities with the cultivation time, agitation and air inlet rates are given in Table 4.46.

The highest volumetric GI activity (U L^{-1}) was obtained at MOT condition (A=1814 U L^{-1}), where glucose isomerase activity was also higher than all the other conditions throughout the process; and the lowest glucose isomerase activity was obtained at LOT as A=1718 U L^{-1} among the investigated oxygen transfer conditions at t=24 h. When cell concentration and volumetric glucose isomerase profiles were compared, a direct proportionality is observed.

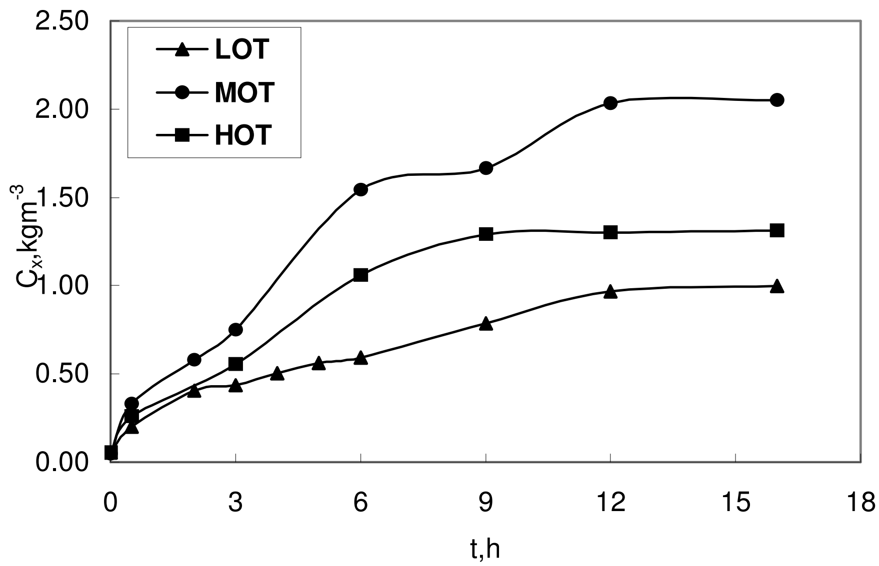

Figure 4.45 The variations in cell concentration with the cultivation time, agitation and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

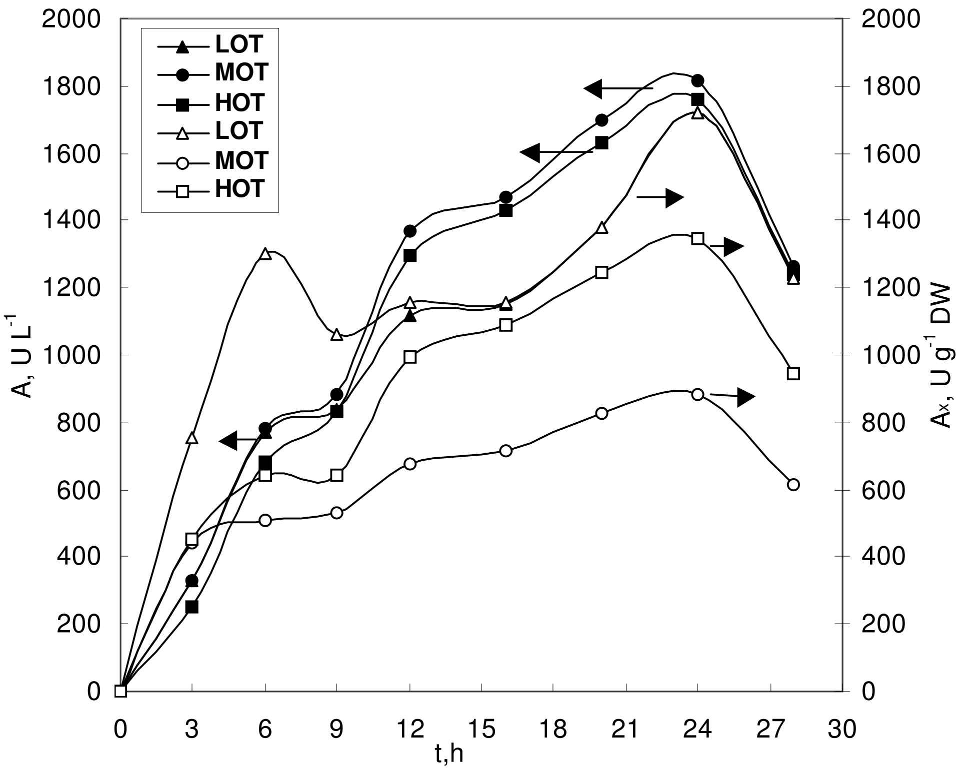

Figure 4.46 The variations in the glucose isomerase volumetric (UL^{-1}) and specific activity (U mg^{-1} DW) with the cultivation time, agitation and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

However when specific glucose isomerase activities were considered, profiles differed remarkably. Highest specific activity was observed at t=24 h of LOT operation (1718 U g^{-1} DW) meaning that at LOT, the highest glucose isomerase activity was attained per gram of *Bacillus thermoantarcticus*. The lowest specific GI activity attained at t= 24 h of MOT condition as 884 U g^{-1} DW.

When glucose isomerase volumetric and specific activity profiles are compared, a remarkable difference is observed. Although closer GI volumetric activity profiles attained at different oxygen transfer conditions, specific glucose isomerase activities were much more affected by the oxygen transfer conditions. A_x was defined as one unit of glucose isomerase unit per gram of cell. This indicates that A_x highly depends on the cell concentration. As cell concentration profiles differed remarkably, specific glucose isomerase profiles changed significantly due to the different oxygen transfer conditions.

4.4.2.4 Xylanase Activity Profiles

The variations of xylanase activity with the cultivation time and pH strategy are given in Figure 4.47.

As seen in Figure 4.47, the highest xylanase volumetric activity was attained as 8.6 U ml^{-1} at t=24 h of MOT. The lowest profile belongs to the LOT operation in which the lowest cell concentration and also glucose isomerase concentrations were attained. Again the relation between xylanase activity and the substrate consumption rate should be considered. Xylanase plays a major role in the digestion mechanism of xylan, which is the sole carbon source used in the fermentation broth of *Bacillus thermoantarcticus.* Higher xylanase activities lead to the higher xylan consumption rates and consequently higher cell formation rates. MOT showed the highest xylanase activity profile in which the highest cell concentration was attained as 2.05 kg m^{-3} at t=16 h of MOT. Higher oxygen transfer condition (HOT) attained a lower xylanase activity as (7.9 U ml^{-1} at t=24 h) than MOT due to the oxygen inhibition on metabolism of the microorganism. While the highest specific xylanase activity profile was attained at LOT operation, the lowest specific xylanase activity profile was obtained at MOT. The highest specific xylanase activity (U mg^{-1}) was attained as 6.8 U mg^{-1} at t=24 h of LOT.

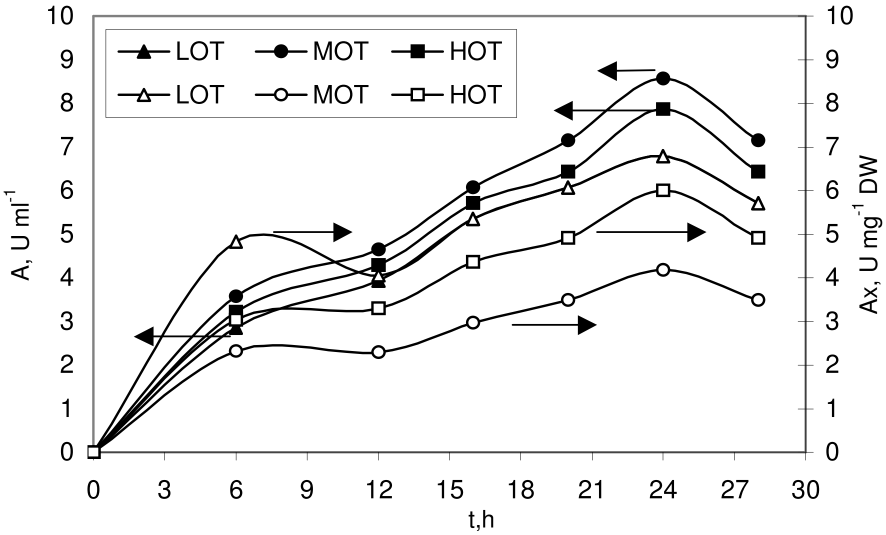

Figure 4.47 The variations in the xylanase volumetric (UL^{-1}) (black label) and specific activity (U g^{-1} DW) (red label) with the cultivation time, agitation and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

4.4.2.5 Oxygen Transfer Characteristics

The variations in K_La, oxygen uptake rate, oxygen transfer rate, the enhancement factor E (=K_La/K_La_o), oxygen uptake rate (OUR), oxygen transfer rate (OTR), maximum oxygen utilization rate (OD), Damköhler number (Da) and effectiveness factor (η) throughout the bioprocesses are given in Table 4.13.

Figure 4.48 and 4.50 show the variation of k_La and E with cultivation time. For MOT operation, k_La and E values were higher in the growth phase of the microorganism than in the glucose isomerase production phase, from 0.0145 to 0.012 s^{-1} and 1.61 to 1.33 respectively. Also, for HOT operation the highest k_La and consequently E were obtained at t=3 h of the bioprocess as 0.033 s^{-1} and 2.70 respectively. Effect of stirrer speed on K_La can be easily seen in Figure 4.49. K_La values increased with the increase in agitation rate. This effect attributed to the rapid breakage of the bubbles into smaller size with an increase in the impeller speed and enhancement in the gas-liquid interfacial area available for mass transfer. As a result, stirring rate can be considered as one of the major effect altering K_La. For example, for operations, LOT, MOT and HOT, k_La takes values as 0.008, 0.0145 and 0.033 s^{-1} for t=0.5 h of the bioprocess. At t <0h, the physical oxygen transfer coefficient K_La_0 was measured in the medium in the absence of the microorganism, and the enhancement factors E (K_La/K_La_0) were calculated throughout the bioprocess at different ozygen transfer conditions. K_La_0 values increased with oxygen transfer conditions as expected. The enhancement factor, E varied between 1.33-2.70. The low E values obtained shows that slow reaction is accompanied with mass transfer, as expected in most of the fermentation processes accomplished in stirred bioreactors.

Oxygen uptake rate (OUR) is another important factor, which depends on the cultivation time and cell concentration. Figure 4.51 and 4.52 show the change of OUR with cultivation time and cell concentration. OUR actually depends on the growth phases of the microorganisms. Firstly, increases during the lag phase and exponential phases of the microbial growth and takes a maximum at the very beginning of the exponential phase as it is the case in this analysis. OUR takes its maximum at t=2nd or 3rd hours of the bioprocesses. The highest OUR is attained at t=2 h (C_x=0.58 kg m-3) of MOT operation as 0.00215 $molm^{-3}s^{-1}$. The lowest OUR was obtained at t=24 h of MOT as 0.0005 $molm^{-3}s^{-1}$. For HOT, OUR had the lowest profile. This indicates that the cell growth and consequently the OUR decreased as a result of oxygen inhibition on the metabolism of *Bacillus thermoantarcticus* at HOT operation. In addition, the variation of OTR with cultivation time is given in Figure 4.53. Although, higher oxygen transfer conditions were present at HOT as a result of higher stirring rate (N=750 min^{-1}) when compared to MOT operation, cell formation and hence, glucose isomerase activity was lower than those of MOT as a result of the inhibition of oxygen on the metabolism. When OUR and OTR curves for LOT operations are analyzed, no curvature is observed for OUR and OTR versus time plot. This is a result of the rapid decrease of dissolved oxygen in the first hour of the process. At the region where oxygen takes values close to 0.009 mol m^{-3}, no measurements for OUR and OTR was performed. At that region OUR and OTR were assumed to be constant at 0.00165 and 0.00086 mol m^{-3} s^{-1} respectively

In order to know the limiting step of the growth process, the maximum possible OUR (OD) and the maximum oxygen transfer rate have been calculated in the course of the fermentation. Relation between both maximum rates is the modified Damköhler number, Da (Calik et al., 2000). Figure 4.54 and 4.55 show the evolution of Da with cultivation time and cell concentration. As seen in the figures, Da highly depends on the stirrer speed. The highest and lowest profiles were achieved at LOT and HOT respectively. For example, Da values found as 4.94, 2.63 and 1.14 for operations LOT (t=3 h), MOT (t=2 h) and HOT (t=3 h) respectively. It is obvious that LOT was a mass-transfer-limited condition, which resulted a higher profile of Da versus time. As the maximum oxygen transfer rate decreased by the increase in mass transfer resistance, Da increased. As HOT attained the highest maximum oxygen transfer rate profile and hence the lowest mass transfer resistance, it had the lowest profile for Da.

Another important dimensionless parameter, effectiveness factor, η, is described as the ratio between the observed biochemical reaction rate and the formal reaction rate, without mass transfer (Calik et al., 2000). As seen in Figure 4.56 and 4.59, η took 1 at the beginning of the bioprocesses (t=0.5 h). This indicates that cells are consuming oxygen with a high rate that maximum possible oxygen uptake rate (OD) value was attained. Then η decreased from 1.00 to 0.7, 0.3 and 0.19 at LOT, MOT and HOT operations respectively, meaning that the microorganisms are utilizing oxygen under the maximum oxygen demand.

The change of maximum oxygen uptake rate, oxygen demand (OD), with cultivation time is given in Figure 4.58. While, LOT attained the highest profile for maximum oxygen utilization rate and HOT attained the lowest profile. For HOT, higher oxygen transfer rate supplied with Q_0/V_R= 0.5 vvm and N=750 min^{-1}, inhibited the growth of the microorganism, lowered its oxygen demand during the bioprocess and finally resulted a dissolved oxygen concentration profile as seen in Figure 4.44. Although OD attained the highest profile at LOT operation due to the highest oxygen demand profile by *Bacillus thermoantarcticus*, cells did not grow as a result of the insufficient oxygen supply to the fermentation broth.

Film effectiveness factor, η, is inversely proportional with Da and this proportionally can be noticed in Figure 4.59. For example for operation HOT, as Da took values, 0.14, 1.06 and 7.57; η k_La took values 1, 0.19 and 0.04 respectively. As the maximum oxygen utilization rate increased, Da increased and consequently, η decreased which is inversely proportional to the maximum oxygen utilization rate.

In this study, oxygen transfer conditions were decided by taking the study of Kocabaş (2004) as a reference. Kocabas studied aromatic amino acid synthesis performance of *Bacillus acidocaldarius*. *Bacillus acidocaldarius* is an aerobic and thermo-acidophilic and chemoheterotroph bacterium (Darland and Brock, 1971). Both *Bacillus thermoantarcticus* (Nicolaus et al.,1996) and *Bacillus acidocaldarius* (Nicolaus et al.,1998) were isolated from the geothermal soil in Antarctica (Mount Melbourne and Mount Rittmann respectively). They have the same optimum growth temperature as 63°C. *Bacillus acidocaldarius* grows in a more acidic medium (pH=4.0) when compared to *Bacillus thermoantarcticus (pH=6.0).* While G+C mole% of *Bacillus acidocaldarius* was found as 64.3, it was found as 53.7 in *Bacillus thermoantarcticus*. Kocabaş (2004) investigated the effects of oxygen transfer at four different conditions at the parameters: air inlet rates of Q_o/V_R = 0.2, and 0.5 vvm, and agitation rates of N= 250, 500, 750 rpm.

As a result, the effect of these parameters on cell growth and product formation in the study of Kocabaş (2004) was useful in deciding the oxygen transfer parameters for *Bacillus thermoantarcticus* growth and glucose isomerase production. Some similar points comprising the oxygen transfer parameters evaluated in the present work and the study performed by Kocabaş (2004) are discussed in order to compare the effects of oxygen transfer conditions on these two thermophilic *Bacillus* species.

Kocabaş (2004) found that K_La firstly increased with time and then decreased as it is found in this study. The volumetric liquid phase transfer coefficients (k_La) took the highest values at the growth phase of *Bacillus thermoantarcticus*. OUR profiles were attained inversely proportional with the agitation rate indicating the inhibitory effect of high dissolved oxygen concentrations on the metabolism of *Bacillus acidocaldarius*. The same trend was obtained in the present work that HOT showed a lower OUR profile than LOT.

Table 4.13 The variations in oxygen transfer parameters with cultivation time, stirring and air inlet rates

Condition	t	k_La (s^{-1})	E k_La/k_La_0	$OTRx10^3$ ($mol\ m^{-3}\ s^{-1}$)	$OTR_{max}x10^3$ * ($mol\ m^{-3}\ s^{-1}$)	$(OTR_{max}x10^3)'$ * ($mol\ m^{-3}\ s^{-1}$)	$OURx10^3$ ($mol\ m^{-3}\ s^{-1}$)	$ODx10^3$ ($mol\ m^{-3}\ s^{-1}$)	Da OD/OTR_{mx}	η OUR/OD
LOT	0.5	0.0080	1.33	0.49	1.44	1.39	1.60	1.60	1.11	1.0
	1	0.0090	1.50	0.86	1.62	1.56	1.65	2.37	1.46	0.70
MOT	0.5	0.0145	1.61	0.62	2.61	2.52	1.30	1.36	0.52	1.0
	2	0.017	1.89	2.07	3.06	2.89	2.15	8.05	2.63	0.3
	16	0.015	1.67	0.98	2.70	2.07	0.90	706.7	281.85	0.001
	24	0.012	1.33	0.51	2.16	1.44	0.50	_	_	_
HOT	0.5	0.03	2.46	0.18	5.40	5.22	1.00	1.00	0.19	1.00
	3	0.033	2.70	0.60	5.94	5.54	1.2	6.3	1.14	0.19
	12	0.021	1.72	1.17	3.78	3.09	1.1	28.6	9.26	0.04
	24	0.013	1.07	0.60	2.34	1.56	0.6	_	_	_

*OTR_{max} values are calculated in two ways. OTR_{max} is equal to the product of K_La and C_{DO}. However, $(OTR_{max})'$ is calculated by the product of K_La and C_{DO} which is measured by assuming the solubility of oxygen, $C_{DO}*$ in the fermentation broth changes in linear with time.

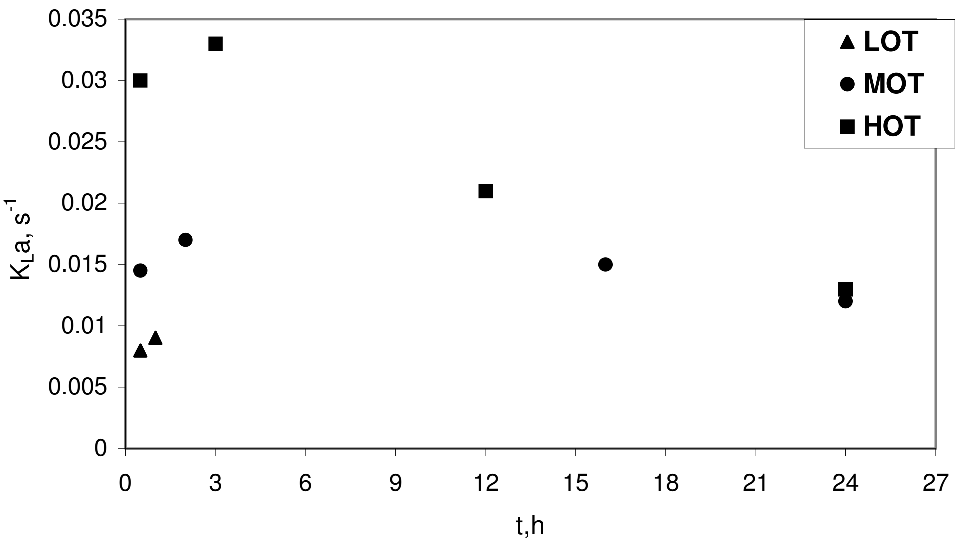

Figure 4.48 The variation of K_La with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

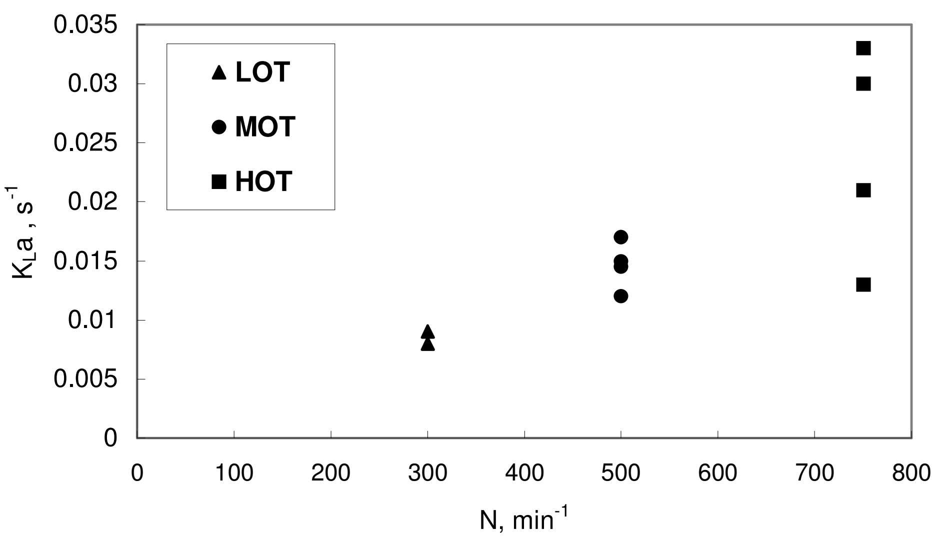

Figure 4.49 The variation of K_La with stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

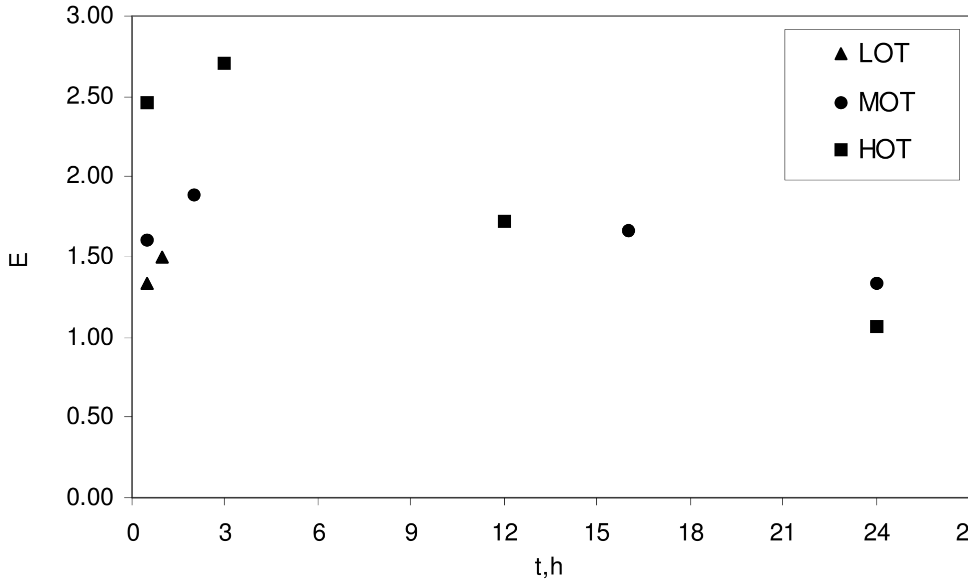

Figure 4.50 The variation of E with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

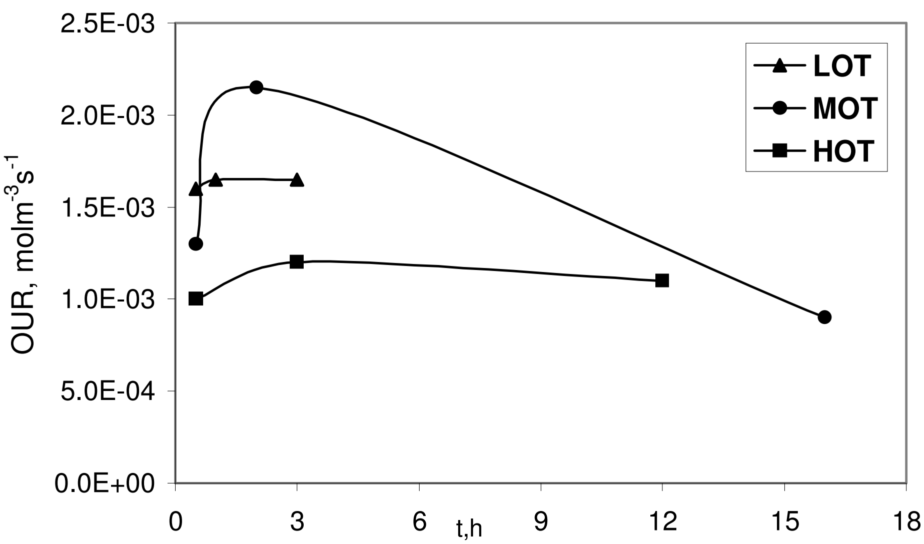

Figure 4.51 The variation of OUR with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

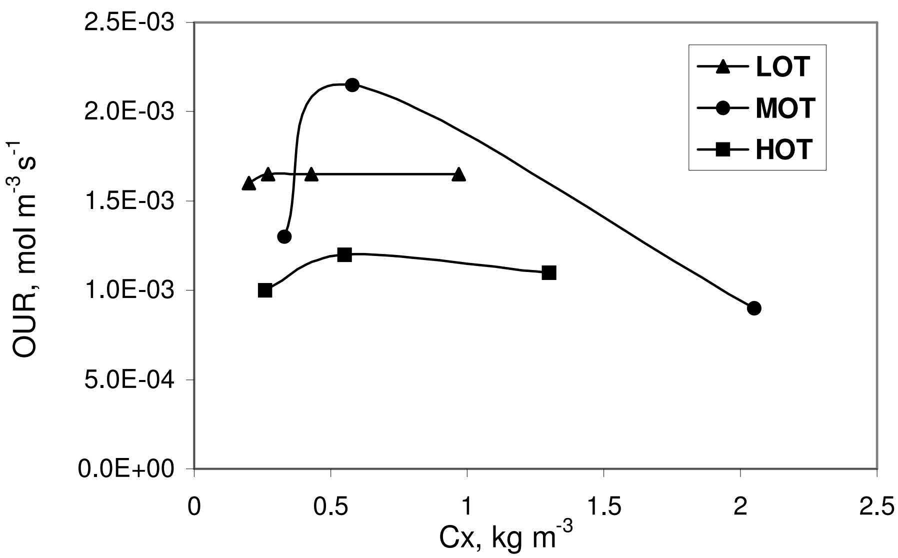

Figure 4.52 The variation of OUR with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

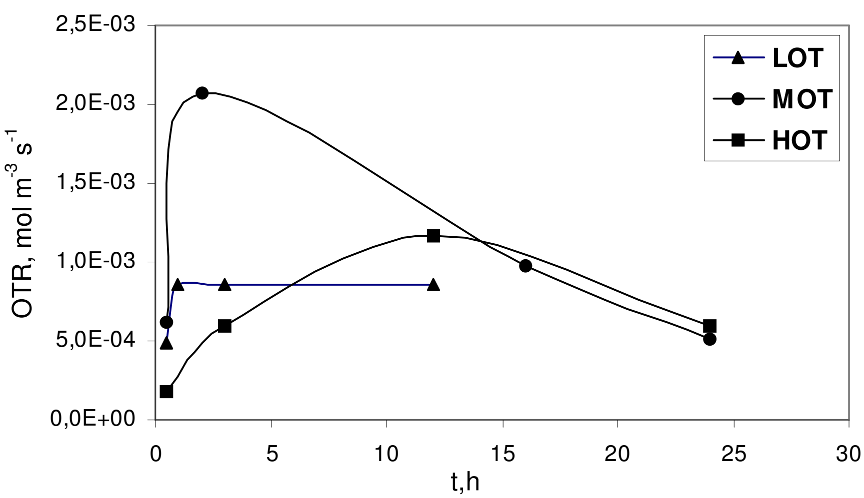

Figure 4.53 The variation of OTR with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

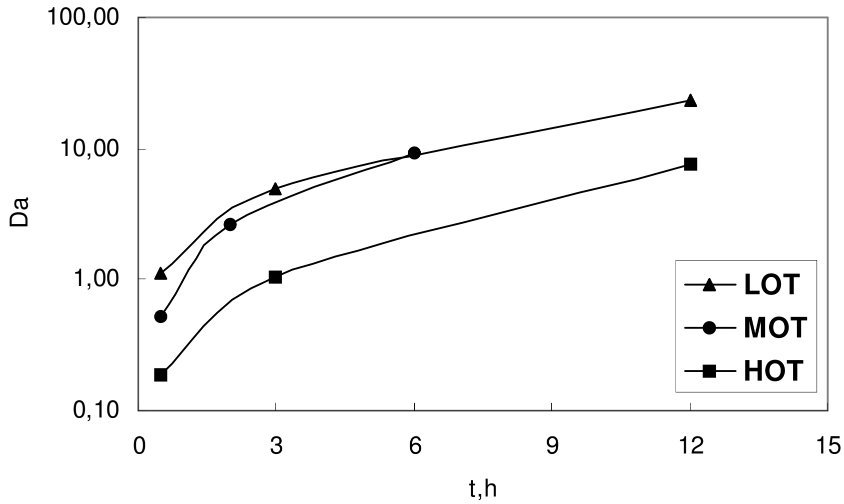

Figure 4.54 The variation of Da (OD/OTR_{max}) with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

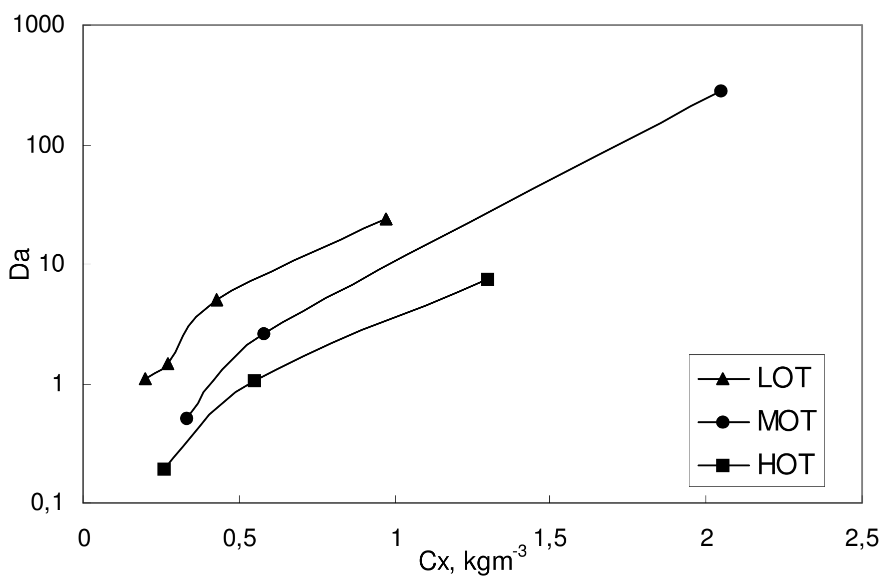

Figure 4.55 The variation of Da (OD/OTR_{max}) with cell concentration, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

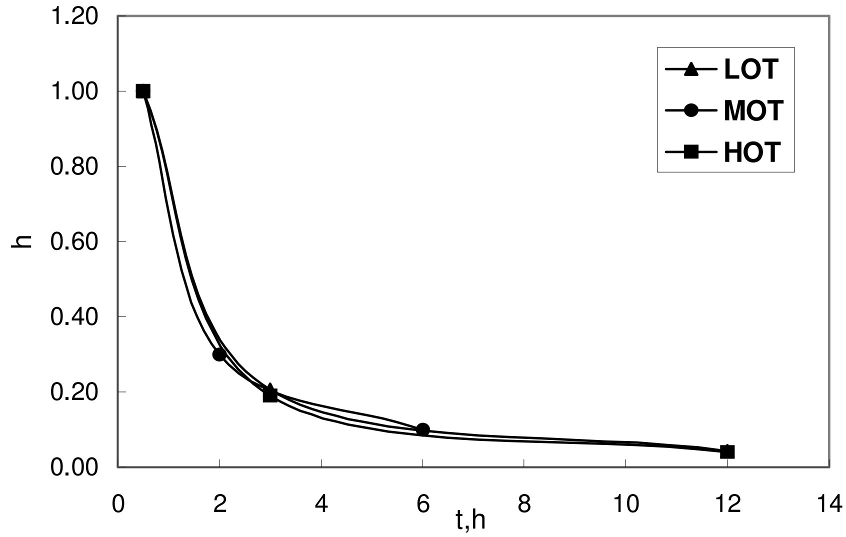

Figure 4.56 The variation of η (OUR/OD) with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

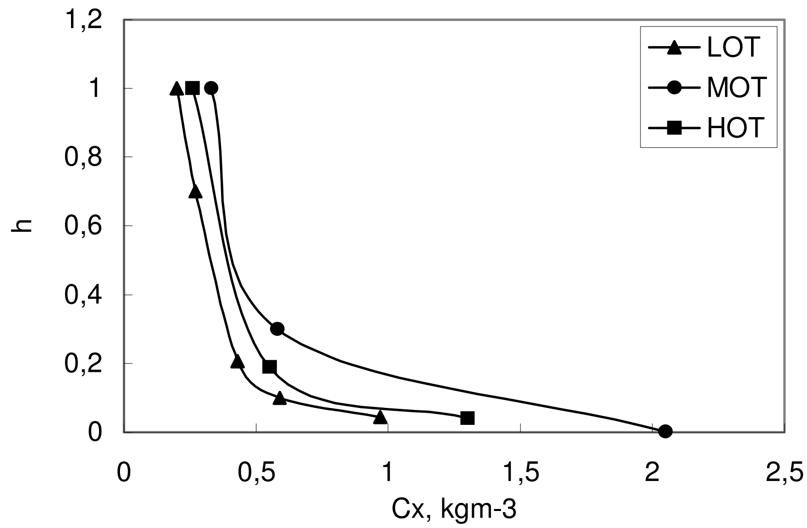

Figure 4.57 The variation of η (OUR/OD) with cell concentration, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

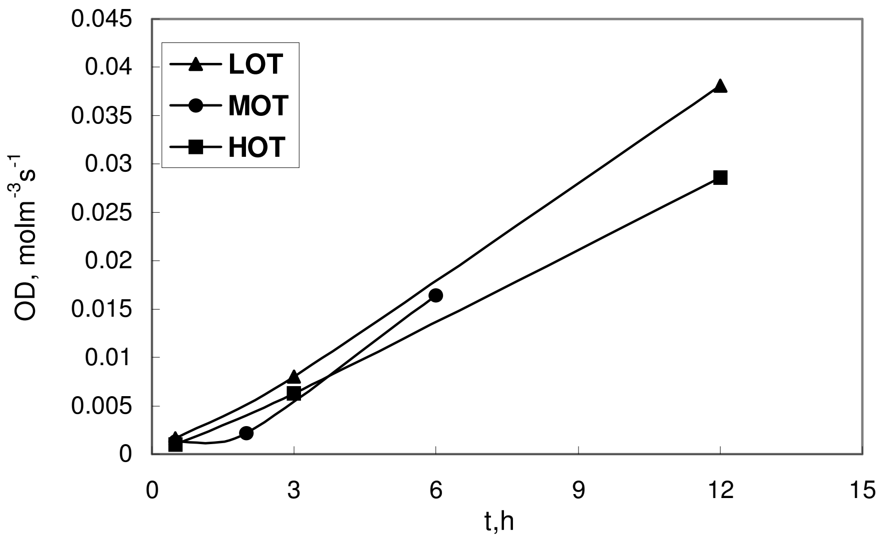

Figure 4.58 The variation of OD with cultivation time, stirring and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

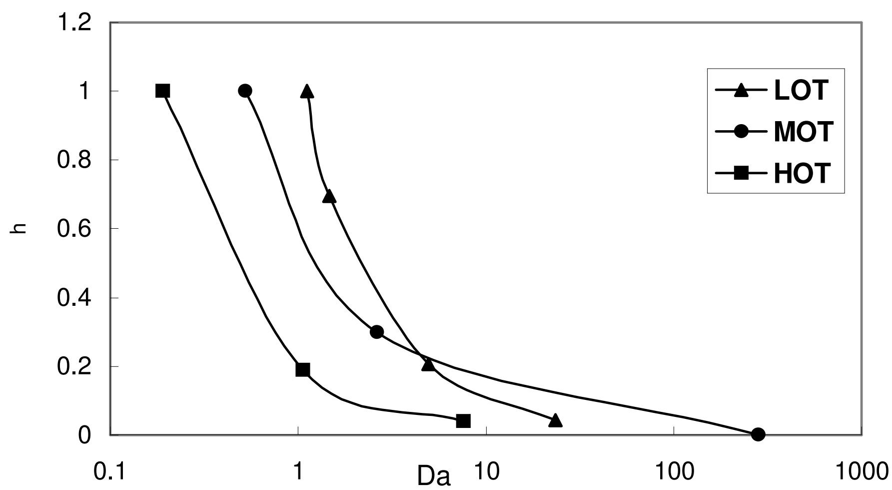

Figure 4.59 The relationship between Da and η at different oxygen transfer conditions at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

4.4.2.6 Specific Growth Rate, Yield and Maintenance Coefficients

The variations in the specific growth rate, μ, the specific oxygen uptake rate, q_0, maintenance coefficients for oxygen, m_0 and m_0' and the yield coefficients with the cultivation time are given in Table 4.14.

Figure 4.60 shows the change of specific growth rate, μ with cultivation time. As seen, specific growth rate, μ, decreases with cultivation time and approaches to 0 as the cells get closer to the stationary phase. The maximum value of μ was attained at t=0.5 h of MOT operation as 1.71 h^{-1} in which highest cell concentration was achieved among the three operation conditions (C_x=2.05 kg m^{-3} at t=16 h).

The variation of specific oxygen uptake rate q_0 with cultivation time, air inlet rate, agitation rate are given in Figure 4.61. Specific oxygen uptake rate was observed to decrease with the cultivation time at all the oxygen transfer conditions. While the highest value q_0 was obtained as 0.46 kg kg^{-1} h^{-1} at t=0.5 h of MOT operation; the lowest value was obtained at t=12 of LOT operation as 0.07 kg kg^{-1} h^{-1}.

As seen in Figure 4.62, $Y_{X/O}$ was observed to decrease with cultivation time. Among all operations, the highest $Y_{X/O}$ was attained as 4.64 kg kg^{-1} at t=0.5 of LOT operation and the lowest $Y_{X/O}$ was obtained as 0.03 kg kg^{-1} at t=12 h of HOT operation.

The change of r_p (U L^{-1} h^{-1}) with cultivation time is seen in Figure 4.63. The highest r_p was attained at t=12 h of MOT operation as 162 U L^{-1} h^{-1}. The variations of q_p with cultivation time, air inlet rate, agitation rate are given in Figure 4.64. As seen in Figure 4.64, in most of the operation conditions, q_p decreased until t=9 and increased sharply. This sharp increase in specific glucose isomerase production rate can be related to the sharp increase of GI activity between hours t=9-12. The highest q_p was obtained as $2.5*10^5$ U kg $cell^{-1}$ h^{-1} at t=3 h of LOT. However, after t=6, the highest specific glucose isomerase production rate was observed at HOT condition that after t=6, q_p favored the highest oxygen transfer conditions. The lowest q_p was obtained as $7.9*10^5$ U kg cell^{-1} h^{-1} at t=16 h of LOT. The variations of $Y_{P/X}$ with cultivation time, air inlet rate, agitation rate are given in Figure 4.65. Until t=12 $Y_{P/X}$ did not vary significantly. However, after t=12 h for all conditions $Y_{P/X}$ changed remarkably. The highest $Y_{P/X}$ was attained as $10*10^6$ U kg cell^{-1} at t=16 of HOT. The lowest $Y_{P/X}$ was observed as $4.7*10^5$ U kg cell^{-1} at t=3 h of MOT.

From the slope of the plot of $1/Y_{X/O}$ versus $1/\mu$, the rate of oxygen consumption for maintenance was obtained, and the variation of m_0 and m_0' with pH strategy are given in Table 4.11. The m_0 varied between 0.119 and 0.272 kg kg^{-1} h^{-1} and m_0' varied between 0.018 and 0.201 kg kg^{-1} h^{-1}. The highest value for m_0 and m_0' were obtained at MOT operation as 0.272 and 0.201 kg kg^{-1} h^{-1} respectively. The calculation procedure of maintanence coefficients, m_0 and m_0' were explained in section 2.4.2.2; the Leudeking-Piret constants calculated for the bioprocesses are given in Table 4.15.

Figure 4.14 The variations in specific growth rate and yield coefficients

Condition	t	μ h^{-1}	r_o kg O_2 $m^{-3}h^{-1}$	q_o kg O_2 kg^{-1} h^{-1}	r_p $UL^{-1}h^{-1}$	q_p U kg $cell^{-1}$ h^{-1}	$Y_{x/o}$ kg cell kg O_2^{-1}	$Y_{p/o}$ U kg O_2^{-1}	$Y_{p/x}$ U kg $cell^{-1}$	m_0 kg kg^{-1} h^{-1}	m_0' kg kg^{-1} h^{-1}
LOT	0.5	1,47	0.18	0.92	-	-	1.60	-	-	0.314	0.036
	1	0.52	0.19	0.70	-	-	0.67	-	-		
MOT	0.5	1.71	0.15	0.46	-	-	3.71	-	-	0.272	0.201
	2	0.46	0.25	0.43	-	-	1.07	_	-		
	16	0.0012	0.10	0.05	24.5	$1.2*10^4$	0.04	$2.4*10^5$	$6*10^5$		
	24	_	0.06	_	29.5	-	-	$5.1*10^5$	-		
HOT	0.5	1.57	0.12	0.44	-	-	3.54	-	-	0.204	0.124
	3	0.3	0.14	0.25	83.4	$1.50*10^5$	1.20	$6.1*10^5$	$5*10^5$		
	12	0.06	0.13	0.10	115.03	$1.2*10^5$	0.63	$1.2*10^6$	$1.9*10^6$		
	24	-	0.07	-	32.57	-	-	$4.7*10^5$	-		

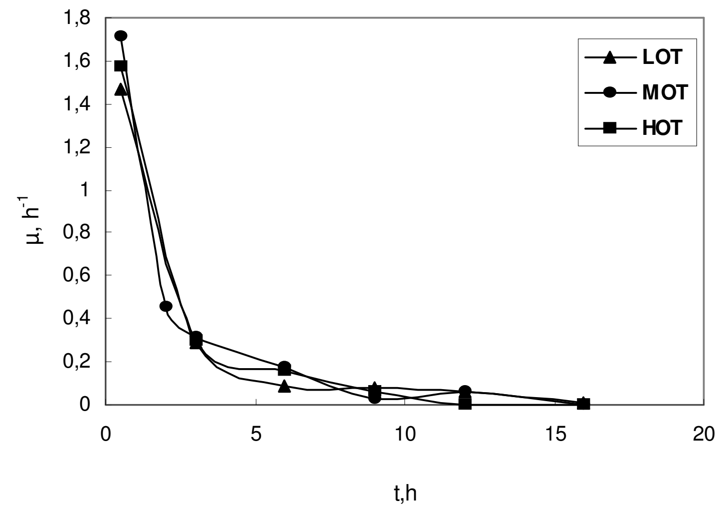

Figure 4.60 The variations in the specific growth rates with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

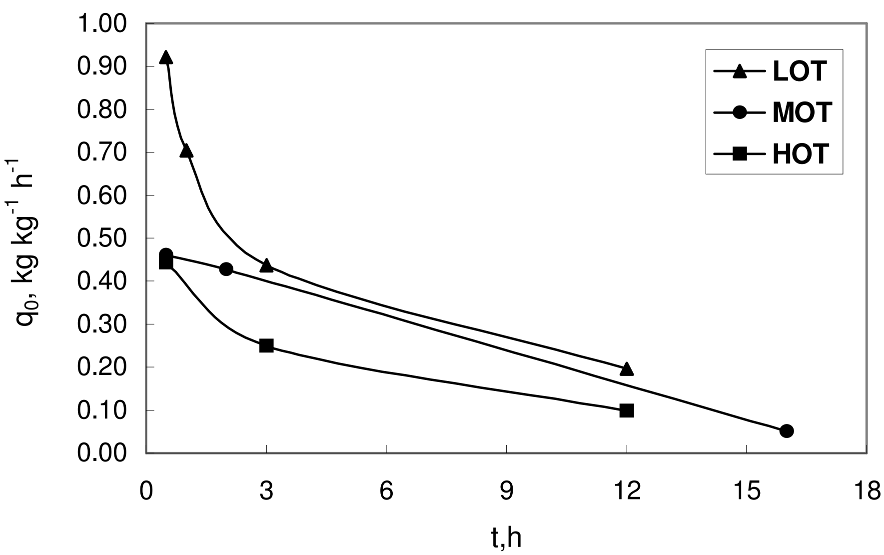

Figure 4.61 The variations in the specific oxygen uptake rates with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

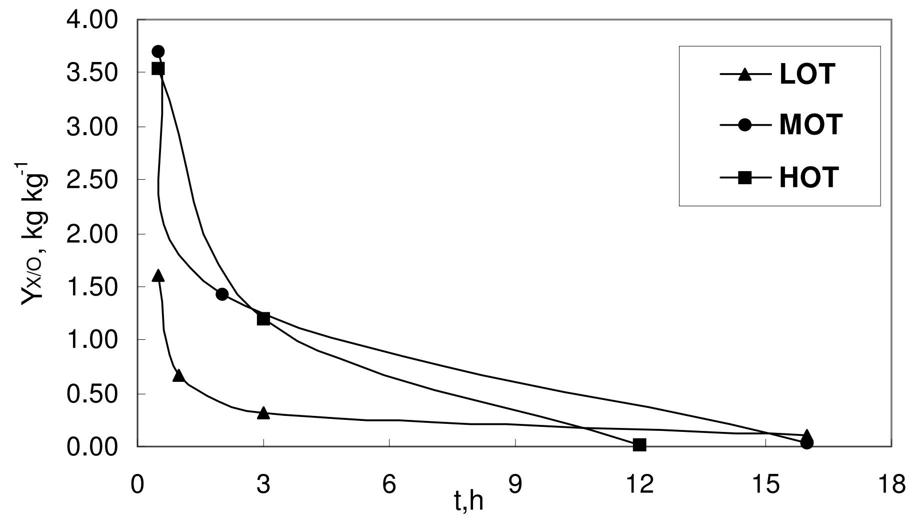

Figure 4.62 The variations in the yield coefficient, $Y_{X/O}$ with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

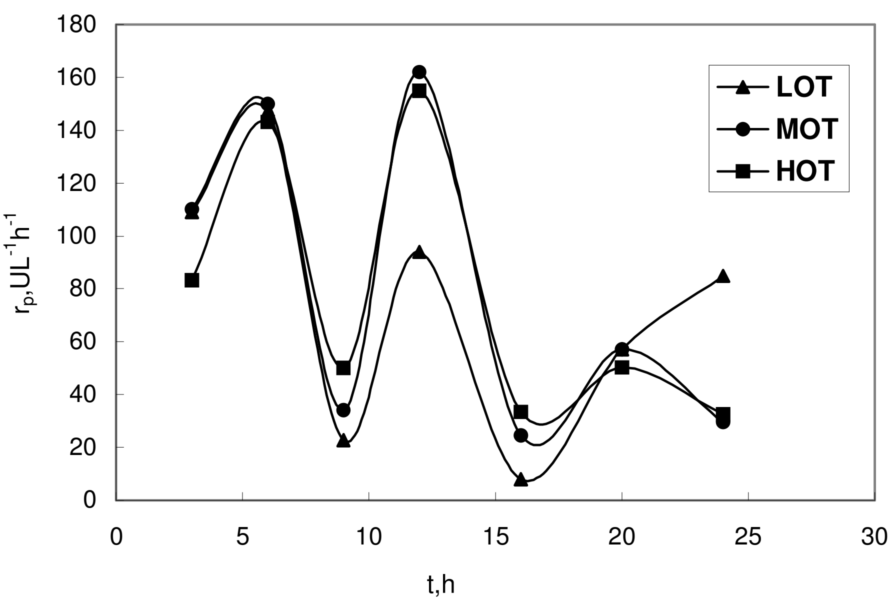

Figure 4.63 The variations in the r_p with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

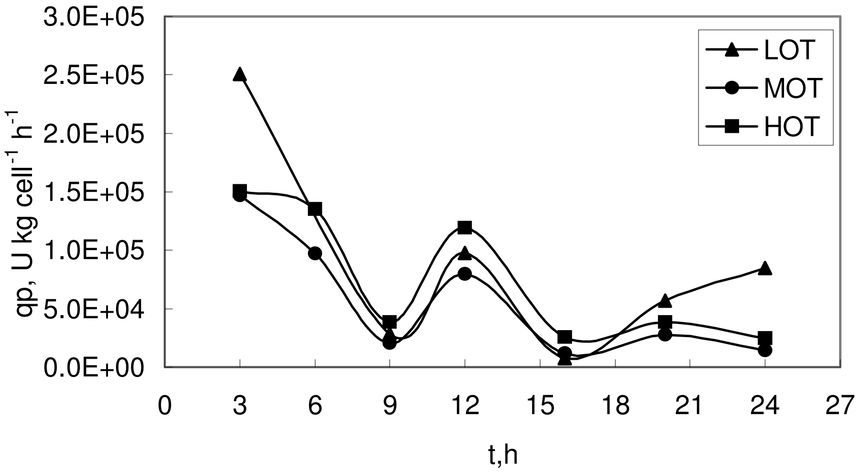

Figure 4.64 The variations in the q_p with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

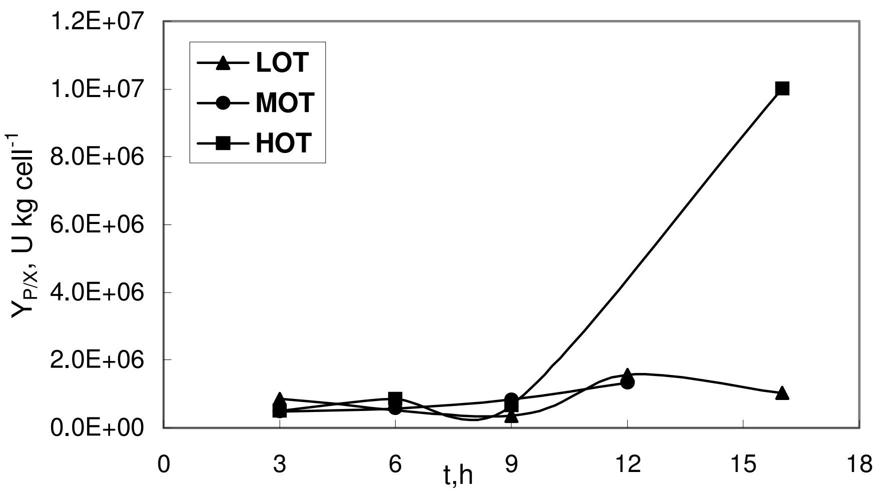

Figure 4.65 The variations in the $Y_{P/X}$ with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

Table 4.15 The variations in Leudeking-Piret constants

Condition	α	β
LOT	0.215	0.133
MOT	0.122	0.031
HOT	0.178	0.040

4.4.2.7 Organic and Amino Acid Concentration Profiles

The variations in the organic acids detected in the fermentation broth with cultivation time and oxygen transfer conditions were given in Tables 4.13, 4.14 and 4.15. As seen in Tables 4.16, 4.17, 4.18 for all oxygen transfer conditions, acetic acid and formic acids are the major by-products in the fermentation medium. While, at MOT and HOT formic acid is the highest organic acid synthesized, acetic acid is the highest organic acid synthesized at LOT. The highest total organic acid concentrations was obtained as 0.721 kg m^{-3} at t=16 h of LOT.

In addition to acetic and formic acids, at all operation conditions moderate concentrations of fumaric and oxalic acids were excreted during bioprocess. Also, at MOT and HOT, high amounts of citric acid were observed to be produced.

Figure 4.66 shows the variation of total organic acid concentration with cultivation time, air inlet rate, agitation rate and pH strategy. As seen in Figure 4.66, while LOT showed the highest profile for organic acid production, HOT shows the lowest profile for organic acid production. In addition, after t=20 h, total amount of organic acid seemed to stay constant at all operations.

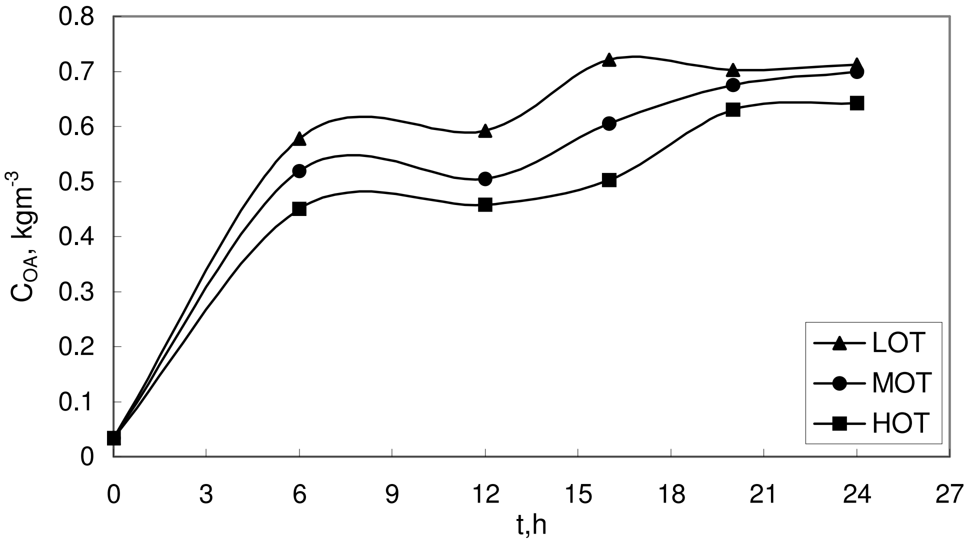

Figure 4.66 The variation of total organic acid concentration with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

Table 4.16 The variations in organic acid concentrations with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

LOT														
Time (h)	Ac	Cit	Form	Fum	Gluc	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc	ΣC_{OA}, kgm^{-3}
0	0.01	-	0.0068	0.0003	-	-	0.0084	0.0039	-	0.0048	-	-	-	0.034
6	0.230	-	0.218	0.0035	-	0.015	-	0.053	-	0.059	-	-	-	0.578
12	0.207	-	0.060	0.0036	-	-	0.190	0.029	-	0.041	-	-	-	0.592
16	0.390	-	0.152	0.0040	-	0.015	-	0.036	0.076	0.047	-	-	-	0.721
20	0.398	-	0.161	0.0039	-	-	-	0.034	0.061	0.045	-	-	-	0.703
24	0.396	-	0.166	0.0040	-	-	-	0.032	0.064	0.052	-	-	-	0.713

Table 4.18 The variations in organic acid concentrations with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

MOT														
Time (h)	Ac	Cit	Form	Fum	Gluc	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc	ΣC_{OA}, kgm^{-3}
0	0.01	-	0.0068	0.0003	-	-	0.0084	0.0039	-	0.0048	-	-	-	0.034
6	0.131	-	0.085	0.0035	-		0.134	0,011	-	0.055	-	-	-	0.519
12	0.163	0.06	0.162	0.0040	-	0.014	-	-	0.057	0.047	-	-	-	0.505
16	0.136	0.16	0.195	0.0042	-	0.015	-	-	0.049	0.053	-	-	-	0.606
20	0.163	0.17	0.215	0.0049	-	0.018	-	-	0.058	0.046	-	-	-	0.675
24	0.175	0.19	0.215	0.0054	-	0.020	-	-	0.061	0.035	-	-	-	0.700

Table 4.18 The variations in organic acid concentrations with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

HOT														
Time (h)	Ac	Cit	Form	Fum	Gluc	αKG	Lac	Male	Mal	Ox	OA	Pyr	Suc	ΣC_{OA}, kgm^{-3}
0	0.01	-	0.0068	0.0003	-	-	0.0084	0.0039	-	0.0048	-	-	-	0.034
6	0,123	0.041	0.159	0.0030	-	0.070	-	-	-	0.054	-	-	-	0.450
12	0.130	0.072	0.158	0.0036	-	-	-	-	0.044	0.050	-	-	-	0.458
16	0.134	0.090	0.176	0,0037	-	-	-	-	0.045	0.054	-	-	-	0.503
20	0.172	0.200	0.213	0.0041	-	-	-	-	-	0.043	-	-	-	0.631
24	0.191	0.197	0.197	0.0043	-	-	-	-	-	0.053	-	-	-	0.643

The variations in the amino acids detected in the fermentation broth with cultivation time and oxygen transfer conditions were given in Table 4.19. At t=0 h for all bioreactor conditions, the following amino acids coming from the precultivation medium were Asp, Glu, Ala, Tyr, Phe, Trp. In general, the major amino acids excreted into the broth during the fermentation are Asp, Glu, Phe and Trp.

In general, the highest total amino acid concentration was observed at the beginning of the operation, at t=6 h. The highest ΣC_{AA} was attained at HOT as 0.326 kg m^{-3} at t=6 h. HOT shows the highest ΣC_{AA} profile when compared to the other operations indicating that higher oxygen transfer conditions resulted higher amino acid profiles.

The major amino acid synthesized among all operation conditions is Tryptophan. The highest tryptophan production is observed at LOT (N=300 min^{-1} and Q_0/V_R =0.5 vvm) as 0.094 kg m^{-3} at t=6h and 12 h. This shows that tryptophan production favored low oxygen transfer conditions. The study done by Kocabas also supports this result. As previously mentioned, Kocabas (2004) studied aromatic aminoacid synthesis of *Bacillus acidocaldarius*. In that study, effects of several oxygen transfer operation on tryptophan production were compared and the highest Trp production was attained at lower oxygen transfer conditions such as N=250 min^{-1}, Q_0/V_R=0.2 vvm (LimOT) and N=250 $min^{-}1$, Q_0/V_R=0.5 vvm (LOT). The maximum tryptophan concentration was attained as 0.32 kg m^{-3} at the condition LimOT.

As seen in Table 4.19 production of the other aromatic amino acids Phenylalanine (Phe) and Tyrosine (Tyr) can be observed. However, they were not produced as much as Tryptophan. Other aminoacids produced significantly during the fermentation are glutamic acid and aspartic acid. The highest glutamic and aspartic acid production was obtained as 0.055 and 0.044 kg m^{-3} of at t=6 of HOT respectively.

Table 4.19 The variations in amino acid concentrations with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0.

LOT												
Time (h)	Asp	Glu	His	Arg	Thr	Ala	Tyr	Val	Met	Phe	Trp	ΣC_{AA}, kgm^{-3}
0	0.00058	0.0007		0.0004		0.0035	0.0069			0.0028	0.0059	0.021
6		0.031	0.036		0.047	0.020	0.030			0. 037	0.094	0.295
12	0.0029	0.017			0.024				0,0026	0.0025	0.094	0.143
16		0.0078			0.024	0.017			0.0028	0.0034	0.089	0.144
20		0.020			0.023	0.016			0,0032	0.035	0.086	0.183
24		0.023			0.023	0.016			0.0035	0.0037	0.083	0.152

MOT												
Time (h)	Asp	Glu	His	Arg	Thr	Ala	Tyr	Val	Met	Phe	Trp	ΣC_{AA}, kgm^{-3}
0	0.00058	0.0007		0.0004		0.0035	0.0069			0.0028	0.0059	0.021
6	0.0071	0.021		0.044		0.028	0.037			0.031	0.057	0.225
12	0.0095	0.041	0.011	0.017		0.023	0,054		0,0039	0.01	0.059	0.228
16	0.018	0.017	0.017	0.0063		0.019		0.03	0.0043	0.0037	0.063	0.178
20	0.015	0.016	0.018								0.065	0.114
24	0.012	0.015	0.019			0.021				0.0046	0.070	0.142

Table 4.19 The variations in amino acid concentrations with the cultivation time, stirring rate and air inlet rates at conditions, T=55°C, V_R= 1.65x10^{-3} m^3, Q_o/V_R=0.5 vvm, N=300, 500, 750 min^{-1}, pH_c=6.0 (Continued)

HOT												
Time (h)	Asp	Glu	His	Arg	Thr	Ala	Tyr	Val	Met	Phe	Trp	ΣC_{AA}, kgm^{-3}
0	0.00058	0.0007		0.0004		0.0035	0.0069			0.0028	0.0059	0.021
6	0.044	0.055			0.045	0.032	0.036			0.047	0.067	0.326
12	0.033	0.038			0.038	0.034	0.036			0.041	0.073	0.293
16	0.018				0.035	0.032	0.032			0.034	0.069	0.220
20	0.017				0.027	0.026	0.028			0.026	0.071	0.195
24	0.016				0.018	0.021	0.021			0.017	0.084	0.177

CHAPTER 5

CONCLUSION

In this study, the effects of bioprocess parameters on glucose isomerase production by *Bacillus thermoantarcticus* were investigated. For this purpose, firstly, the production medium was designed in terms of its carbon and nitrogen sources in laboratory scale bioreactors. For determining the optimum combination of medium components that yields the highest glucose isomerase activity, the statistical approach, Response Surface Methodology (RSM) was employed. Thereafter, using the optimized medium the effects of bioreactor operation parameters, i.e., pH and oxygen transfer conditions were investigated in the pilot scale bioreactor.

5.1 Experiments performed in laboratory scale bioreactors

The following conclusions were drawn for the experiments performed in laboratory scale bioreactors:

1. The effects of carbon sources, glycerol, xylan and xylose and salt solution with the given composition, 0.25 kg m^{-3} $MgSO_4.7H_2O$, 0.001 kg m^{-3} $FeSO_4.7H_2O$, 0.001 kg m^{-3} $ZnSO_4.7H_2O$, 0.000075 kg m^{-3} $MnSO_4.\ H_2O$, 0.00001 kg m^{-3} $CuSO_4.5H_2O$ were investigated in the medium containing: 10 kg m^{-3} of carbon source, 6 kg m^{-3} of yeast extract, 3 kgm^{-3} of NaCl, 2.5 kg m^{-3} of peptone, 1.5 kg m^{-3} of beef extract (reference medium) between t=0-9 h of the bioprocess. The medium containing 10 kgm^{-3} xylan with 33 μl of salt solution attained the highest glucose

isomerase volumetric activity and cell concentration as 720 UL^{-1} and 0.61 kgm^{-3} respectively at t=9 h.

2. The effects of organic nitrogen sources, beef extract and peptone, were examined in the medium containing: 10 kg m^{-3} of xylan, 6 kg m^{-3} of yeast extract, 3 kg m^{-3} of NaCl, 2.5 kg m^{-3} of peptone, 1.5 kg m^{-3} of beef extract with 33 µl salt solution between t=12-24 h of the bioprocess. The highest GI volumetric activity was attained in the medium containing: 10 kg m^{-3} of xylan, 6 kg m^{-3} of yeast extract, 3 kg m^{-3} of NaCl with 33 µl salt solution, as 1330 U L^{-1} at t=24 h of the bioprocess.

3. The effects of inorganic nitrogen sources 6 kg m^{-3} of $(NH_4)_2HPO_4$, NH_4Cl, $(NH_4)_2SO_4$ and yeast extract on glucose isomerase volumetric activity were investigated in the medium containing: 10 kg m^{-3} of xylan, 3 kgm^{-3} of NaCl with 33 µl salt solution between t=0-13 h of the bioprocess. The highest GI volumetric activity was obtained in the medium containing 6 kg m^{-3} of $(NH_4)_2SO_4$ as 932 U L^{-1} and the highest cell concentration was attained as 0.81 kg m^{-3} at t=13 h.

4. The effect of NaCl on GI volumetric activity was evaluated in the medium containing: 10 kg m^{-3} of xylan, 6 kg m^{-3} of yeast extract, 3 kg m^{-3} of NaCl with 33 µl salt solution between t=0-13 h of the bioprocess. The highest GI volumetric activity was obtained in the medium without NaCl as 1032 U L^{-1} and the highest cell concentration was attained as 0.83 kg m^{-3} at t=13 h.

5. The variation of GI volumetric activity with cultivation time was investigated in the medium containing: 10 kg m^{-3} of xylan, 5 kg m^{-3} of yeast extract, 5 kg m^{-3} of ammonium sulfate with 33 µl salt solution. The highest GI volumetric activity was obtained as 1618 U L^{-1} at t=24 h. As a result, the following experiments peformed in laboratory scale bioreactors were carried out at t=24 h.

6. For the medium design experiment with RSM, approximate ranges of xylan, yeast extract and $(NH_4)_2SO_4$ were obtained as 7-13, 3-7, 4-8 kgm^{-3} respectively. The experiment performed with RSM resulted the optimum combination of medium components: 10.64 kg m^{-3} of xylan, 5.66 kg m^{-3} of yeast extract and 5.92 kg m^{-3} of $(NH_4)_2SO_4$ with a GI volumetric activity of 1630 U L^{-1} at t=24 h. This value was 1.6 fold higher than the glucose isomerase activity obtained in the reference medium at t= 24 h (1020 U L^{-1}).

7. The optimum initial pH was evaluated in the designed medium and obtained as pH_0=6.0. The glucose isomerase activity at t=24 h and cell concentration at t=9 h of the cultivation were found as 1630 U L^{-1} and 0.75 kg m^{-3} at pH_0=6 respectively.

8. The effect of xylan types, birchwood, oat spelts and beechwood on GI volumetric acitivity was examined in the designed medium and the highest activity was obtained in the medium containing birchwood xylan as 1630 U L^{-1} at t=24 h.

9. The effects of carbon sources glucose, fructose, sucrose and xylan on GI volumetric activity and xylanase activity were carried out in the designed medium. The medium containing sucrose attained cell concentration of Cx=0.65 kg m^{-3} at t=9 h and GI volumetric activity of 1460 U L^{-1} at t=24 h which is close to the GI volumetric activity obtained in the xylan containing medium (1630 U L^{-1}). However, sucrose containing medium showed 0.62 U ml^{-1} xylanase activity which is about 10% of the xylanase activity obtained in the medium containing xylan (6.3 U ml^{-1}).

5.2 Experiments performed in pilot scale bioreactor

Using the defined medium the effects of bioreactor operation conditions, pH and oxygen transfer conditions were investigated. Following conclusions were drawn for the experiments performed in pilot scale bioreactor:

5.2.1 The influence of controlled-pH and uncontrolled-pH operations on glucose isomerase production.

1. In order to investigate the effect of pH control, pH=6 uncontrolled and controlled operations at an air inlet rate of Q_0/V_R=0.5 vvm and agitation rate of N=500 min^{-1} were applied in pilot scale batch bioreactor. The medium contained 10.64 kg m^{-3} of xylan, 5.66 kg m^{-3} of yeast extract and 5.92 kg m^{-3} of $(NH_4)_2SO_4$ which was designed by RSM in the laboratory scale bioreactors.

2. The highest GI volumetric activity was attained as 1838 U L^{-1} at t=24 h of MOT-pHuc condition in which the highest cell concentration was obtained, Cx=2.26 kg m^{-3}. On the other hand, at MOT-pHc GI volumetric activity was attained as 1814 U L^{-1} in which the cell concentration was 2.05 kgm^{-3}. However, the highest specific GI activity was obtained as 885 U g^{-1} DW at t=24 h of MOT-pHc. In addition, the highest xylanase volumetric activity was attained as 9.3 U ml^{-1} at t=24 h of MOT-pHuc and 8.6 U ml^{-1} at t=24 h of MOT. The highest specific xylanase activity was attained as 4.2 U mg^{-1} at t=24 h of MOT-pHc.

3. Dissolved oxygen profile of uncontrolled-pH operation was higher than controlled-pH operation. Dissolved oxygen decreased sharply in the first 3 hours of the process in both conditions due to the high metabolic activity of cells and increased after t=9–12 h of the bioprocess depending on the period of growth phases of the microorganism. In general, the oxygen uptake rates (OUR) were higher at growth phase of the process, where the cell formation and the substrate consumption rates, i.e., metabolic activity of the cells were high (Calik et al., 2006). OUR profile of MOT-pHc was higher than that obtained at MOT-pHuc which is in accordance with C_{DO} profile. pH control increased OTR slightly higher depending on the difference between the equilibrium concentration and the dissolved oxygen concentration in the medium.

4. K_La and consequently the biological enhancement factor, E was higher in in the growth phase of the bioprocess than in the glucose isomerase

production phase. In addition, higher K_La values were obtained at the controlled pH operation.

5. Damköhler number (Da), effectiveness factor (η) were determined to compare the mass transfer and biochemical reaction rates. MOT-pHc showed slightly higher Da profile, which indicated that mass transfer resistances were more effective at the controlled pH operation.

6. The maximum value of μ was attained as 1.72 h^{-1} and 1.71 h^{-1} at t=0.5 h of MOT-pHuc and MOT-pHc in which the highest cell concentrations were obtained as 2.26 and 2.05 kgm^{-3} respectively.

7. The cell yield on oxygen, $Y_{X/O}$ decreased—beginning from its maximum value 4.18 and 3.71 kg cell kg O_2^{-1} at MOT-pHuc and MOT-pHc operations respectively. This decrease resulted from the increase of OUR in the growth phase of the bioprocesses and the decrease of cell formation rate (dCx/dt) throughout the bioprocesses. The highest product yield on cell, $Y_{P/X}$ was obtained at t=16h of MOT-pHuc as $6*10^6$ U kg $cell^{-1}$ due to the higher volumetric glucose isomerase activity obtained at the uncontrolled-pH operation.

8. Specific oxygen uptake rate (q_0) was higher at growth phase of bioprocesses; reaching the highest value at t=0.5 h of MOT-pHc as 0.46 kg kg^{-1} h^{-1} and the lowest value at t=15 h of MOT-pHuc as 0.04 kg kg^{-1} h^{-1}. Similarly, specific glucose isomerase production rate (q_P) was higher at growth phase of bioprocesses; having the highest value at t=2 h of MOT-pHuc as $2.2*10^5$ U kgm^{-3} cell h^{-1}.

9. The rate of oxygen consumption for maintenance, m_0, varied between 0.195-0.272 kg kg^{-1} h^{-1}, and the highest value was obtained as 0.272 kg kg^{-1} h^{-1} at MOT-pHc, when the oxygen consumption for by-product production was neglected. However when oxygen consumption for by-product production is considered, the rate of oxygen consumption for maintenance attained as 0.201 kg kg^{-1} h^{-1} at MOT-pHc.

10. Aromatic amino acids, Phenylalanine (Phe) and Tyroptaphan (Trp) were obtained as the major amino acid excreted into the fermentation broth at both operations.

11. The accumulation of total organic acids was obtained at the lowest value at MOT-pHuc condition. This should be considered as an advantage in a bioprocess that by-product formation is minimized. This result is also compatible with higher GI volumetric activity and cell concentration obtained at MOT-pHuc condition.

5.2.1 The influence of oxygen transfer conditions on glucose isomerase production.

1. The effects of oxygen transfer conditions were carried out at three different conditions: air inlet rates of Q_0/V_R=0.5 vvm, and the agitation rates of N=300, 500, 750 min^{-1}. The medium contained 10.64 kg m^{-3} of xylan, 5.66 kg m^{-3} of yeast extract and 5.92 kg m^{-3} of $(NH_4)_2SO_4$ which was designed by RSM in the laboratory scale bioreactors.

2. Among all operations, the highest GI volumetric activity was attained as 1814 U L^{-1} at t=24 h of MOT in which the highest cell concentration was obtained, Cx=2.05 kg m^{-3} at t=16 h. On the other hand, the lowest GI volumetric activity was obtained as 1718 U L^{-1} at t=24 h of LOT in which the lowest cell concentration was obtained as 1.0 kg m^{-3}. However, highest specific GI activity was observed at t=24 h of LOT operation as 1718 U g^{-1} DW and the lowest specific GI activity was observed at t=24 h of LOT operation as 884 U g^{-1} DW. In addition, the highest xylanase volumetric activity was attained as 8.6 U ml^{-1} at t=24 h of MOT. The highest xylanase specific activity was obtained as 6.8 U mg^{-1} at t=24 of LOT.

3. Dissolved oxygen concentration depends on the extent of OTR to the media and the OUR of the cells. At LOT operation, dissolved oxygen concentration dropped much more rapidly (t=1 h) and stayed contant below 0.01 mol m^{-3} as a result of lower oxygen transfer conditions (no

accumulation of C_{DO} after t=1 h). However at HOT, the lowest dissolved oxygen concentration was attained as 0.07506 mol m^{-3} at t=8.5 h due to the higher oxygen transfer conditions. After t=16 h of all operations, as the OTR values were close to the OUR values, C_{DO} did not change considerably throughout the process.

4. The highest and lowest k_La and E were obtained as 0.033-0.008 s^{-1} and 2.46-1.33 at t=3 h and t= 0.5 h of HOT and LOT respectively. It is observed that k_La values increased with an increase in stirring rate. An increase in stirring rate resulted in a rapid breakage of gas bubbles and enhancement in the gas-liquid interfacial area available for mass transfer. As a result, higher k_La profiles were obtained at higher mass transfer conditions.

5. At the beginning of the bioprocesses, biochemical reaction limitations were effective (Da<1); however, after t=2 h of the bioprocesses, mass transfer resistances became more effective (Da>>1). At the beginning of the operations (t=0.5 h), η was 1.0 at all operations indicating that the cells consumed oxygen at a high rate that the maximum possible oxygen utilization (OD) value was attained.

6. The highest specific growth rate, μ was obtained as 1.71 h^{-1} at MOT where the highest cell concentrations was obtained as 2.05 kg m^{-3}.

7. Cell yield on oxygen, $Y_{X/O}$ values decreased with cultivation time. At the beginning of the bioprocesses a sharp decrease was observed due to the increase in oxygen uptake rate during the exponential phase of the microbial growth. The highest and the lowest $Y_{X/O}$ were attained as 3.71 and 0.03 kg kg^{-1} at t=0.5 h of MOT and t=12 h of HOT operations respectively. The product yield on cell, $Y_{P/X}$ attained the highest and lowest values at t=16 h and t=3 of HOT and MOT as $10*10^6$ and $4.7*10^5$ U kg $cell^{-1}$ respectively. $Y_{P/X}$ showed an increasing profile throughout the bioprocesses due to the decrease of cell formation rate (dCx/dt) as the cells reach stationary phase of the growth.

8. Specific oxygen uptake profile, q_0 attained the highest and lowest values as 0.92 and 0.05 kg kg^{-1} h^{-1} at t=0.5 h and t= 16 h of LOT and MOT operations respectively. q_0 took higher values at the growth phase of the microorganism due to the higher metabolic activity of cells at the growth phase. Specific glucose isomerase production rate, q_P attained the highest and lowest values as 2.5*10^5 and 7.9*10^3 U kgm^{-3} cell h^{-1} at t=3 h and t=16 h of LOT operation. q_P also shows a similar fashion as q_0 that it took higher values at the growth phase of the bioprocesses.

9. The rate of oxygen consumption for maintenance, m_0, varied between 0.036-0.314 kg kg^{-1} h^{-1}, and the highest value was obtained as 0.314 kg kg^{-1} h^{-1} at LOT, when the oxygen consumption for by-product production was neglected. However when oxygen consumption for by-product production is considered, the rate of oxygen consumption for maintenance attained as 0.036 kg kg^{-1} h^{-1} at LOT. As a result, when the by-product formation was taken into account, m_0 took lower values such as from 0.272 to 0.201 and 0.204 to 0.124 kg kg^{-1} h^{-1} at MOT and HOT respectively.

10. Tryptophan was found as the major amino acid excreted during the bioprocesses. The highest tryptophan concentration was obtained at LOT operation. This result is in accordance with the study performed by Kocabaş (2004) who examined the effect of oxygen transfer conditions on tryptophan production. In both studies, tryptophan production preferred low oxygen transfer conditions.

11. The accumulation of total organic acids was obtained at the highest value at t=16 h of LOT. The lowest profile for organic acid production was attained at HOT. Acetic acid and formic acid are the major organic acids excreted into the fermentation medium. Also, moderate concentrations of fumaric and oxalic acids were excreted during bioprocesses.

When all pH and oxygen transfer conditions are analyzed together the following suggestions can be made:

1. MOT-pHuc showed the highest GI volumetric activity and cell concentration as 1838 U L^{-1} and 2.26 kg m^{-3} respectively at t=24 h of the bioprocess. When pH was controlled, GI volumetric activity and cell concentration were obtained as 1814 U L^{-1} and 2.05 kg m^{-3} respectively at t=24 h of the bioprocess. As the specific GI activity profile of MOT-pHc (885 U g^{-1} DW at t=24 h) was higher than MOT-pHuc (814 U g^{-1} DW at t=24 h), oxygen transfer conditions were analyzed by controlling the pH at pH=6.0. pH control during a bioprocess indicates the control of pH of the extracellular medium of the microorganism. Microorganisms are able to maintain the intracellular pH at a constant level, but they use energy for maintaining the proton gradient across the cell membrane. So, by controlling the pH, expenditure of this energy can be lowered. Even though pH control seems as an advantage, it lowered the GI volumetric activity in the present study. However, when the by-product formation was taken into account, uncontrolled-pH is considered as advantageous that the lowest organic acid production profile was attained at MOT-pHuc. When these results are born in mind, future studies comprising different conditions of pilot scale bioreactor experiments should be continued with a different pH strategy. Although, the highest volumetric GI activity was attained at an initial pH_0=6 at laboratory scale bioreactors, the effect of different uncontrolled-pH conditions were not performed in the pilot scale bioreactor. In this context, the effect of different uncontrolled-pH conditions can be analyzed in pilot scale bioreactors. After determining the optimum pH strategy in pilot scale bioreactor, the combination of different air inlet rates (0.2–0.7 vvm) and agitation rates can be applied in order to maximize glucose isomerase activity.

2. Effect of xylan sources such as corncobs, wheat bran and rice bran as carbon sources can be tested for the production of glucose isomerase. Use of agricultural by-products can be an alternative way for induction of the production of the glucose isomerase. Because use of cheaper carbon sources supports the suitability of the fermentation conditions for commercial glucose isomerase production. Although the use of $(NH_4)_2SO_4$ in the fermentation broth was effective for improvement of glucose isomerase activity , utilization of inorganic nitrogen sources can be minimized which are not convenient to use commercially.

3. It is concluded that *Bacillus thermoantarcticus* is a potential producer of xylanase and also β-xylosidase. These two enzymes gain their importance as the major enzymes in the digestion of xylan which is the sole carbon source used in the GI production medium of *Bacillus thermoantarcticus*. As RSM is able to define the relation between several independent variables and several responses, a medium design experiment considering the activities xylanase and β-xylosidase together can be performed. As a result, individual activities of these two enzymes can be taken into account for developing a better bioprocess for glucose isomerase production.

4. The production of glucose isomerase from *Bacillus thermoantarcticus* is advantageous when the downstream process for the production of the enzyme is considered. The utilization of xylan and also yeast extract in the fermentation medium can be considered as a disadvantage that it is much more difficult to recover glucose isomerase in a complex medium than in a defined medium. However, glucose isomerase from *Bacillus thermoantarcticus* is an intracellular enzyme that can be easily recovered simply by centrifuging the cells. As a result the type of the fermentation medium does not affect the recovery of glucose isomerase from *Bacillus thermoantarcticus.*

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APPENDIX A

Calibration of *Bacillus thermoantarcticus* Concentration

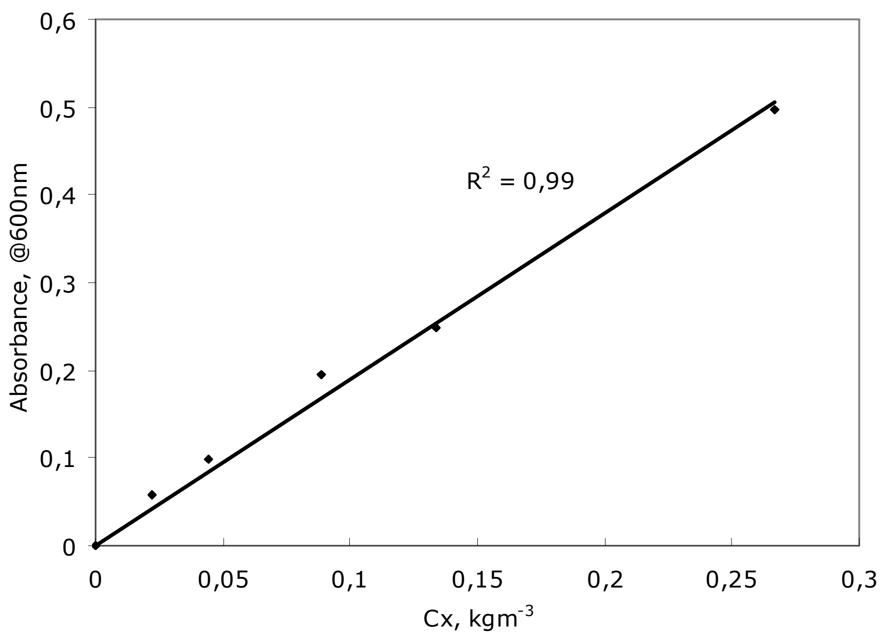

Figure A.1 Calibration curve for *Bacillus thermoantarcticus* concentration

Slope of the calibration curve, m=1.90 1/kg m-^{3} (λ=600 nm)

$$Cx = \frac{\text{Absorbance}}{1.90} \times \text{Dilution Rate}$$

APPENDIX B

Calibration of Fructose

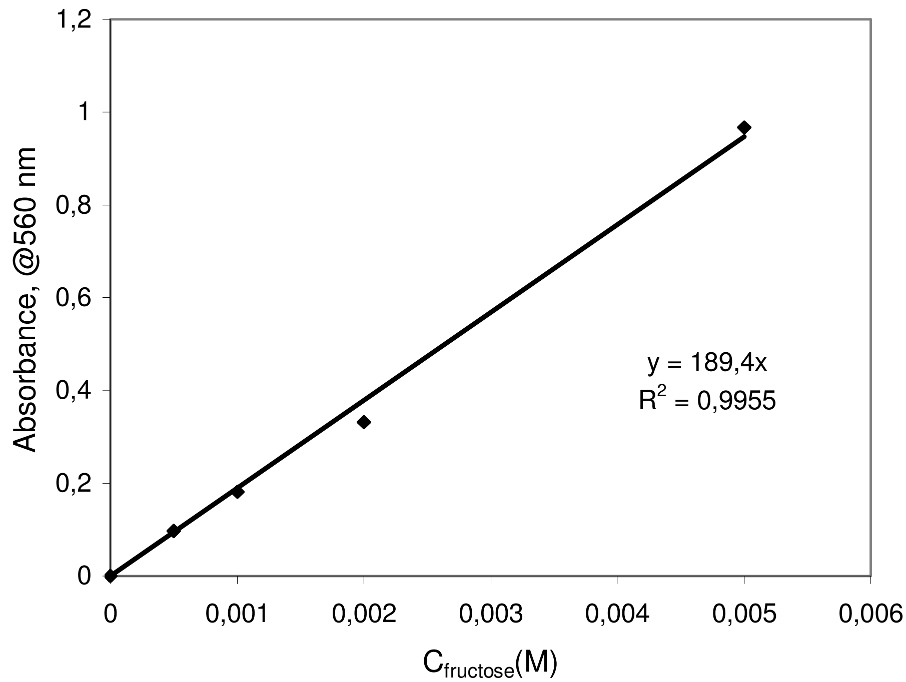

Figure A.2 Calibration curve of the D-Fructose in 0.02 M phosphate buffer, pH=7.0, T=80°C, λ=560nm

One unit of glucose isomerase activity was defined as the amount of enzyme required to yield 1 μmol of fructose at 80°C and pH 7.0 in one minute. The reaction was carried out at a volume of 60 μL for 10 minutes at 80°C. The activity, U L^{-1} is given by,

$$A(UL^{-1}) = \frac{A_\lambda}{189.4 A_\lambda M^{-1}} \times \frac{10^6\,\mu mol}{1 mol} \times \frac{1L}{10^6\,\mu L} \times \frac{60\,\mu L}{10\min} \times \frac{1m^3}{10^3 L} \times \frac{1}{\text{sample volume in L}}$$

APPENDIX C

Calibration of Reduced Sugar Concentration

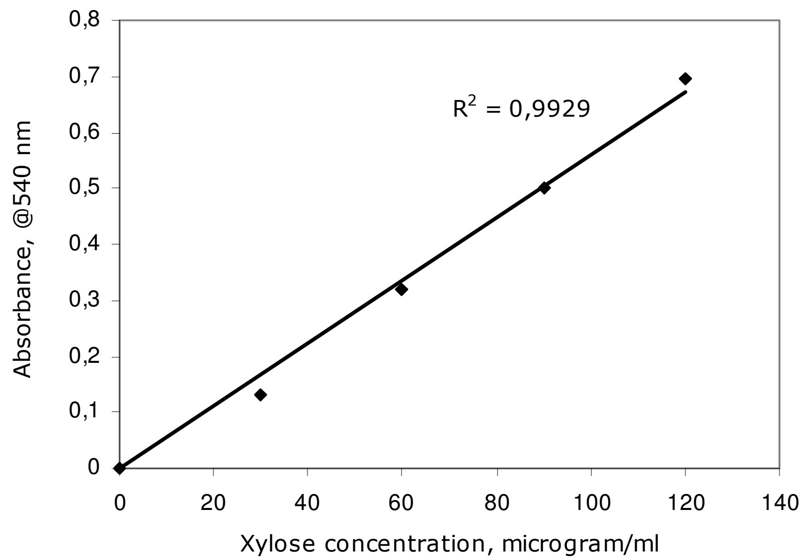

Figure A.3 Calibration Curve of the DNS solution

APPENDIX D

Calibration of Organic Acids

D.1 Acetic acid

Empower software

Untitled

Reported by User: System   Project Name: OrganicacidGI

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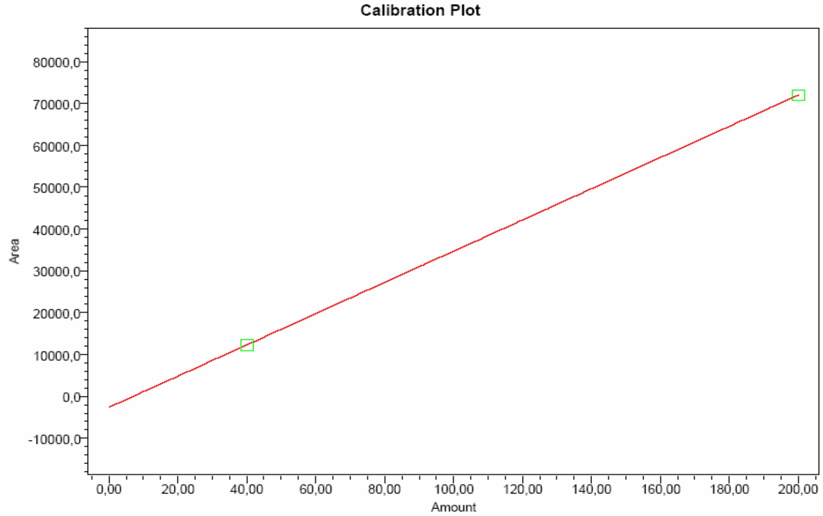
Processing Method ID:	1600	System:	RI_UV
Calibration ID:	1606	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:49:07	Proc. Chnl. Descr.:	****

A -2,607989e+003
B 3,731626e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



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2	Peak1 2487Channel 1	2	200,000000	72024,536287	200,000000	0,000	No	No

Report Method: Untitled   Printed 15:16:17   18.02.2007   Page: 1 of 1

D.2 Citric acid

Untitled

Reported by User: System

Project Name: OrganicacidGl

Processing Method:	citric	Project Name:	OrganicacidGl
Processing Method ID:	1466	System:	RI_UV
Calibration ID:	1472	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:03:17	Proc. Chnl. Descr.:	****

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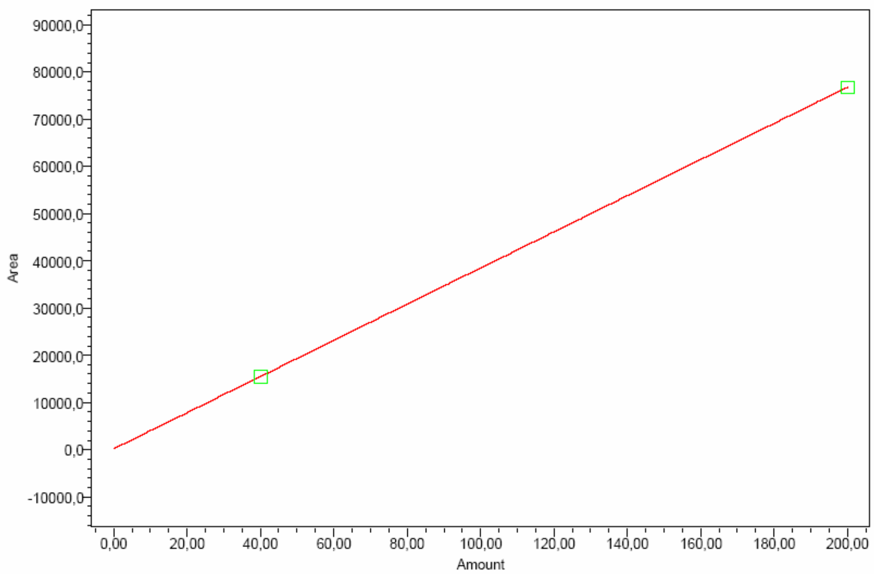
B 3,827949e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

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2	Peak1 2487Channel 1	2	200,000000	76748,920145	200,000000	0,000	No	No

Report Method: Untitled Printed 15:11:45 18.02.2007 Page: 1 of 1

D.3 Formic acid

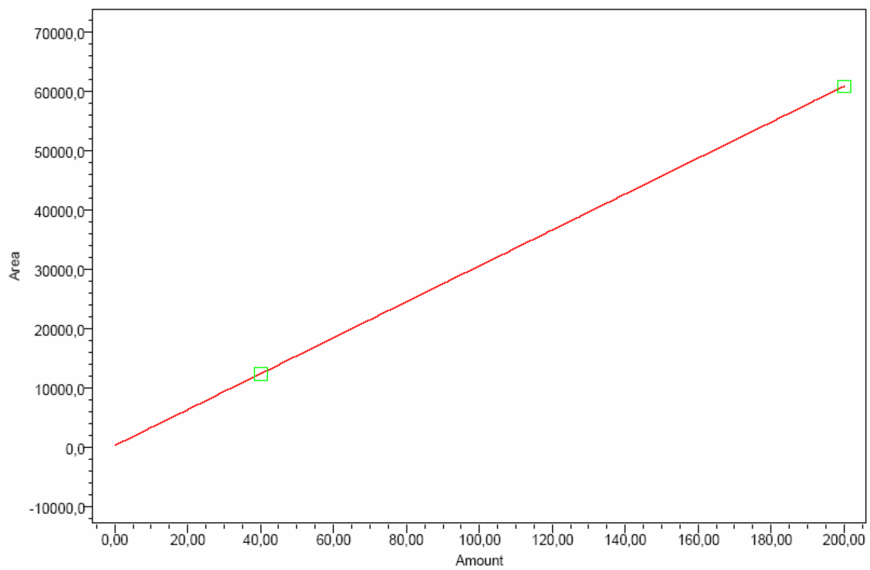
Untitled

Reported by User: System | Project Name: OrganicacidGI

Processing Method:	formic	Project Name:	OrganicacidGI
Processing Method ID:	1546	System:	RI_UV
Calibration ID:	1552	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:34:55	Proc. Chnl. Descr.:	****

A 3,331157e+002
B 3,028167e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

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Report Method: Untitled Printed 15:14:48 18.02.2007 Page: 1 of 1

D.4 Fumaric acid

Empower software

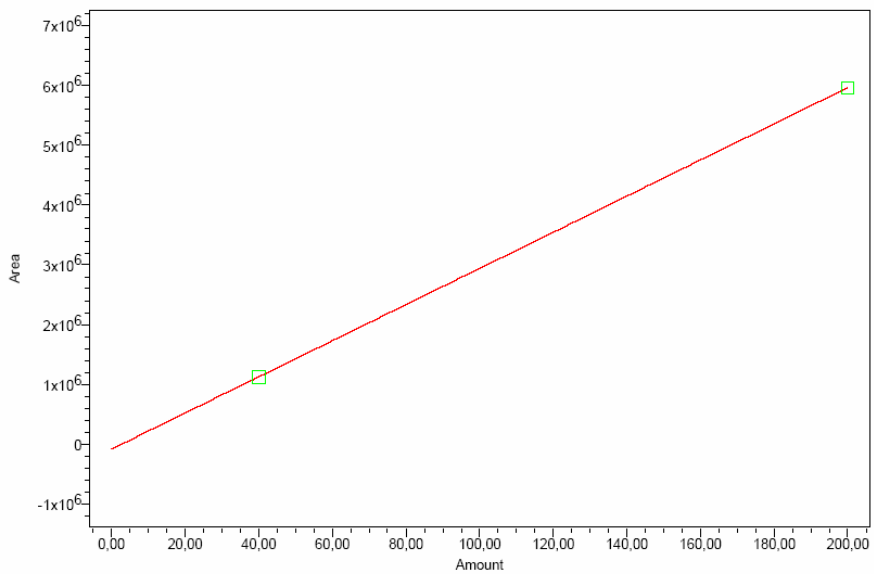
Untitled

Reported by User: System | Project Name: OrganicacidGI

Processing Method:	fum	Project Name:	OrganicacidGI
Processing Method ID:	1478	System:	RI_UV
Calibration ID:	1484	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:08:35	Proc. Chnl. Descr.:	****

A -7,904401e+004
B 3,020930e+004
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
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2	Peak1 2487Channel 1	2	200,000000	5962815,000314	200,000000	0,000	No	No

Report Method: Untitled   Printed 15:12:49   18.02.2007   Page: 1 of 1

D.5 Gluconic acid

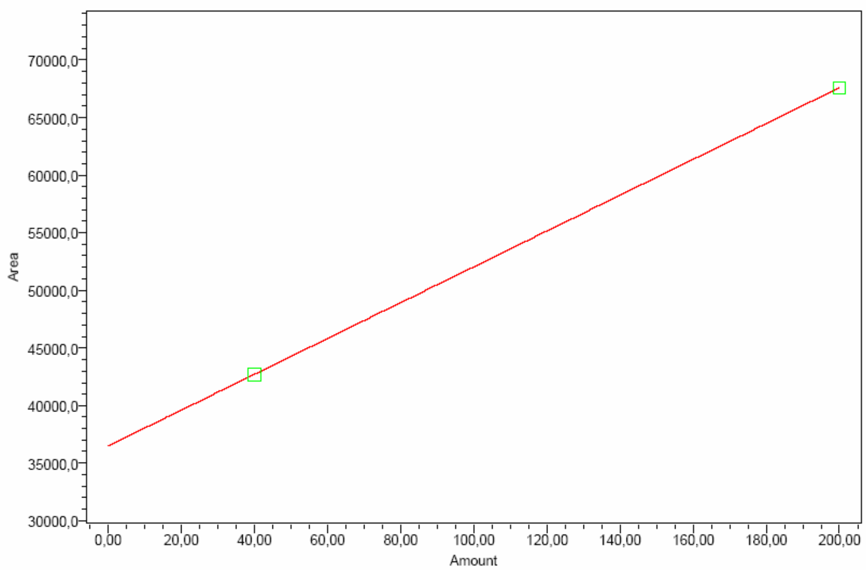
Empower software **Untitled**

Reported by User: System Project Name: OrganicacidGI

Processing Method:	gluconic	Project Name:	OrganicacidGI
Processing Method ID:	1652	System:	RI_UV
Calibration ID:	1660	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 15:22:30	Proc. Chnl. Descr.:	****

A 3,648688e+004
B 1,555339e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

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Report Method: Untitled Printed 15:22:50 18.02.2007 Page: 1 of 1

D.6 Glutaric acid

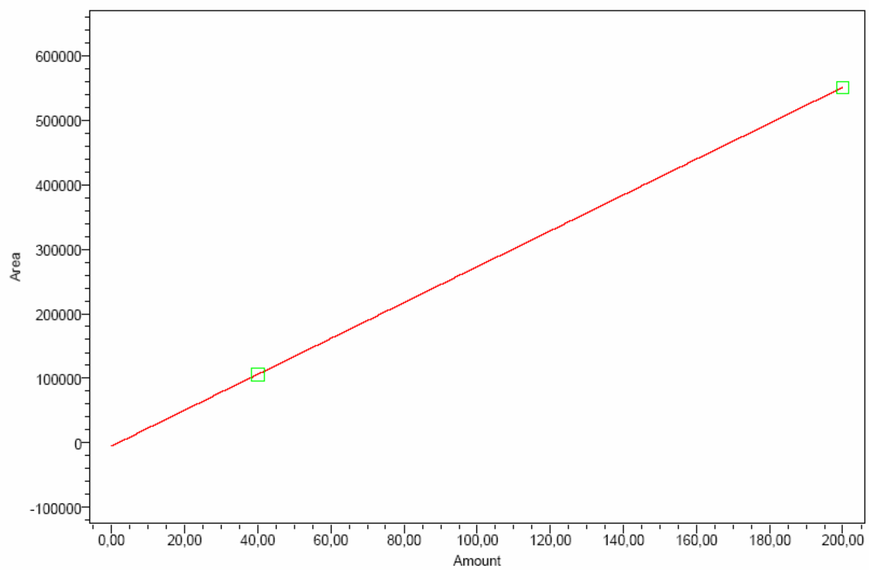
Empower software — **Untitled**

Reported by User: System   Project Name: OrganicacidGI

Processing Method:	glut	Project Name:	OrganicacidGI
Processing Method ID:	1488	System:	RI_UV
Calibration ID:	1494	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:13:09	Proc. Chnl. Descr.:	****

A -5,023916e+003
B 2,779234e+003
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak2 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Peak2 2487Channel 1	1	40,000000	106145,430166	40,000000	0,000	No	No
2	Peak2 2487Channel 1	2	200,000000	550822,814353	200,000000	0,000	No	No

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D.7 Lactic acid

Empower software

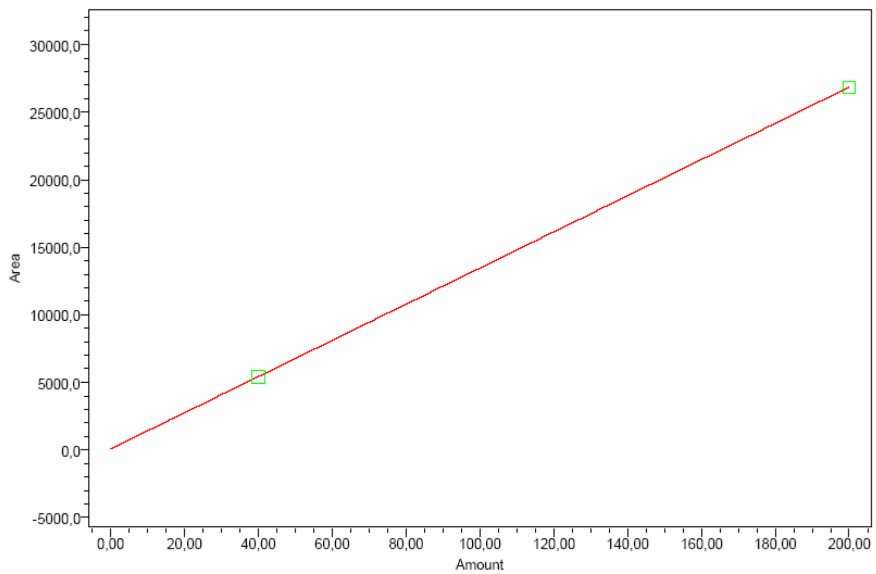
Untitled

Reported by User: System | Project Name: OrganicacidGI

Processing Method:	lac	Project Name:	OrganicacidGI
Processing Method ID:	1556	System:	RI_UV
Calibration ID:	1562	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:37:38	Proc. Chnl. Descr.:	****

A 7,518227e+001
B 1,338900e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Peak1 2487Channel 1		40,000000	5430,781816	40,000000	0,000	No	No
2	Peak1 2487Channel 1		200,000000	26853,179998	200,000000	0,000	No	No

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D.8 Maleic acid

Untitled

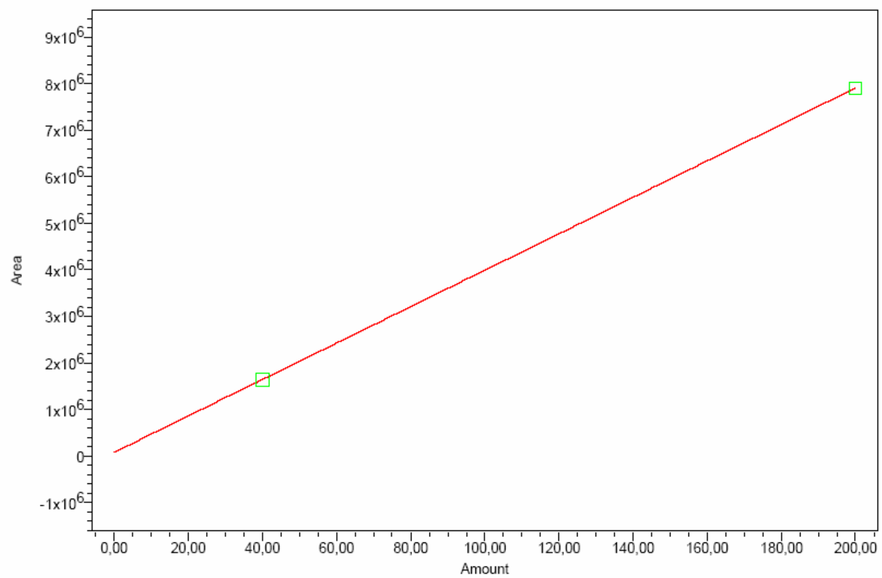
Reported by User: System

Project Name: OrganicacidGI

Processing Method:	maleic	Project Name:	OrganicacidGI
Processing Method ID:	1508	System:	RI_UV
Calibration ID:	1514	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:20:30	Proc. Chnl. Descr.:	****

A 7,842343e+004
B 3,914000e+004
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Peak1 2487Channel 1	1	40,000000	1644023,304110	40,000000	0,000	No	No
2	Peak1 2487Channel 1	2	200,000000	7906422,800963	200,000000	0,000	No	No

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D.9 Malic acid

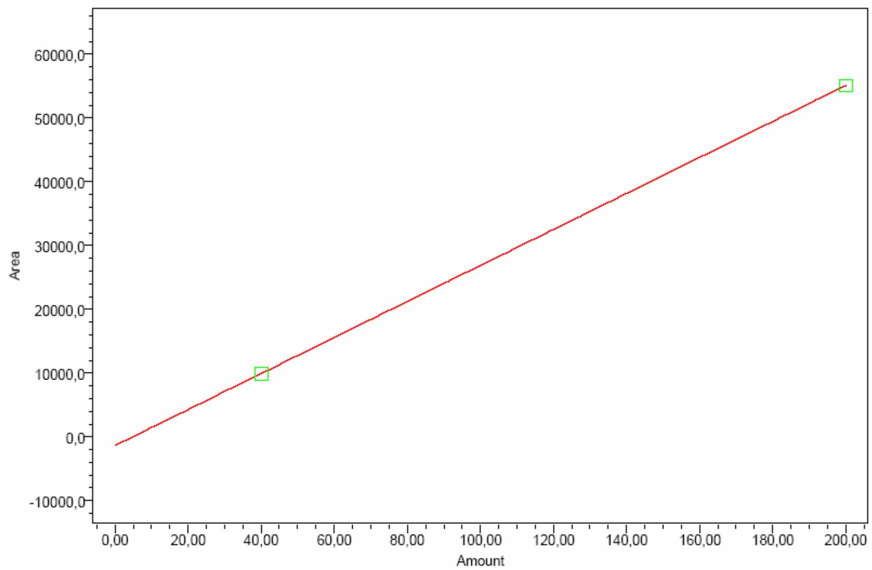
Untitled

Reported by User: System   Project Name: OrganicacidGI

Processing Method:	malic	Project Name:	OrganicacidGI
Processing Method ID:	1566	System:	RI_UV
Calibration ID:	1572	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:40:25	Proc. Chnl. Descr.:	****

A -1,352829e+003
B 2,822618e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Peak1 2487Channel 1		40,000000	9937,643943	40,000000	0,000	No	No
2	Peak1 2487Channel 1		200,000000	55099,535261	200,000000	0,000	No	No

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D.10 Oxalic acid

Empower software

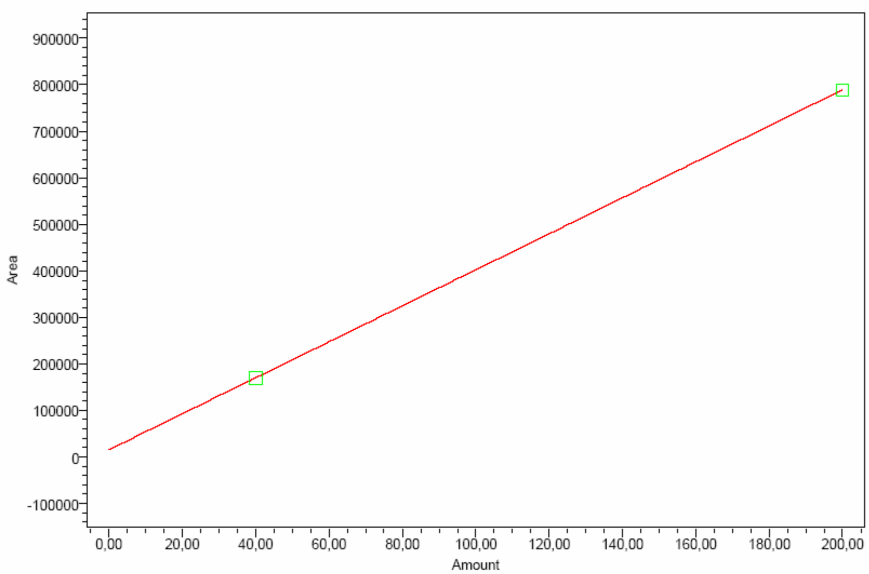
Untitled

Reported by User: System   Project Name: OrganicacidGI

Processing Method:	oxa	Project Name:	OrganicacidGI
Processing Method ID:	1498	System:	RI_UV
Calibration ID:	1504	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:17:16	Proc. Chnl. Descr.:	****

A 1,569526e+004
B 3,866608e+003
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Peak1 2487Channel 1		40,000000	170359,566185	40,000000	0,000	No	No
2	Peak1 2487Channel 1		200,000000	789016,808444	200,000000	0,000	No	No

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D.11 Pyruvic acid

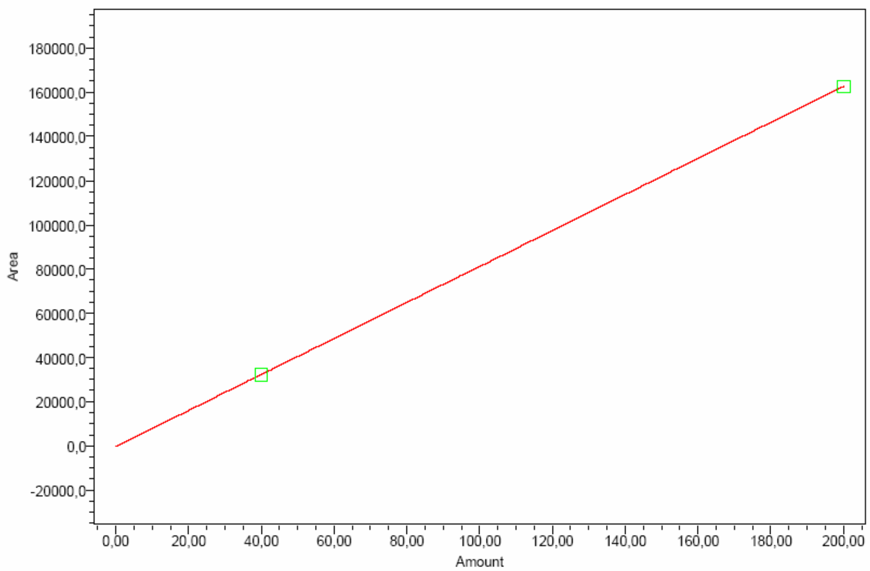
Untitled

Reported by User: System Project Name: OrganicacidGI

Processing Method:	pyru	Project Name:	OrganicacidGI
Processing Method ID:	1536	System:	RI_UV
Calibration ID:	1542	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:31:31	Proc. Chnl. Descr.:	****

A -3,074948e+002
B 8,149089e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Peak1 2487Channel 1	1	40,000000	32288,860850	40,000000	0,000	No	No
2	Peak1 2487Channel 1	2	200,000000	162674,283496	200,000000	0,000	No	No

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D.12 Succinic acid

Empower software

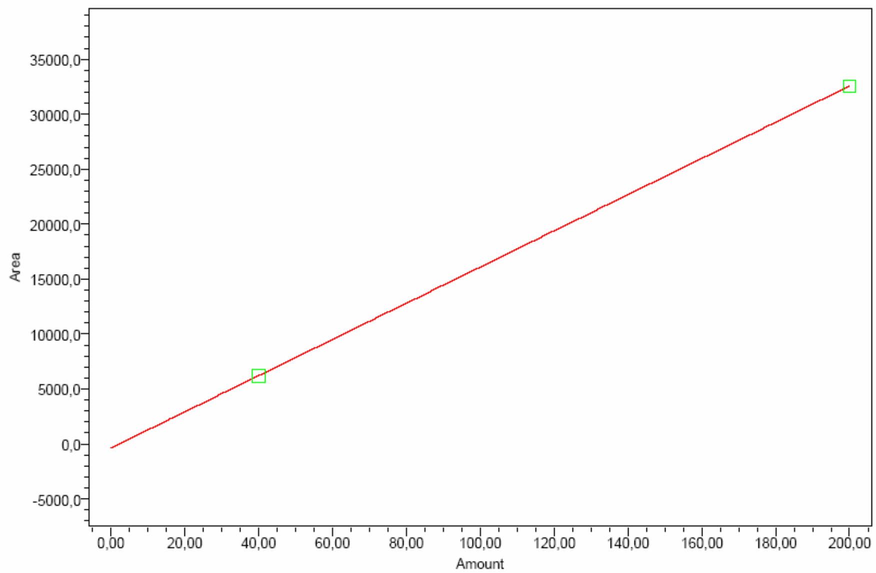
Untitled

Reported by User: System Project Name: OrganicacidGI

Processing Method:	suc	Project Name:	OrganicacidGI
Processing Method ID:	1518	System:	RI_UV
Calibration ID:	1524	Channel:	2487Channel 1
Date Calibrated:	18.02.2007 14:22:56	Proc. Chnl. Descr.:	****

A -3,632964e+002
B 1,646887e+002
C 0,000000e+000
D 0,000000e+000
R^2 1,000000

Calibration Plot



Peak: Peak1 2487Channel 1

	Name	Level	X Value	Response	Calc. Value	% Deviation	Manual	Ignore
1	Peak1 2487Channel 1		40,000000	6224,250806	40,000000	0,000	No	No
2	Peak1 2487Channel 1		200,000000	32574,439504	200,000000	0,000	No	No

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