FERMENTATION CHARACTERISTICS OF FOUR GENETICALLY ENGINEERED *Saccharomyces cerevisiae* STRAINS

by

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This thesis is dedicated to my two highly developed eucaryotic "micro" organisms;

Uluç and *Defne*

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ABSTRACT

The production of ethanol from starch has been investigated in four genetically modified *Saccharomyces cerevisiae* strains (YPG/AB,YA7, YPG/MM and YPB-G). Three of the four strains produce the *Aspergillus awamori* glucoamylase together with either the *Bacillus subtilis* (YPG/AB, YA7) or the mouse (YPG/MM) α-amylase as separately secreted polypeptides. YPB-G, on the other hand, secretes a bifunctional fusion protein that contains both the *B.subtilis* α-amylase and the *A.awamori* glucoamylase activities. Substrate utilisation, biomass growth, ethanol production and plasmid stability were all studied in both starch and glucose-containing media in shake flasks and in a New Brunswick fermenter. Much higher growth rates were found when any of the four strains was grown on glucose. YPG/AB showed the most efficient utilisation of starch for ethanol production with the lowest levels of reducing sugars accumulating in the medium. This strain produced 18.8 g/L and 43.8 g/L ethanol from 50 g/L and 100 g/L starch respectively. The superior performance of YPG/AB and YA7 as compared to YPB-G was found to correlate with its higher level of α-amylase activity in starch containing media. YPB-G, which secretes the bifunctional fusion protein, could produce 35.2 g/L ethanol in media with starch concentrations above 100 g/L, while YPG/MM did not produce ethanol from starch because of its negligible secretion of glucoamylase. Furthermore, a simple structured mathematical model was proposed to analyze the growth and enzyme secretion kinetics of the recombinant strain (YPB-G) in a complex medium containing glucose. The parameters obtained by the nonlinear estimation techniques were validated against experiments that were not used in the calibration of the model.

ÖZET

Genetik mühendisliği teknikleri kullanılarak hazırlanmış dört farklı rekombinant *Saccharomyces cerevisiae* suşunun nişastadan başlayarak etanol üretimi incelenmiştir. Bu suşların üç tanesi (YPG/AB, YA7, YPG/MM) *Aspergillus awamori* glikoamilaz geni ile *Bacillus subtilis* (YPG/AB,YA7) veya fare pankreası (YPG/MM) α-amilaz genini barındırmakta ve protein salgılamaları farklı promoterlar altından sağlanmaktadır. Bununla birlikte, YPB-G suşu *A. awamori* glikoamilaz geni ve *B. subtilis* α-aamilaz geni barındırmakta ve aynı promoter altından füzyon protein olarak salgılamaktadır. Substrat kullanımı, biyokütle ve etanol üretimi, plazmid kararlılığı, nişasta veya glikoz içeren besi ortamlarında çalkalayıcı ve New Brunswick fermentör koşullarında incelenmiştir. Dört suş için de yüksek büyüme hızları glikozlu ortamda gözlenmiştir. YPG/AB suşu, parçalanmış şekerleri ortamda en az düzeyde biriktirmek suretiyle nişastayı etanole en verimli şekilde çevirmiştir. 50 g/L ve 100 g/L nişastadan sırasıyla 18.8 g/L ve 43.8 g/L etanol üretmiştir. YPG/AB ve YA7 suşlarının üstünlüğü nişastalı besi ortamında α-amilaz aktivitesinin yüksekliğine de bağlıdır. Füzyon protein salgılayan YPB-G suşu, ancak 100 g/L nişastadan 35.2 g/L etanol üretmiştir. YPG/MM suşu ise çok düşük düzeylerdeki glikoamilaz aktivitesi nedeniyle nişastayı etanole çevirememiştir. Ayrıca, basit yapısal bir matematiksel model ortaya konarak, YPB-G hücrelerinin glikozlu ortamda büyüme ve enzim salgılama kinetiği incelenmiş, doğrusal olmayan kestirim yöntemi ile hesaplanan parametreler modelleme sırasında kullanılmayan deneysel verilerle doğrulanmıştır.

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LIST OF SYMBOLS

Latin Letters

A	adsorbence
A_{exp}, A_{model}	α-amylase activity (U/mL)
c	concentration (g/L)
D	dilution rate, dilution factor (hr^{-1})
D_{crit}	critical dilution rate (hr^{-1})
$E_n(x)$	error
f	function
G, G_{exp}, G_{model}	glucoamylase activity (U/mL)
h	time increment between the measurements
J	objective function
K	constant in model equations (mg/g)
k_1	constant in model equations (mg/g)
k_2	constant in model equations (mg/L/hr)
K_1	constant in model equations (mg/g/hr)
k_a	constant in model equations (U/mg.hr)
K_a	constant in model equations (U/mL)
k_g	constant in model equations (U/mg.hr)
K_g	constant in model equations (U/mL)
k_m	constant in model equations (1/g/hr)
k_p	constant in model equations (mg/g)
k_s	constant in model equations (g/g.hr)
K_s	constant in model equations (mg/L)
K_{ss}	constant in model equations (g/g)
M	time intervals
OD	adsorbence
P	protein concentration(mg/L)

$q_{O_2}x$	oxygen uptake rate (mmol O_2/L/hr)
S	internal substrate (g/L)
S_{intm}, S_{model}	substrate concentration (g/L)
S_o	external substrate concentration (g/L)
t	time, growth time (hr, sec)
t_{lag}	lag phase period, (hr)
t_m	maturation time (hr)
t_s	lag time (hr)
X	biomass formation (g/L)
X, X^*, $X_{t\text{-}tm}$, X_{exp}, X_m	biomass concentration(g/L)
X_i	set of measurements
$X_{initial}$, X_{final}	initial and final biomass (g/L)
$y_\theta(x_i)$	simulated values
y_i	experimental values
$Y_{p/s}$	mg protein formed per g glucose consumed (g/g)
$Y_{p/x}$	mg protein formed per g cells formed (g/g)
$Y_{sx}X$	consumed substrate (g/L)
$Y_{x/s}$	g cells formed per g glucose consumed (g/g)

Greek Letters

ε	adsorption coefficient (l/mmol/cm)
ν	degrees of freedom
ΔA	adsorbance difference
μ_{max}	maximum specific growth rate (hr^{-1})
μs	dilution factor

1. INTRODUCTION

Sccharomyces cerevisiae is a well known eucaryotic microorganism possessing many of the biosynthetic and general metabolic features of the mammalian (including human) cells. Recent developments in recombinant DNA technology have opened new areas for the production of valuable enzymes, proteins and metabolites through bioprocesses. In the ethanol production, in order to achieve high productivity from raw starch materials without pretreatment, many attempts have been made for the construction of recombinant yeast strains.

Although there is enough information on the cloning, construction and preparation of recombinant yeast cells in the literature, there is a lack of information on the growth and fermentation behaviors of these strains. In fact, the reliability of the constructs for large scale production of either single cell proteins or ethanol can only be tested under carefully defined and controlled conditions, which requires parametric studies leading to the development of process models. These reasons encourage studies on the fermentation characteristics of recombinant yeast strains.

The optimization of the expression and secretion of the functional starch hydrolyzing enzymes, namely α-amylase and glucoamylase in genetically modified *Saccharomyces cerevisiae* was considered to be very important for the one step bioconversion of starch rich materials to ethanol or single cell protein. In most of these constructs heterologous α-amylase (AMY) or glucoamylase (STA2) genes from various organisms have been expressed and excreted. Co-expression of AMY and STA2 genes in recombinant *Saccharomyces cerevisiae* has resulted in the production of higher amylolitic activity (Steyn et al., 1992). The expression of a bifunctional protein from the fusion of the complete reading frames of both the α-amylase and the glucoamylase cDNAs from the filamentaous fungus, *Aspergillus shirousamii* (Shibuya et al., 1992a) in *S.cerevisiae* was a more effective approach, and this construct displayed a higher level of activity with raw starch as substrate than a mixture of the two native enzymes (Shibuya et al., 1992b).

Finally, de Moraes et al. (1995) prepared eight different constructions, these include strains that produce *Bacillus subtilis* α-amylase (BsAAase), mouse pancreatic α-amylase (MAAase) or *Aspergillus awamori* glucoamylase (GAase), either singly or in combination, as well as strains that produce either BsAAase/GAase or MAAase/GAase fusion enzymes. They have observed good performances not only in digesting soluble and corn starch but also in using all of the hydrolysis products for growth.

In the present work, four recombinant *Saccharomyces cerevisiae* strains, (i) YPB-G which expresses the *Bacillus subtilis* α-amylase - *Aspergillus awamori* glucoamylase (BsAAase/GAase) as a fusion protein, (ii) YPG/AB bearing a double cassette plasmid to produce separately *Bacillus subtilis* α-amylase (BsAAase) and *Aspergillus awamori* glucoamylase (GAase), (iii) YPG/MM that expresses mouse pancreatic α-amylase - *Aspergillus awamori* glucoamylase (MAAase/GAase) and also (iv) YA7 bearing another double cassette plasmid to produce separately *Bacillus subtilis* α-amylase (BsAAase) and *Aspergillus awamori* glucoamylase (GAase), were examined. Fermentation experiments were performed either in shake flasks or in New Brunswick Bioflo III batch/continuous fermenter. A series of experiments were conducted for the determination of the growth characteristics of the different recombinants. In these experiments, the time course of cell growth, substrate consumption, accumulation of sugars, α-amylase and glucoamylase secretion, extracellular protein and ethanol production and plasmid stability were determined in various media compositions beginning from a minimal medium through a complex medium.

Several models were tested to correlate the biomass formation and protein production in yeast minimal medium. Furthermore, a simple structured model was proposed to analyze the growth kinetics of the recombinant strain (namely YPB-G) in a complex medium containing glucose.

A detailed literature survey is given in Chapter 2. The various experimental studies carried out, the procedures and the equipment used are described in Chapter 3. The results of this study are presented and discussed in Chapter 4. Chapter 5 contains the major conclusions that can be drawn from this study and the recommendations for future work.

2. THEORETICAL BACKGROUND

2.1. Yeast

Fungi have been exploited by mankind for many thousands of years with perhaps the earliest recorded examples being the use of yeast to make ethanol, a process known to the Sumerians and the Babylonians before 6000 BC. In the ensuring 8000 years one particular genus of yeast, namely, *Saccharomyces*, has played a central role in the commercial exploitation of fungi by mankind. These facultative anaerobes utilize the Embden-Meyerhof pathway to convert sugars to pyruvic acid, with each molecule of pyruvic acid then being reductively decarboxylated to give rise to one molecule each of ethanol and carbon dioxide (**Figure 2.1**). This simple, efficient way of fermenting glucose to ethanol and carbon dioxide has provided the foundation for the two of major food industries, brewing and baking (Oliver and Tuite, 1991).

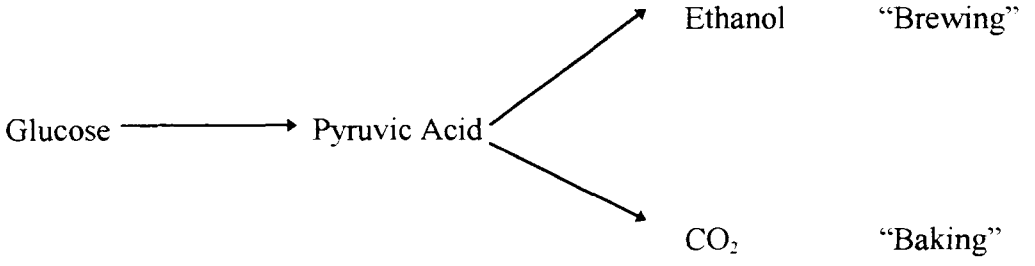


FIGURE 2.1. The Exploitation of Yeast Metabolism

Yeasts are unicellular micro-organisms that posses many of the biosynthetic and general metabolic features of mammalian, including human, cells (Wiseman, 1992). Among yeasts, *Saccharomyces cerevisiae* has been used for the production of heterologous proteins in a number of cases since it has been used extensively in fermentation, and there is a comprehensive body of knowledge on its genetic and biological background.

Furthermore, this microorganism is very suitable as a host because of an absence of pyrogenic toxins. For these reasons, *S.cerevisiae* is an attractive host organism for the production of useful commercial proteins (Park et al., 1992). Brewers have for centuries exploited the large scale growth of yeast in fermenters (culture vessels) to produce alcoholic beverages by the rapid breakdown of sugars such as maltose and glucose in the yeast growth medium. The breakdown of these sugars, obtained from the starch in plant materials, is achieved within the yeast by a complex mixture of enzymes, the catalysts made by the yeast for converting glucose to ethanol and carbon dioxide. This biodegradation of sugars by metabolic processes in the yeast cell is coupled to the harnessing of the chemical energy released to achieve biosynthetic reactions. These chemical synthetic reactions are mediated by adenosine triphosphate (ATP) made during some of the metabolic processes (Wiseman, 1992).

In general, yeasts grow well at 25-30°C in media rich in nutrients that include sugars and also protein digests. These media contain all of the approximately 20 amino acids required to build the proteins (including enzymes) needed for yeast growth. Yeasts grow rapidly in stirred aerated growth media. In addition, most yeasts, especially those selected and grown by brewers, achieve high growth rates without the special need for stirring and aeration. They metabolize the sugars in the growth medium by enzyme-catalyzed formation of glucose followed by splitting of the glucose molecule (glycolysis) and conversion of the products to ethanol and carbon dioxide. Brewers seek to develop genetically engineered yeasts that will directly metabolize starches (Wiseman, 1992).

2.2. Regulation of Sugar Utilization

The conversion of glucose to ethanol and carbon dioxide by yeast was the first biochemical pathway to be studied in detail. The initial observation that this process is catalyzed by an extract of yeast led to the discovery of enzymes and coenzymes and laid the foundation for modern biochemistry (van Dam, 1996).

During growth, cells use up the substrates to meet three major requirements: metabolic intermediates to form cellular material, redox potential and energy (e.g. ATP). Yeast obtains the energy required for growth from two catabolic pathways: glycolysis and respiration. Glycolysis involves the transformation of one molecule of hexose into two molecules of pyruvate with formation of two molecules of ATP. Pyruvate can be excreted from the cells after decarboxylation to ethanol. Degradation of sugar to ethanol is called alcoholic fermentation. Alternatively, pyruvate can be oxidized to CO_2 and H_2O in respiration. In this case, O_2 is required and the amount of energy formed ranges from 24 to 34 moles of ATP per mole of sugar, depending on the coupling between the respiratory chain and the oxidative phosphorylation (Lagunas, 1986). Fermentation is very active in yeast, even in the presence of O_2, whereas respiration occurs very slowly (De Deken, 1966).

Yeast expends as much as 70 to 75% of the total sugar consumed in the production of ATP. This high expenditure to meet energy requirements is due to the preferential use of fermentation, whose ATP yield is very low. From 10- 20% of sugar is assimilated to form cellular structures, and from 5-10% in the maintenance of redox potential and other functions (Lagunas, 1986).

The description of the yeast energy metabolism very often reported are misleading and in contradiction to present knowledge. However, yeast does not behave as expected for this type of microorganism. Facultative anaerobes are usually defined as having three peculiar characteristics: (i) the ability to grow aerobically or anaerobically using oxygen (respiration) and organic compound (fermentation) as final acceptor of electrons produced in catabolism; (ii) the preferential use of oxygen, if available due to the greater energy yield of respiration versus fermentation; (iii) the smaller rate of breakdown of sugars in aerobiosis than in anaerobiosis as a consequence of the great difference in the ATP yield. This is also studied as an inhibition of fermentation by respiration, the so-called Pasteur effect (Entian, 1992).

S.cerevisiae meets the above criteria in: (i) It is true that yeast can grow in the presence as well as in the absence of air. However, during anaerobiosis, yeast requires an

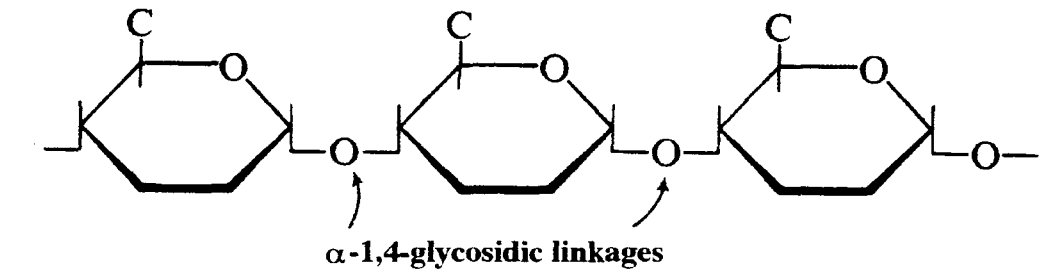
external supply of ergosterol and non-saturated fatty acids (Hunter and Rose, 1970). The dependence of biosynthesis of these compounds on oxygen has been very often overlooked in the interpretation of certain experimental result. (ii) The preferential use of respiration is not true in the case of yeast. In contrast, yeast prefers fermentation whenever a fermentable substrate is available. In these cells, sugars produce a repression of the respiratory enzymes that strongly decrease their ability to respire. Only when sugars are exhausted, or present in very low concentration, respiratory enzymes are derepressed and respiration plays an important role in their catabolism. (iii) Neither is it true, as discussed above, that a relevant Pasteur effect occurs during growth of these cells. These facts demonstrate that yeast only meets one of the three criteria employed to describe the behavior of facultative anaerobes. Therefore, it can be concluded that either *S.cerevisiae* should not be considered as a facultative anaerobe or other criteria should be formulated to define this kind of microorganism (Lagunas, 1986).

2.3. The Utilization of Starch by Yeast

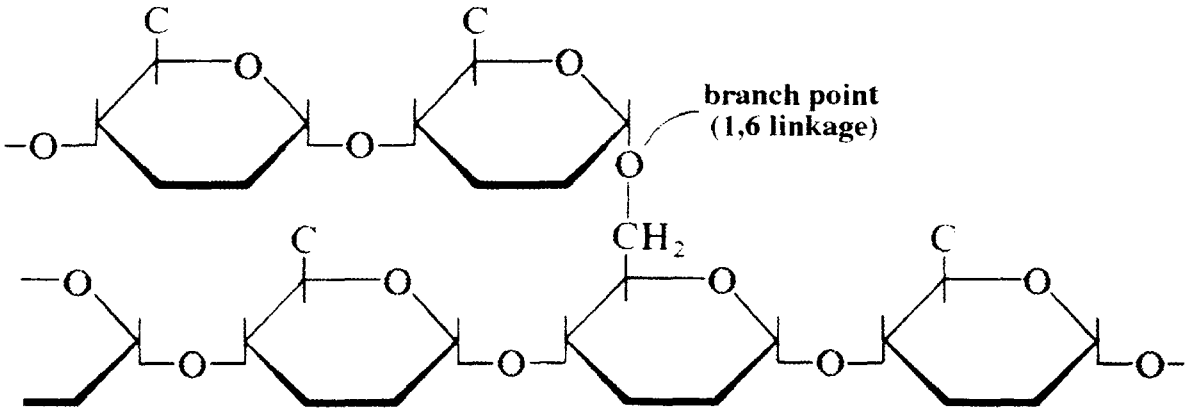
Starch is a mixture of amylose, essentially of linear (1-4) linked α-D-glucopyranose residues and amylopectin, which is similar but also branched from (1-6) linkages (Guilbot and Mercier, 1985). The ratios of these two constituents vary between starches from different plants and from different samples from the same kind of plant (Fogarty, 1979; Guilbot and Mercier, 1985) (**Figure 2.2**).

The production of ethanol by yeast fermentations, for use as a fuel or chemical feed-stock, plays an important role in the economy of a number of developing countries, e.g. Brazil and India (de Moraes et al. 1995; Rosilio-Calle et al., 1992). Traditionally, these fermentations have relied on sugar rich substrates, such as cane juice. However, sugar cane is a seasonal crop and requires high-quality agricultural land for its growth. Hence, there are both economical and social advantages to extending the substrate range of yeast so that

ethanol may be produced from starchy crops such as cassava (Marzola and Bartholomew, 1979; Coomb, 1984; Rosillo-Calle et al., 1992). Since *Saccharomyces cerevisiae* is unable to utilize starch during the vegetative growth phase, it is necessary to add expensive enzymes or to manipulate the yeast genetically such that it is able to hydrolyze starch itself (de Moraes et al., 1995). Another possible way to achieve direct fermentation of starch is to use mixed microorganisms such as an amylolytic microorganism and an ethanol-fermenting microorganism which will simultaneously saccharify and ferment starch molecules (Dostalek and Haggstrom, 1983; Han and Steinberg, 1987; Kurasawa et al., 1989; Tanaka et al., 1986).



(a)



(b)

FIGURE 2.2. Starch Molecule (a) Amylose and (b) Amylopectin

Developments in recombinant DNA technology resulted in many constructs. In most of the constructs, heterologous α-amylase or glucoamylase genes from various organisms have been expressed and excreted. The optimization of the synthesis and secretion of these two enzymes in genetically modified *S.cerevisiae* is thus central to the efficient one-step bioconversion of starch-rich materials to ethanol or single cell protein (SCP). One of the first attempts were made by Astolfi-filho et al., in 1986. They inserted mouse pancreatic α-amylase complementary DNA into a shuttle vector after the *S.cerevisiae* Mfα1 promoter and secretion signals coding sequences. In later studies, co-expression of the *Bacillus amyloliquefaciens* α-amylase and the *Saccharomyces diastaticus* glucoamylase genes in recombinant *S. cerevisiae* resulted in the production of higher amylolytic enzymes (for review Steyn et al., 1991). The expression of a bifunctional protein from the fusion of the complete reading frames of both the α-amylase and the glucoamylase cDNAs from the filamentaous fungus, *Aspergillus shirousamii* (Shibuya et al., 1992a) in *S.cerevisiae* was a more effective approach. This construct displayed a higher level of activity with raw starch as substrate than a mixture of the two native enzymes (Shibuya et al., 1992b). Recently, de Moraes et al., (1995) prepared eight different constructs, including strains that produce *Bacillus subtilis* α-amylase (BsAAase), mouse pancreatic α-amylase (MAAase) or *Aspergillus awamori* glucoamylase (GAase) either singly or in combination, as well as strains that produce either BsAAase/GAase or MAAase/GAase fusion enzymes. They have observed good performances not only in digesting soluble and corn starch but also in using all of the hydrolysis products for growth. In addition to that, Nakamura et al. (1997) constructed by integrating a glucoamylase producing gene (STA1) into the chromosome of *S.cerevisiae*. The glucoamylase was constitutively produced by the recombinant yeast and starch was properly converted into ethanol with a yield of 0.48.

2.4. Ethanol Production From Starch

The production of ethanol from biomass resources has been widely studied for the purpose of developing an alternative energy source to fossil fuels. Starch is one of the major biomass constituents, and thus has been a main substrate for biomass conversion technology (Mori and Inaba, 1990). In the fermentation of ethanol from raw starchy materials, reduction of the cooking energy required for liquefaction and hydrolysis of raw materials is receiving increasing attention because the cost of cooking is a considerable portion of the total cost for producing ethanol (Hoshino et al., 1989). The production of ethanol directly from starch without cooking, requires new fermentation techniques involving a combination of a raw-starch-digesting amylase and a yeast strain (Hoshino et al., 1990; Hoshino et al., 1989), a mixed culture of a microorganism producing enzymes for liquefying and hydrolyzing and a strain converting sugar to ethanol (Kurasawa et al., 1989; Dostalek and Haggstrom, 1983; Han and Stenberg, 1987; Lee et al., 1983; Tanaka et al., 1986) or glucoamylase and/or α-amylase producing recombinant yeast strains (Inlow et al., 1988; Nakamura et al., 1996; de Moraes et al., 1995). However, in methods using two microorganisms, it is difficult to establish the optimum culture conditions for the production of ethanol (Hoshino et al., 1990).

In the case of a culture medium containing raw starch without cooking, enzymes and/or microorganisms immobilized on an insoluble carrier such as a gel of a polysaccharide cannot act effectively by diffusion limitation within the carrier. Moreover, if the immobilized system were used in a packed or fluidized bed, clogging of the substrate or destruction of the gel would take place. The membrane systems also have a problem with clogging, which causes an unstable flux through the membrane, and it is necessary to provide a facility for washing. To overcome these problems, a system was proposed by Hoshino et al. in 1989 and 1990. In their work, a raw starch-digesting amylase immobilized on a reversible soluble-autoprecipitating (S-AP) carrier was used. The immobilized enzyme (D-AS) acted on raw starch in soluble form at pH 5.0 and could be recovered in insoluble form at pH 4.0 from the reaction medium by self-sedimentation

(Hoshino et al., 1989). Later, the same group proposed another reactor for the recycling-use of D-AS and flocculating yeast cells in order to improve ethanol productivity from raw starch (Hoshino et al., 1990). One year later, Sanroman et al. (1991) proposed a new technology based on the use of pulsed reactors. They have developed a type of pulsing device giving a see-saw-type of disturbance, and a decrease was observed in the diffusional difficulties encountered in the operation of fixed-bed bioreactors. They have also tested the applicability of their technique in the hydrolysis of concentrated starch solutions by glucoamylase immobilized on chitin slabs. Another application of bioreactors for the production of ethanol from starch was the use of a pervaporation membrane bioreactor using *Clostridium thermohydrosulfuricum* to attain both high productivity and efficient recovery of ethanol from the fermentation broth (Mori and Inaba, 1990).

Furthermore, yeasts strains capable of fermenting starch and dextrin to ethanol were isolated from samples collected from Brazilian factories in which cassava flour is produced. They have observed considerable alcohol production (Laluce et al, 1988; Banerjee et al., 1988). Strains of about 150 species of yeast have been reported to be able to utilize starch as the sole carbon source for aerobic growth (Barnett et al., 1983; Barnett et al., 1985; Kreger-van Rij, 1984). Yeast amylases are mostly α-amylase and glucoamylase (Shibuya et al., 1992a). Other enzymes may be involved in hydrolyzing the oligosaccharides formed when starch is broken down. Isomaltase hydrolyses the (1-6) bonds of isomaltose (6-o-α-D-glucopyranosyl-D-glucopyranose) and other oligosaccharides; α-glucosidase hydrolyses maltose. Reliable studies on yeasts which synthesize both enzymes have necessitated the purification the enzymes. For this reason alone, many publications on starch breakdown by yeasts have been of little value (McCann and Barnett, 1986).

Dostalek and Haggstrom (1983) studied the conversion of starch into ethanol in a mixed culture of an amylolitic yeast, *Saccharomycopsis fibuliger*, and an anaerobic bacterium, *Zymomonas mobilis*, and they obtained a maximum ethanol concentration of 9.7 g/L and a production rate of ethanol of 0.54 g/L/h in a fermentation of 30 g/L starch. Tanaka et al. (1986) investigated the production of ethanol from starch by a coimmobilized mixed culture system of aerobic and anaerobic microorganisms, *Aspergillus awamori* and

Zymomonas mobilis, in Ca-alginate gel beads, and they obtained a maximum ethanol concentration of 22.0 g/L with a production rate of 0.61 g/L/h in the fermentation of 100 g/L starch. Recently, Nakamura et al. (1997) were able to produce 24.9 g/L of ethanol with a production rate of 0.66 g/L/h in the direct fermentation of 100 g/L starch by using recombinant *Saccharomyces cerevisiae* strains.

2.5. Amylases

Amylases are extensively applied enzymes which can hydrolyze the glycosidic bonds in starch and related glucose-containing compounds (Bailey and Ollis, 1986). α-Amylase and glucoamylase are two of the oldest enzymes to be extensively studied (Bezbaruah et al., 1991). α-Amylase (AAase) (EC 3.2.1.1. α-1-4-glucan-4-glucano-hydrolase) catalyzes the endoamylolitic cleavage of α-(1-4)-glycosidic linkages in starch and releases short oligoshaccharides and α-limit dextrins, in a random manner, while hydrolysis of amylopectin is limited by its (1-6) linkages. The initial stage of action of these enzymes is characterized by a rapid decrease of the molecular weight of the substrate and consequent break down of the starch structure, especially that of amylose. Glucoamylase (GAase) (EC 3.2.1.3, α-1-4; 1,6-glucan glucohydrolase) catalyzes the exoamylolytic cleavage of starch and releases glucose (Shibuya et al., 1992a).

Starch contains straight-chain glucose polymers called amylose and a branched component known as amylopectin. The branched structure is relatively more soluble than the linear amylose and is often effective in rapidly raising the viscosity of starch solution. The action of α-amylase reduces the solution viscosity and this enzyme is often called the *starch-liquefying enzyme*. On the other hand, glucoamylase attacks primarily the nonreducing ends of starch, dextrins and maltose and is called a *saccharifying enzyme* (Bailey and Ollis, 1986).

There are three hypotheses that attempt to explain the action of α-amylases on the linear chain of polysaccharides. *Unique Chain Hypothesis*: after the formation of the enzyme-substrate complex, the hydrolysis occurs progressively through the chain until it is totally degraded. *Multiple Chain Hypothesis*: the action is random, an enzyme-substrate complex is formed each time an effective interaction occurs, resulting in the hydrolysis of a linkage per complex formed. *Multiple Attack Hypothesis*: in this model, the enzyme-substrate interaction is random, and each time the complex is formed, multiple bonds are hydrolyzed.

Glucoamylase, on the other hand, is an exo-enzyme and its mode of action is to bind to the substrate and cleave the bonds progressively from the non-reducing end, hydrolyzing several bonds per enzyme-substrate complex formed. Because of these differences in action, the α-amylase attack is hampered by its association to the glucoamylase; in other words, glucoamylase is suppressing in some way the action of the α-amylase (de Moraes, 1994).

Several *Bacillus* species are versatile producers of α-amylase, but only a few of them are capable of secreting amylase activity at high temperature. Generally, the optimum activity of α-amylase was recorded at a pH range of 4.0-7.0 and a temperature range of 30-70°C (Bezbaruah et al., 1991; Simones-Mendes, 1984; Thomsen, 1983; Gasperik et al., 1991; Moseley and Keay, 1970; Paquet et al., 1991). *Clostridium thermosaccharolyticum* and *Aspergillus niger* Glucoamylases, on the other hand, had an optimum pH range of 4.0-6.0 and a temperature range of again 40-70°C (Specka et al., 1991; Stoffer et al., 1993). Downstream processing techniques were applied for the purification of these enzymes from different sources (Bezbaruah et al., 1991; Simones-Mendes, 1984; Thomsen, 1983; Gasperik et al., 1991; Moseley and Keay, 1970; Paquet et al., 1991; Specka et al., 1991; Stoffer et al., 1993).

2.6. Recombinant DNA Technology in Yeast

The procedure for the growing of cells from a single cell transformed by the entry of foreign DNA is often referred to as cloning, and the same word is used also by many authors to describe the production of many extra copies therefore of the foreign gene DNA in the recombinant cells of yeasts, bacteria and of mammalian cells in culture vessels (Wiseman, 1992). Foreign DNA can be made to enter the yeast cell by carefully arranged procedures, and it then lodges within the cell nucleus. These techniques of genetic engineering make use of the natural ability of yeast strains to take up circular lengths of the DNA polymer chain. This DNA is usually the two micron size (2μ) "plasmid" that can become part of, or associated with, the chromosomal material in the yeast cell nucleus. The 2μ plasmid DNA is a 6300-bp, circular, double-stranded molecule present in most *Saccharomyces* strains at 50-100 copies per cell. It is isolated, often by centrifugation techniques, from yeast. Highly specific enzymes, referred to as restriction endonucleases are then used to break open the DNA circle at only one position. The required piece of appropriate DNA is often obtained by excision from the foreign DNA using the same restriction endonuclease. This is then "stitched in" using enzymes that reform the phosphodiester bonds that are needed to hold together the correct sequence of DNA component. The recircled modified plasmid is now a suitable vector for entering into the yeast cell which is usually used in the form of yeast protoplast that has had its carbohydrate cell wall dissolved away, using an appropriate mixture of hydrolytic enzymes. Once inside the nucleus of the yeast cell, the modified plasmid is able to reproduce itself and also displays the normal ability of genes to express the molecular information which is inherent in their characteristic sequence of DNA bases. Thus DNA can specify the biosynthesis of messenger RNA (mRNA) that specifies the sequence of the amino acid residues in the corresponding protein in the yeast cell.

2.6.1. Yeast Vectors

Yeast vectors (plasmids) are suitable for introduction of foreign genes into yeast are made in several different forms, each with different characteristic features of efficiency and stability. Almost all yeast plasmid vectors are, in fact, both yeast and *E.coli* vectors. They usually comprise all or a part of an *E.coli* vector such as pBR322 and a yeast replication and selection system. This arrangement allows *E.coli* to be used as the preparative organism for the various manipulations associated with recombinant DNA procedures (Oliver and Tuite, 1991).

Common yeast vectors are named according to their ability to integrate into the yeast chromosome or replicate independently: Yeast integrating plasmids (YIp) are incapable of autonomous replication and are therefore maintained only after insertion into a chromosome by homologous recombination. Yeast replicating plasmids (YRp), which can contain yeast chromosomal DNA that allows autonomous replication of the plasmid, and the derivatives of the natural plasmid from yeast (YEp -yeast episomal plasmid) give a much higher frequency of transformation than do the YIps. The YIp vectors yield only a few transformants per microgram of DNA, whereas the YRp and YEp vectors yield between 10^2 and 10^5. Typically, the YEp and YRp plasmids are unstable; this property can be used advantegous in some circumstances but may be a disadvantage in others. The YEp plasmids replicate at a high copy number (e.g. about 50 per cell) and can therefore be used to increase the gene copy number of cloned DNA. Lastly, YRp plasmids can carry a yeast centromere and, in this conformation, be stable maintained in low copy numbers (Davis et al., 1986).

An important consideration at the cellular level is the influence of foreign gene expression on host growth. High expression of foreing gene is desired; however, the synthesis of a cloned gene product places additional stress on the cells. This can result in lower growth rate, lower cellular yield, and plasmid instability. The combined effect is that the overall production rate of the gene product is sometimes reduced. To lessen these

negative effects of cloned gene expression, plasmids with inducible promoters may be effective in maximizing gene production, because the timing and level of cloned gene product synthesis can be controlled. Inducible promoters in *S.cerevisiae* such as SUC2, PHO5, GAL1, GAL7 and GAL10 have been developed (Park et al., 1993). There are also strong promoters such as Mfα1, PGK and ADH1 (de Moraes et al., 1995).

2.7. Plasmid Stability

The productivity of recombinant cell systems depends on many factors ranging from the interactions between the host cell and the plasmid to the downstream processing of the product. One important consideration at the cellular level is the influence of plasmid gene expression on the host. High expression levels are desired; however, the synthesis of a cloned gene product places additional stress on the cells. This can result in lower growth rate, yield, and plasmid stability and can reduce overall productivity (Da Silva and Bailey, 1991). Two types of plasmid instability have been observed. One is due to defective partitioning resulting in the loss of entire plasmid and is called *segregational instability,* and the other type is due to deletion, insertion or rearrangement of DNA and is called *structural instability* (Park et al., 1991). Cell growth and cloned gene product synthesis depend on the copy number and stability of the plasmid utilized. Ideally, higher copy numbers lead to higher expression levels. An optimum number of plasmids usually exists, however, beyond which the detrimental effects of high expression outweigh the positive effects of increasing the number of gene copies. The level of transcription of each gene is as important as the absolute number of genes and may depend on many factors. Plasmid stability also plays an important role in determining productivity (Da Silva and Bailey, 1991). The media may be formulated to favor the growth of plasmid-carrying cells over plasmid-free cells, or selective pressure against plasmid-free cells may be employed using auxotrophic mutants or antibiotic-resistant plasmids (Parker and DiBiasio, 1987; Park et al., 1993).

Many workers have studied the plasmid stability in *Saccharomyces cerevisiae* (Ayub et al., 1992; Parker and DiBiasio, 1987; Schwartz et al., 1988; Yang and Shu, 1996; Guerrini et al., 1991). Yang and Shu (1996) reported that, GM-CSF production increased by recombinant yeast cells using a fibrous-bed bioreactor giving rise to plasmid stability. Da Silva and Bailey (1991a) studied the influence of plasmid promoter strength using the yeast GAL1, GAL10 and hybrid GAL10-CYC1 promoters and found that the rate of increase in β-galactosidase specific activity after induction in batch and continuous cultures was 3-5 times higher with the GAL1 promoter. Ayub et al. (1992) also worked on the superoxyde dismutase production by recombinant yeast cells and reported that PGK^- plasmid containing cells had a selective advantage during the respiratory phase of batch growth since they could utilize both glycerol and ethanol. Parker and DiBiasio (1987) have shown that plasmid stability substantially increased at higher growth rates. Similarly, Da Silva and Bailey (1991b) examined the effect of dilution rate and induction of cloned gene expression in continuous cultures of recombinant yeast. They have observed low plasmid stability both after induction and at low dilution rates. Some of the workers concentrated mainly on the choice of the fermentation media (Schwatz et al., 1988; Wang and Da Silva, 1993). Schwatz et al. (1988) determined the plasmid loss kinetics for *S.cerevisiae* transformed with the 2μm DNA-based plasmid in selective and nonselective media. They have simulated the experimental data in both media using proposed mathematical models. While, Wang and Da Silva, (1993) investigated the expression and secretion of invertase for the autoselection strain in batch culture for three different media. And they have observed that, biomass yields and invertase productivity increased with the complexity of the medium.

The production and secretion of cloned gene products place a substantial burden on the host cell. Therefore, the stable maintenance of the plasmid-bearing cell population must be encountered through a selection mechanism. Despite their success, traditional methods of applying selective pressure, including antibiotic addition and genetic complementation, are not desirable on a production scale. Antibiotic addition is too expensive (and environmentally undesirable) on a large scale, and traditional genetic complementation restricts the type of nutrient media which can be used. Other methods, such as inducing a promoter at late stages of batch culture, restrict optimization of the bioreactor operating strategy. To overcome these difficulties, autoselection systems have

been developed. With these systems, plasmid retention is essential for cell viability regardless of the nutrient medium; thus, all media are selective and complex antibiotic-free medium can be utilized. For yeast, autoselection systems include the *ura3fur1* double mutant developed by Loison et al. (1986) for which a URA3 containing plasmid is necessary for viability, and the srb1-1 mutant/SRB1 plasmid system reported later (Rech et al., 1992). Later, Wang and Da Silva, (1993) developed a strain representing an extension of the system described by Loison et al. (1986) and contained three mutations: *ura3*, *fur1*, and *urid-k*. The *ura3* mutation blocks an essential step in the pyrimidine biosynthetic pathway, while the *fur1* and *urid-k* mutations block the utilization of extracellular uracil, cytosine, uridine and cytidine. Therefore, three mutations in combination effectively block all pyrimidine biosynthetic and salvage pathways, and a plasmid-encoded URA3 gene is essential for cell survival regardless of the nutrient medium.

2.8. Factors Affecting Protein Secretion into the Medium

The secretion of heterologous proteins from recombinant microorganisms has emerged as an important process which has the potential to greatly reduce separation costs in industrial fermentations since a secreted protein will be contaminated with fewer other proteins. Secretion can also eliminate many of the problems associated with overproduction of recombinant proteins in microbial hosts such as toxicity. These problems may include the formation of inclusion bodies (which require unfolding and refolding processes for biological activity), the degradation of produced proteins by cellular proteases (Marten et al., 1995).

2.8.1. Cell Wall Porosity

The cell wall of *Saccharomyces cerevisiae* consists of glucans, mannoproteins and a small amount of chitin (Cabib et al., 1982). The glucans determine the rigidity of the cell wall and the mannoproteins determine its porosity (Zlotnik et al., 1984). The mannoproteins can be subdivided into (a)SDS-soluble mannoproteins, and (b)SDS-insoluble, but glucanase-soluble mannoproteins. The SDS-soluble mannoproteins represent almost 80% of the wall protein. In contrast, the glucanase-soluble mannoproteins are 20% of the wall protein (De Nobel et al., 1990b). A simple view of the cell wall is that of a sieve with fairly large holes, through which compounds can diffuse readily if they are not too big (De Nobel and Barnett, 1991). Porosity is an important property of the cell wall, because it limits the secretion of homologous e.g. periplasmic proteins and heterologous proteins (De Nobel et al., 1989). It might also affect the efficiency by which yeast cells are transformed by heterologous DNA (De Nobel et al., 1990a). Scherrer et al. (1974) stated that only molecules smaller than 700 Da can pass through the wall, but numerous examples of secretion of larger molecules into the medium are known, such as the heterologous proteins prochymosin and Ig chains. De Nobel et al. (1990a) reported that yeast cell walls are, in principle, permeable to globular proteins with a molecular mass up to 400 kDa. Also, it has been shown that the cell wall porosity of batch-grown *S.cerevisiae* was maximal in the early exponential phase and fell off rapidly to lower levels in later growth phases (De Nobel et al., 1990b). Evidence that the cell wall may act as an ion exchanger has also been reported (Shaeiwitz et al., 1989).

2.8.2. Environmental Factors

Environment is an important factor affecting gene stability, specific gene expression and cell concentration (Hardjito et al., 1993). Direct comparison of the published data regarding the capability of budding yeast to release secreted proteins into the growth medium is difficult because of differences in strains, growth conditions and the physiological states of the cultures (Rossini et al., 1993). Rossini et al. (1993) investigated that conditions loosening cell wall structure, including higher growth temperature, growth in rich medium and the presence of reducing agents, which most likely act by breaking cell wall disulfide bridges, improved β-galactosidase excretion by systematically changing the growth medium composition and temperature. They have observed that alterations in growth temperature and growth medium composition had only minor effects on excretion efficiency of α-galactosidase and glucoamylase II, the yeast proteins that are known to be released efficiently into the growth medium. These observations suggest that the wall does not act simply as a molecular sieve. Different kinds of chemical interactions may contribute to retention of proteins in the periplasmic space. Several charged groups are present in the wall components; their level and type may change as a function of the growth conditions, which are known to affect cell wall structure and composition (De Nobel et al., 1990a,b) and play a role in retention of wall and periplasmic proteins (Rossini et al., 1993).

2.8.2.1 Effect of Temperature. Different steps in the secretory pathway may be differentially affected by the growth temperature. For instance, growth at low temperatures may partially restore the secretion defect brought about by underglycosylation of acid phosphates (Riederer and Hinnen, 1991), possible because improved folding of the underglycosylated enzyme at the lower temperature improves the post-endoplasmic reticulum steps (Rossini et al., 1993). Marten et al. (1995) investigated the effects of temperature on the kinetics and efficiency of the secretion of cloned invertase in a recombinant yeast system at temperatures ranging from 25-45°C. They have observed both the amount of invertase produced and the rate of invertase secretion to the periplasm showed maxima at 35°C.

2.8.2.2 Effect of pH. Ruohonen et al. (1991) have studied the optimization of *Bacillus* α-amylase production and observed that in standard non-buffered medium, α-amylase was rapidly inactivated but stabilization of the pH at 6.0 led to stable accumulation of α-amylase in the culture medium. On the other hand, Kunze et al. (1988) studying *B. amyloliquefaciens* α-amylase were unable to detect enzyme activity in the yeast liquid culture medium due to the low pH. Interestingly, in this report secretion of the α-amylase from the yeast cells was readily demonstrated by a plate assay, where holas were formed around yeast colonies producing α-amylase on a starch-containing plate. It seems that the pH on the plate does not decrease below the critical level (Rossini et al., 1993).

2.8.2.3 Effect of Medium Composition. Hardjito et al. (1993) increased the β-galactosidase production rate and cell production rate with a feeding policy. They have recorded that the final volumetric productivity, as determined by both cell concentration and gene expression, was strongly affected by the time course of the glucose levels in the bioreactor (Hardjito et al., 1993). Similarly, Wang and Da Silva (1993) observed that when the complexity of the medium increased, biomass yields and invertase productivity also increased in a stable recombinant yeast strain.

The use of an enriched medium has been shown to correlate with decreasing product proteolysis as well as increased cell yield, and thus to substantially improved productivity of the secreted recombinant protein (Wang and Da Silva, 1993).

2.9. Models for Yeast

Most literature models are concerned with the primary metabolism of yeast since this has a direct relation to the important industrial processes. A reasonable fit of experimental data can be obtained with crudely structured models. However, these models can not describe the well-documented phenomenon of spontaneous oscillations observed in continuous cultures of *S. cerevisiae*. In such a situation, it is necessary to apply a

segregated population model. Models describing spontaneous oscillations are, therefore, mathematically quite complex (Nielsen and Viladsen, 1992).

2.9.1. Simple Structured Models for Growth of Yeast

The successful application of the steady-state chemostat is illustrated by the large amount of fermentation data that can be for *S.cerevisiae* in the open literature. The steady state data provide fundamental knowledge of the primary metabolism of yeast, and the continuos accumulation of this type of data also adds valuable information concerning the growth cycle of yeast (Nielsen and Viladsen, 1992).

In an aerobic glucose-limited chemostat with *S.cerevisiae*, two distinct growth regimes are observed: (1) at low dilution rates D, all of the glucose is converted to biomass and carbon dioxide, a by-product of the energy-forming reactions, and (2) at high dilution rates ethanol is formed in addition to biomass and carbon dioxide (Meyenburg, 1969). The shift in metabolism at the critical dilution rate, D_{crit}, was traditionally referred to as the Crabtree effect, i.e. an inhibition of the oxidative system by high glucose concentrations, but it is now generally accepted that the formation of ethanol at aerobic conditions is a consequence of a bottleneck in the oxidation of pyruvate (Alexander and Jeffries, 1990).

Considering the complexity of the aerobic sugar metabolism by yeast, and the ability of yeast to grow on ethanol in the absence of sugar in addition to the complexity different of the fluxes through the metabolic pathways at anaerobic conditions, it is clearly impossible to simulate growth and product formation by *S.cerevisiae* using an unstructured model, at least if the model shall have any predictable strength outside the growth conditions at which the model parameters were determined (Nielsen and Viladsen, 1992).

One of the first structured models for *S.cerevisiae* was formulated by Bijkerk and Hall (1977). Since their model did not predict lag phases, Pamment et al. (1978) modified

the original model by introducing separate enzyme systems for glucose and ethanol metabolism. Later on, Barford and Hall (1981) proposed a metabolically structured model based on an intracellular ATP balance. Another metabolically structured model was derived by Bellgardt et al. (1982). In the model considered, the intracellular glucose, pyruvate, acetyl-CoA, acetaldehyde, ethanol, NADH, ATP, and the most important energy-generating pathways were described. However, until 1992, the most widely accepted yeast model was that of Sonnleitner and Kappeli (1986). Their model described a decrease in the oxidative capacity with decreasing oxygen concentration, the well-known Pasteur effect. By a combination of the compartment model concept with an intracellular ATP balance, Viladsen and Nielsen (1990) derived a structured model for *S.cerevisiae* as an extension of the two-compartment model for lactic acid fermentation. As for *E.coli*, there have been attempts to set up highly structured models for *S.cerevisiae* (Hall and Barford, 1981; Steinmeyer and Shuler, 1989; Liao and Lightfoot, 1988; Delgado and Liao, 1991; Schalien et al., 1995; Vanrolleghem et al., 1996).

2.9.2. Segregated Population Models for Yeast

The cell division is asymmetric with the formation of a so-called *mother* and a so-called *daughter* cell. The daughter cell is converted to a mother cell within the time period t_1. Thereafter, a new bud emerges on the mother cell, and after the time period $t_1 + t_2$, the cell divides. t_1 and t_2 are functions of the environmental conditions and the cellular composition (Nielsen and Viladsen, 1992).

The asymmetric division of budding yeast has been modeled by Hjortso and Bailey (1982). They have calculated the steady state distribution function for exponential growth and for linear growth of the individual cells at different specific growth rates. Later, they have also examined the transient situation (Hjortso and Bailey, 1983). Porro et al (1988) found that the oscillatory regimes in a chemostat are determined by the dilution rate and

the dissolved oxygen concentration. Also, Strassle et al. (1988) showed that the oscillations were strongly dependent on the experimental equipment. The cell cycle was determined by the sequential attainment of two thresholds: a critical cell size required for budding and a critical cell size for cell division (Martegani et al., 1990). Later on, Hjortso and Nielsen (1994 and 1995) have developed a conceptual model of autonomous oscillations in microbial culture. The model development for oscillating cultures is important since it is possible to use them for increasing the production of compounds which cells only synthesize during a part of the cell cycle.

2.9.3. Models for Recombinant Yeast

Until 1992, there were fewer structured models for *S.cerevisiae* than for the recombinant bacteria. Hjortso and Bailey (1984a) extended their segregated-population model to predict plasmid stability at steady-state growth. It was assumed that the culture was under selection pressure, whereby, only plasmid-containing cells can survive in the environment. Only the plasmid-containing cells were considered, not the copy number distribution (Nielsen and Viladsen, 1992). Their later attempt was to extend to dynamic conditions, e.g. shifting to a non-selective medium (Hjortso and Bailey, 1984b). Wittrup and Bailey (1988) and Wittrup et al. (1990) also discussed a segregated population model for recombinant yeast.

In a compartment model for recombinant yeast, Coppela and Djurjati (1990) proposed very complex parameters (48 parameters), and the primary metabolism was described in a far more empirical fashion than in most of the models.

To model recombinant S.*cerevisiae* it seems better to extend one of the models discussed above, since most of these models are known to describe the primary metabolism quite well. With added kinetic expressions for plasmid replication and

productivity, one has to determine only the parameters of these expressions from experiments with the recombinant strain, possibly combined with some adjustment of the parameters in the kinetics for the primary metabolism (Nielsen and Viladsen, 1992).

At first glance, microorganisms appear to be very different, but a closer study reveals that they have a number of basic functions in common. They all need catabolism of substrate to create energy for growth and maintenance of cell functionality, and entire pathways are identical in many microorganisms. With these and other similarities of several microorganisms in mind, one can expect that a simple structured model which only considers major intracellular reactions can be used as a basis for description of different fermentation processes (Nielsen et al., 1991).

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Microorganisms and Plasmids

Saccharomyces cerevisiae host strain GRF18 (MATa, his3-11, his3-15, leu2-3, leu2-112, can1, mal) and four recombinant *Saccharomyces cerevisiae* strains, (i) YPB-G which expresses the *Bacillus subtilis* α-amylase - *Aspergillus awamori* glucoamylase (BsAAase/GAase) as a fusion protein, (ii) YPG/AB bearing a double cassette plasmid to produce separately *Bacillus subtilis* α-amylase (BsAAase) and *Aspergillus awamori* glucoamylase (GAase), (iii) YPG/MM that expresses mouse pancreatic α-amylase - *Aspergillus awamori* glucoamylase (MAAase/GAase) as separate proteins and also (iv) YA7 bearing another double cassette plasmid to produce separately *Bacillus subtilis* α-amylase (BsAAase) and *Aspergillus awamori* glucoamylase (GAase), were used in this study.

The strains were kindly provided by Dr. Stephen Oliver (Department of Biochemistry and Applied Molecular Biology, UMIST, England) and the plasmids pPB-G, pPG/AB, pPG/MM and pA7 were constructed and used to transform the *Saccharomyces cerevisiae* host strain GRF18 to obtain YPB-G, YPG/AB, YPG/MM and YA7 respectively by Dr. Lidia M. de Moraes (Department of Biochemistry and Applied Molecular Biology, UMIST, England). **Figures 3.1-3.3** show the maps of the plasmid constructions.

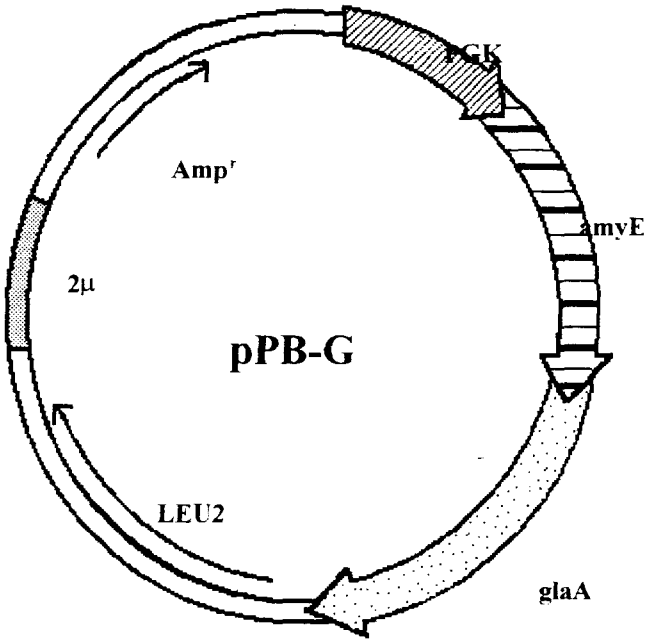


FIGURE 3.1. Structure of Plasmid pPB-G

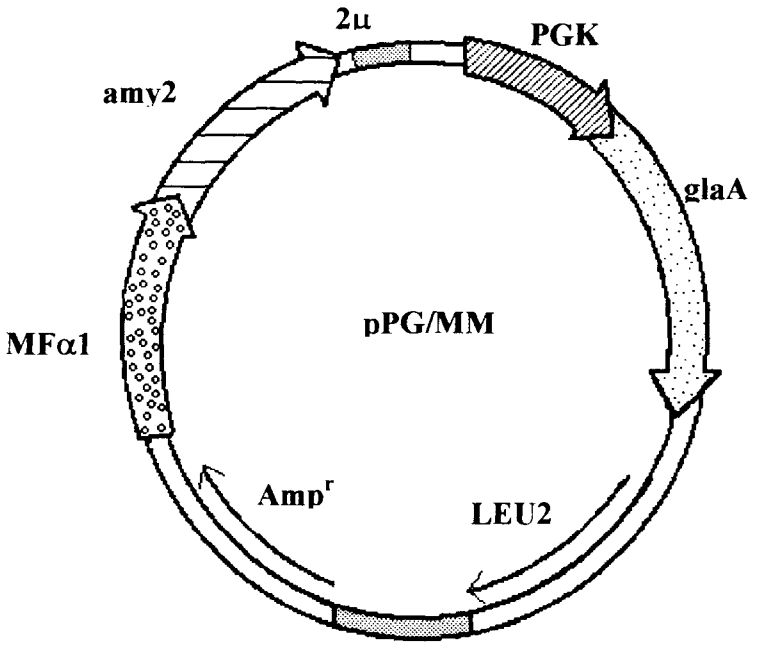


FIGURE 3.2. Structure of Plasmid pPG/MM

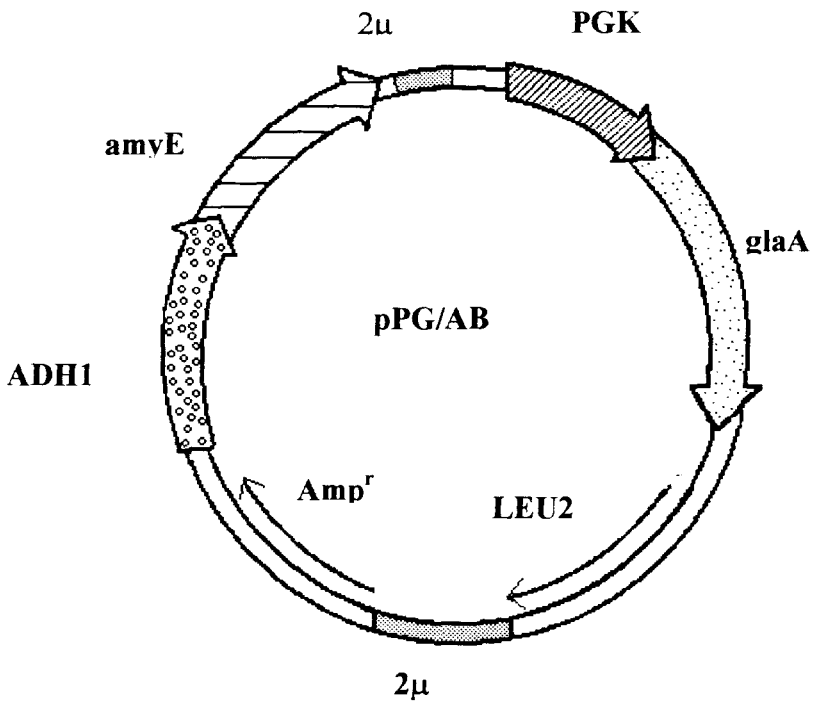


FIGURE 3.3. Structure of Plasmid pPG/AB

3.1.2. Maintenance

For maintenance, single colonies of *S.cerevisiae* strains were inoculated into 5 mL of YNB-G medium and were grown overnight. An aliquot (1 mL) of the culture was mixed with an equal volume of 30% (v/v) glycerol and stored at -80°C for long term storage. Frozen cultures were used by streaking the cells on YNB-G agar plates for the preparation of master plates which were used to remove a single isolated colony for the preparation of a sub-master plate. In order to maintain high percentage of viability and avoid any risk of contamination, new glycerol cultures were prepared from fresh agar plates every six months. The master plates were prepared once every two months and were kept at +4 °C.

3.1.3. Culture Media

The liquid media used throughout this study were YNB-G, YNB-GS, YEP-Sg, YEP-S, YEP-G, YPS and YPG.

The solid media was prepared by the addition of 2%(w/v) agar to the liquid media.

YNB-G (Yeast Nitrogen Base without aminoacids and Glucose Medium)

Yeast Nitrogen Base without aminoacids	3g
$(NH_4)_2SO_4$	5g
Histidine	0.02g

pH = 6.0-6.5

The medium was supplemented with 5g-50g of glucose per liter of deionized and distilled water.

For solid medium, 2%(w/v) agar was added, when the glucose concentration was 20g/L in the YNB-G liquid medium.

YNB-GS Medium (Yeast Nitrogen Base without aminoacids, Glucose and Starch Medium)

Yeast Nitrogen Base without aminoacids	3g
$(NH_4)_2SO_4$	5g
Histidine	0.02g
Glucose	20g
Soluble Starch	10g

per liter of deionized and distilled water.
For solid medium, 2%(w/v) agar was added.

YEP-Sg (Yeast Extract, Peptone, Starch and Glycerol Medium)

Yeast Extract	5g
Peptone	10g
Histidine	0.02g
Soluble Starch	10g (unless stated)
Glycerol	50mL

pH = 6.0-7.0

per liter of deionized and distilled water.

YEP-S (Yeast Extract, Peptone and Starch Medium)

Yeast Extract	5g
Peptone	10g
Histidine	0.02g

pH = 5.0-6.0

YEP-S medium was supplemented with different concentrations of soluble starch per liter of deionized and distilled water.

YEP-G (Yeast Extract, Peptone and Glucose Medium)

Yeast Extract	5g
Peptone	10g
Histidine	0.02g
Glucose	50g

pH = 6.0-7.0

per liter of deionized and distilled water.

YPS (Yeast Extract, Peptone and Starch Medium)

Yeast Extract	10g
Peptone	20g

Histidine	0.02g

pH = 6.0-7.0

YPS medium was supplemented with 5-50g of soluble starch per liter of deionized and distilled water.

For solid medium, 2%(w/v) agar was added when the starch concentration was 10g/L in the YPS liquid medium.

YPG (Yeast Extract, Peptone and Glucose Medium)

Yeast Extract	10g
Peptone	20g
Histidine	0.02g

pH = 6.0-7.0

YPG medium was supplemented with 5-50g of glucose per liter of deionized and distilled water.

For solid medium, 2%(w/v) agar was added, when the glucose concentration was 20g/L in the YPG liquid medium.

3.1.4. Chemicals

All chemicals and solutions used in this study were purchased from MERCK (Germany) , SIGMA (U.S.A), DIFCO (U.S.A) and FLUKA (Switzerland) unless otherwise stated in the text.

Commercial enzymes (α-amylase and glucoamylase) were from BOEHRINGER MANNHEIM.

3.1.5. Buffers and Standard Solutions

All the buffers and solutions were filter sterilized and stored in sterile bottles.

3.1.5.1 Buffers and Solutions in α-Amylase Assay

Buffer

Na-Acetate	50mM

pH was adjusted to 5.9 either by glacial acetic acid or by 5M NaOH.

Substrate

Soluble Starch	0.2g
Na-Acetate (pH=5.9)	100mL

Iodine Solution

KI	5g
I_2	0.5g
dH_2O	100mL

stored in a dark bottle.

3.1.5.2 Buffers and Solutions in Glucoamylase Assay

Buffer

Na-Acetate	50mM

pH was adjusted to 4.5 either by glacial acetic acid or by 5M NaOH.

Substrate

Soluble Starch	0.5g
Na-Acetate (pH=4.5)	100mL

3.1.5.3 Storage Buffer

Trisma base	0.2M

pH was adjusted to 9.0 by glacial HCL or 5M NaOH.

3.1.5.4 Buffers and Solutions used in the Plasmid DNA Isolation

Yeast Recovery Buffer

NaCl	100mM
Tris-HCl	10mM,pH8.0
EDTA	1mM
SDS	1%(w/v)

Rnase	(Dnase free), 10μg/mL
Phenol	TE Saturated, pH was adjusted to 8.0.
Chloroform:Isoamylalcohol	24:1(v/v)

Tris HCL

Tris-HCl	10mM
EDTA	0.1mM

pH was adjusted to 8.0 by glacial HCL or 5M NaOH.

3.1.5.5 Solutions in RNA Analysis

Reagent I (Ferric Chloride Reagent)

$FeCl_3 6H_2O$	100mg
Glacial HCL	100mL

stored in a dark bottle.

Reagent II (Ethanolic Orcinol Reagent)

Orcinol	6.0g
Ethanol	100mL

stored in a dark bottle.

3.1.5.6 Solution in Reducing Sugar Analysis (DNS Method)

NaOH	13.2g
dH_2O	944mL
3.5-Dinitrosalicilic Acid	7.06g
K-Na Tartarate	204g
Phenol	5.06mL
Nametabisulphite	5.52g

were mixed in the indicated order and stored in a dark bottle.

3.1.5.7 Solution in Starch Analysis

KI	0.5g
I_2	0.15g
dH_2O	100mL

were mixed in the indicated order and stored in a dark bottle.

3.1.6. Laboratory Equipment

Agarmatic	Bench -Top Agar Sterilizer, New Brunswick, England
Autoclaves	Medexport, CIS, C.W.I.S. Eyela, Model MAC-601, Japan
Balances	Precisa 80A-200M, Switzerland Gec Avery
Camera	Polaroid DS-34 Direct screen Instant Camera, USA
Centrifuges	SORVALL RC-5B Refrigerated Superspeed Centrifuge, DUPONT, USA Biofuge 28RS HERAUS, Germany Centrifuge 5415 C EPPENDORF, Germany Centrifuge NF 615, NÜVE, Turkey
Cold Room	VWR SCIENTIFIC, VCR 422DBA, USA
Deepfrezers	-80 C, Hetofrig CL 89, HETO, Denmark -20 C, BOSCH, Germany
Electrophoresis	Horizon 58, Model 200, Horizontal Gel Electrophoresis Apparatus, BRL, USA Miniprotean II, BIORAD, USA Power / Pac 300, BIORAD, USA
Fraction Collector	Model 2110, BIORAD, USA

Fermenter	Bioflo III Batch/Continuous Fermenter, New Brunswick, England
Filtration Unit	MILLIPORE Vacuum filter,USA
Freeze Dryer	Chem. Lab. Instruments Ltd. Model SB6, England
Gas Chromatograph	SHIMADZU GC-8A, Japan
Gel Dryer	HETO Dry GD-I, Denmark
Glucose Analyzer	YSI 2700, YSI, USA
Hot Plate	NÜVE 318, Turkey
Laminar Flow Cabinet	Holten-Lamin Air, HBB 2460, Denmark
Ice Machine	SCOTSMAN, AF-30, UK
Incubators	EN500, NÜVE,Turkey FN500, NÜVE, Turkey Lab-Line AMBI-HI-LOW Chamber, 3554-18, USA
Microscope	Olimpus 3061,Japan
Orbital Shakers	GFL 3032, Germany INNOVA 4340, New Brunswick Co.,England
pH meter	HANNA Instruments, HI 8521, Singapore
Pumps	Masterflex Computerized Drive, Cole Parmer Ins.,USA Ismatecsa, Switzerland

Refrigerators	+4 C, ARÇELİK, Turkey +4 C, Ultra 1500, SİMTEL, Turkey
Sonifier	Model 250/450 Sonifier Branson Ultrasonic Co., USA
Spectrophotometers	DU 640 BECKMAN, USA SHIMADZU UV-150-02 Double Beam Spectrophotometer, Japan
Thermo-cyclers	Thermal Reactor TR1, HYBAID, UK
Transilluminator	Reprostar II, CAMAG, Switzerland
UV Monitor	Econo-UV Monitor, BIORAD, USA
Vortex	ELEKTROMAG, Turkey
Water Baths	HETO, CB 8-30e, Denmark HETO DT Hetotherm, Denmark NÜVE, BM 102, Turkey
Water Distillation System	MILLIPORE, Milli Ro Plus,USA , MILLIPORE, QVF Plus, USA GFL 2004, Germany

3.2. Experimental Methods

3.2.1. Sterilization

Sterilization is a treatment that frees the treated object of almost all living organisms. Throughout this study, it was carried out either by removing them by filtration or by killing them using steam or chemicals. All the buffers and solutions described in 3.1.5 were filter sterilized.

3.2.1.1 Sterilization of Media. Different media were used for culture growth. The media were prepared in Erlenmeyer flasks such that the total volume of the medium was always one third of the flask. The flask was sealed with a cotton stopper and wrapped with Al-foil. YNB-G, YNB-GS media were sterilized by autoclaving at 15 psig and 121°C for 15 minutes and thereafter left to cool down before using. On the other hand, YEP-G and YPG media were similarly sterilized without glucose, and glucose was sterilized separately in sterilized water by autoclaving at 15 psig and 121°C for 3 minutes to avoid its degradation. All the solutions of the media were allowed to cool down after autoclaving, then they were combined aseptically.

YEP-Sg, YEP-S, YPS media were sterilized by autoclaving at 15 psig and 121°C for 30 to 45 minutes depending on the size of the culture to promote starch degradation.

3.2.1.2 Sterilization of the Fermenter. Motor drive was removed from the top of the vessel and placed on the motor mount at the top of the cabinet. Air lines and all probe cables were removed. Jacket and exhaust condenser water lines were disconnected. Sampler rubber bulb was also removed and glass wool was inserted into the port and the sampler valve was opened to allow the release of pressure inside the vessel. Since the vessel should not be sterilized empty, 100 mL of sterilized water was put into it to allow

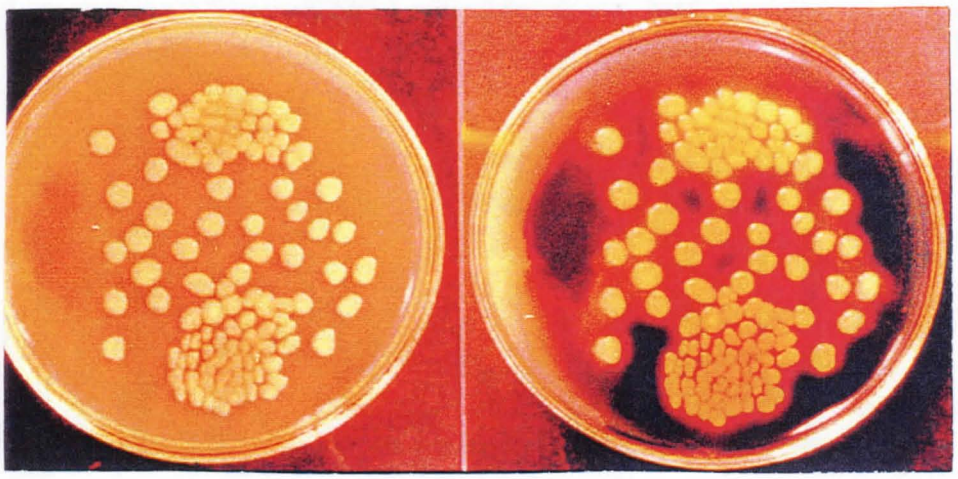
the probe tips to be moisturized during sterilization. The sterilization was performed at a temperature of 121°C and at 15 psig for 25 minutes.

The pH probe was calibrated prior to autoclaving, while the DO probe was calibrated after autoclaving by the dynamic gassing out method.

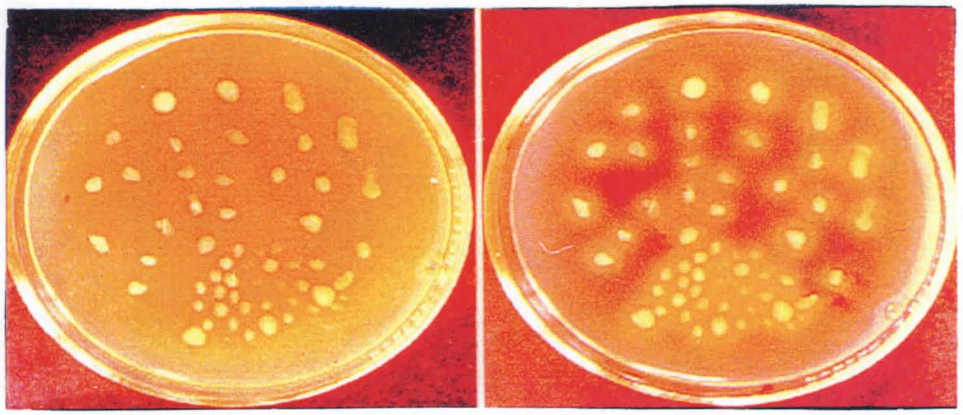
3.2.2. Preparation of Preculture

To eliminate structural instability as described in Wei et al. (1989), single colonies grown on YNB-G agar plates were transferred to YNB-GS agar plates. After allowing growth into single colonies, the plates were I_2 stained. The colony which shows a white amylolitic hola was chosen for further growth in YNB-G medium (**Figure 3.4**).

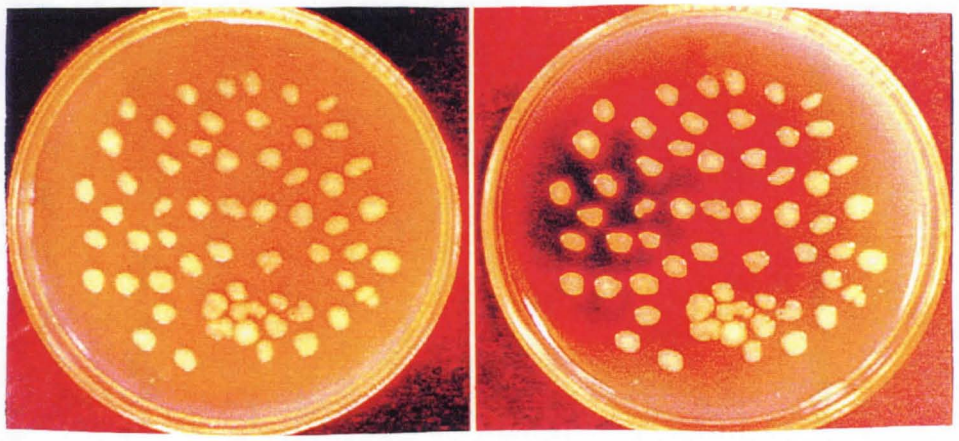
Depending on the size of the inoculum, 50 - 200 mL of sterile YNB-G liquid nutrient medium was inoculated with a single colony by an inoculating needle which was flamed to sterilize and cooled on the agar plate. The preculture was incubated in an orbital shaker at 30°C until late-exponential phase for about 24 hours. This culture was used as an inoculum on a 10% (v/v) basis unless otherwise stated.



(a)



(b)



(c)

FIGURE 3.4. Colonies Showing Amylolytic Activity - I_2 Staining Test. (a) YPG/AB, (b) YPB-G, (c) YPG/MM

3.2.3. Growth Conditions

Since the experiments were performed in different culture media, the volumes of cultures also differed depending on the experiment. In shake flask experiments with YNB-G medium, the total volume of the culture was kept at 1 L. The rate of agitation was 180 rpm and the temperature was at 30°C in the GFL orbital shaker used for these experiments. The pH was allowed to follow its natural course. The inoculum size was 2%(v/v).

In shake flask experiments with YEP-Sg, YEP-S, YEP-G, YPS and YPG, the volume of culture medium was kept at either 1.5 L or 2 L. The rate of agitation was again 180 rpm and the temperature was at 30°C in Innova orbital shaker used in these experiments. Again, the pH was allowed to follow its natural course. The size of inoculum was 10%(v/v) to initiate the growth in such complex media.

In fermenter experiments with YEP-S, YEP-G, YPS and YPG , the volume of culture medium was kept at 1.5 L. The control of various parameters such as temperature, pH, dissolved oxygen concentration, aeration and agitation were performed by the control system of the New Brunswick Bioflo III fermenter. The agitation speed was 400 rpm, the temperature was at 30°C, and the pH was kept constant at 5.6 by buffering the system either with 12.5% (v/v) NH_4OH or with 3% (w/v) succinic acid. Foam control was achieved by the 10% (v/v) silicone antifoaming agent. In the experiments with aeration, filtered air was supplied to the system at a flowrate of 0.5 L/min.

3.2.4. Partial Purification of Enzymes

A partial purification protocol described by Bezbaruah (1991) was applied to the culture supernatants in some of the experiments. Culture supernatants separated from cells

by centrifugation at 10000 rpm for 15 minutes were adjusted to pH of 7.0 with 1N HCL or by 5M NaOH. After bringing down the temperature to 4°C, enzyme grade solid ammonium sulphate was added with stirring until the saturation point reached 60%. The precipitates were collected by centrifugation at 4°C and 18000 rpm for 30 minutes. Each precipitate was dissolved in 10 mL of distilled water and dialyzed against several changes of distilled water at 4°C. The dialyzed solution was next adjusted to pH 7.0, and double the volume of cold acetone (-5°C) was added with stirring. The precipitates were collected by centrifugation at 4°C and were dissolved in 10 mL 0.2M Tris buffer pH 6.0. The precipitates were subsequently collected by centrifugation at 18000 rpm and 4°C for 30 minutes, dissolved in 0.2M Tris buffer and stored at 4°C until used.

Another procedure that was applied was the direct precipitation of the enzymes by using double volume of cold acetone (-5°C). The precipitates were again collected by centrifugation at 4°C and were dissolved in 10 mL 0.2M Tris buffer pH 6.0.

3.2.5. Analyses

3.2.5.1 Determination of the Growth Curve. The growth of cells was followed either by measuring the optical densities at 600nm or by determining the dry cell weight from known volumes of culture samples .

In the liquid media containing glucose such as YNB-G, YEP-G, YPG, the growth of the cells was followed by measuring the optical densities of the culture samples at 600nm in a DU640 Beckman Spectrophotometer. A calibration chart was prepared to correlate the dry weights to optical densities. A known volume of cell suspension was filtered through predried and preweighed filter paper of 0.22μ pore size under suction. The filter paper containing the cells was then dried at 60°C for one day and reweighed. The

difference in weights gave the mass of cells in the sample of the culture. For example, in YNB-G medium, the correlations of dry weights to optical densities were

OD = 0.53* (Dry Weight) -0.05 for YPG/MM
OD = 0.39 *(Dry Weight) -0.03 for YPG/AB
OD = 0.40* (Dry Weight) -0.026 for YA7 strains.

In the liquid media containing soluble starch such as YEP-S, YPS, since the contribution of the starch particles affected the optical densities of the culture samples, only the dry weights of the cells were measured. The known volumes of samples were centrifuged at 5000 rpm for 15 minutes, washed several times with deionized sterile water to get rid of starch and centrifuged prior to each wash. Finally, after suspending them in 1 mL sterile water, they were transferred to 1.5 mL preweighed eppendorf tubes. Cell precipitate was collected by centrifugation at 14 000 rpm for 10 minutes using an Eppendorf 5415 C bench-top centrifuge and then dried at 60°C for one day and reweighed.

3.2.5.2 Determination of Residual Starch Concentration. In the determination of starch concentration in the culture, aliquots of the culture supernatant was taken at certain time intervals. Known volumes of the aliquots were mixed with 5 mL of Iodine solution (0.5%KI and 0.15%I_2).These samples were diluted to the final volume of 15 mL with dH_2O. The absorbancy was read at 550 nm. against blank containing 5mL of Iodine solution and 10 mL of dH_2O.

Using the calibration chart prepared by known amounts of starch, absorbancies were converted to grams of starch concentration. **Figure 3.5** shows a sample calibration chart correlating absorbancies and grams of starch.

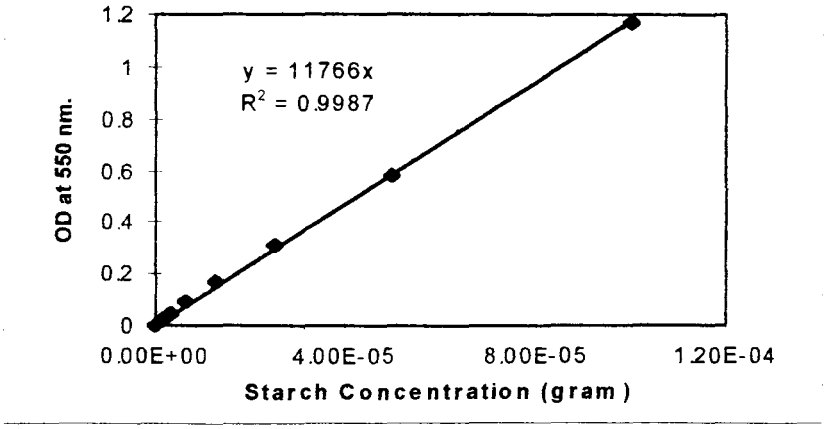
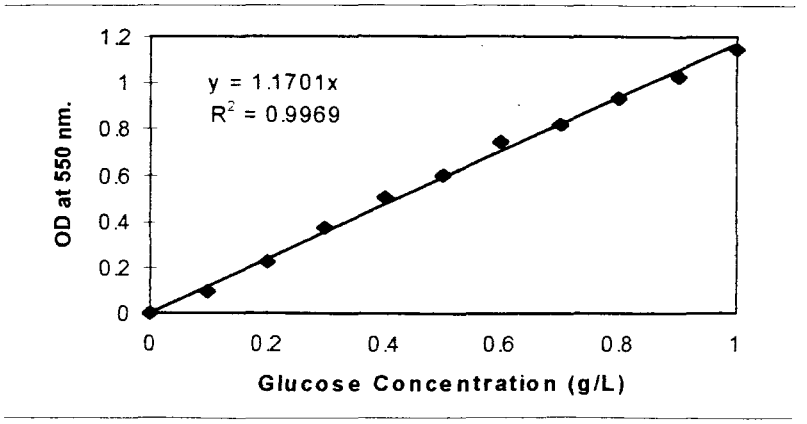


FIGURE 3.5. Calibration of Starch Analysis

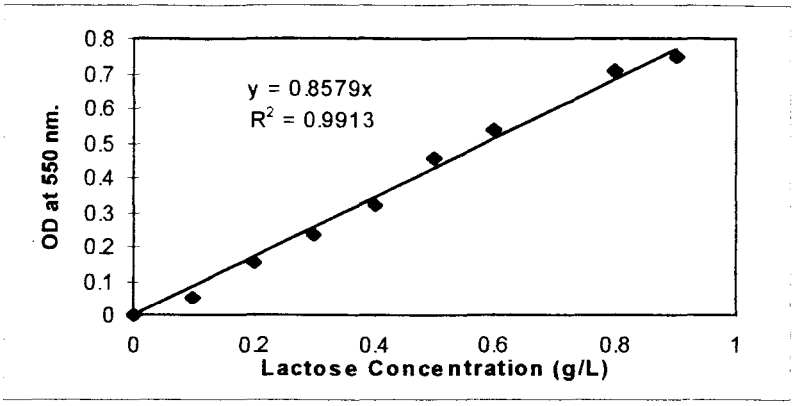
3.2.5.3 Determination of Reducing Sugar Concentration (DNS Method). The DNS Solution was prepared as described in Section 3.1.5.6. In order to be in the range of assay performance (up to 1g/L reducing sugar), the assay was performed by adding 1 mL of appropriately diluted samplesinto 3 mL of DNS Solution. After vortexing the samples for good mixing they were placed in a boiling water bath for 5 minutes. Finally, they were cooled down to room temperature under tap water and 6 mL of dH_2O was added and mixed. The absorbancy of the samples were measured at 550 nm against blank containing 3 mL of DNS solution and 7 mL of dH_2O treated as the samples.

Using a calibration chart prepared by known amounts of glucose, maltose and lactose, absorbencies were correlated with g/L of reducing sugars (**Figures 3.6 (a), 3.6 (b), 3.6 (c)**).

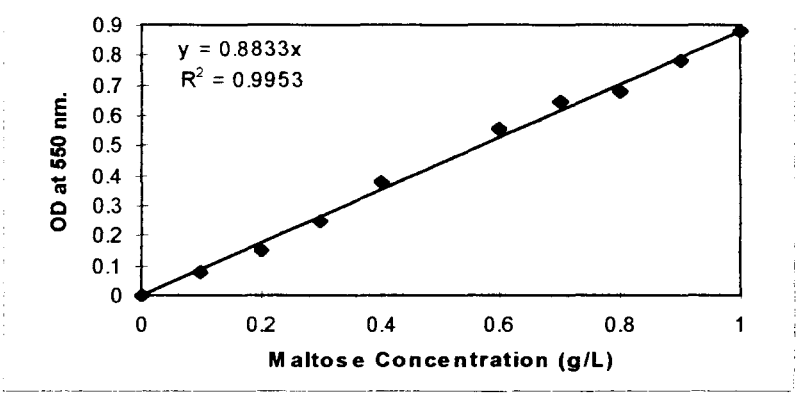
3.2.5.4 Determination of Glucose Concentration. The amount of residual glucose concentration in the medium was measured by using the D-Glucose Kit of Boehriger Mannheim as described by the manufacturer.



(a)



(b)



(c)

FIGURE 3.6. Calibration of Reducing Sugar Analysis based on (a) glucose, (b) lactose and (c) maltose.

D-Glucose is phosphorylated to glucose-6-phosphate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP).

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidized by nicodinamide-adenine dinucleotide phosphate (NADP) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH).

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-Glucose. The increase in NADPH is measured by means of its absorbancy at 340 nm.

The test combination contains Solution I: consisting of triethanolamine buffer, pH 7.6, NADP, ATP, magnesium sulfate and stabilizers, and Suspension II: consisting of hexokinase and glucose-6-phosphate dehydrogenase as well as Solution III: as D-glucose standard.

Samples were placed into a water bath at 80°C for 15 minutes to stop enzymatic reactions. They were centrifuged and the supernatants (diluted accordingly) were used for the assay. In a cuvette, 0.1 mL of sample, 1.0 mL of solution I and 1.9 mL of redistilled water were pipetted. In another cuvette as blank, 1.0 mL of solution I and 2.0 mL of redistilled water were pipetted. They were mixed and absorbencies (A_1) were read at 340 nm. against air after about 3 minutes. Then, the reaction was started by the addition of 0.02 mL of suspension II and mixing. After about 10 -15 minutes, which corresponded to the end of the reaction, absorbencies (A_2) were read again at the same conditions both for the sample and for the blank.

The absorbancy difference of the blank (A_2 - A_1) was subtracted from the absorbancy difference of the samples (A_2 - A_1) , thereby obtaining $\Delta A_{D\text{-glucose}}$. The concentration of D-glucose was calculated by the following equation:

$$c_{D\text{-glucose}} = D \times (5.441 / \varepsilon) \times \Delta A_{D\text{-glucose}} \text{ (g D-glucose / L sample)}$$

where ε = adsorption coefficient of NADPH at 340 nm. = 6.3 (l /mmol/cm)
D = Dilution factor of the samples

3.2.5.5 Determination of Ethanol Concentration. Two separate methods were used for ethanol determination. In one method, the amount of ethanol concentration in the medium was measured by GC. Shimadzu gas chromatograph (model GC-8A) equipped with CR-1B Chromatopac Processor and Recorder (model R-111 M) was used with a thermal conductivity detector (TCD). Helium supplied by BOS was the carrier gas for the analysis. A stainless steel column, 3 mm in inner diameter, 3 m in length and packed with 50/80 mesh Porapak Q was used. A calibration chart was also prepared (**Figure 3.7**).

The amount of ethanol was also determined by a second method, using an enzymatic kit the Boehringer Mannheim Ethanol Kit. Ethanol is oxidized to acetaldehyde in the presence of the enzyme alcohol dehydrogenase (ADH) by nicotinamide-adenine dinucleotide (NAD). The equilibrium of this reaction lies on the side of ethanol and NAD. It can, however, be completely displaced to the right at alkaline conditions and by the trapping of the acetaldehyde formed. Acetaldehyde is oxidized in the presence of aldehyde dehydrogenase (Al-DH) quantitatively to acetic acid. NADH is determined by means of its light absorbancy at 340 nm.

The test combination contains Mixture I: consisting NAD and aldehyde dehydrogenase and stabilizers, Suspension II: consisting of ADH, approximately 7000u, Solution III: as ethanol standard.

Samples were placed into a water bath at 80°C for 15 minutes to stop the enzymatic reactions. They were centrifuged and the supernatants (diluted accordingly) were used for the assay. 0.1 mL of sample and 3.0 mL of mixture I were pipetted into a cuvette. In another cuvette as blank, 3.0 mL of mixture I and 0.1 mL of redistilled water were pipetted. They were mixed and absorbencies (A_1) were read at 340 nm. against air after about 3 minutes. Then, the reaction was started by the addition of 0.05 mL of suspension II and mixing. After about 10 -15 minutes, at the end of the reaction, absorbencies (A_2) were read again at the same conditions both for the sample and for the blank.

The absorbancy difference of the blank (A_2 - A_1) was subtracted from the absorbancy difference of the samples (A_2 - A_1) , thereby obtaining $\Delta A_{ethanol}$. The concentration of ethanol was calculated by the following equation:

$c_{ethanol} = D \times (0.7256 / \varepsilon) \times \Delta A_{ethanol}$ (g ethanol / L sample)

where ε = adsorption coefficient of NADPH at 340 nm. = 6.3 (l /mmol/cm)
D = Dilution factor of the samples

The results of the two analysis methods i.e. the chromatographic method and the Boehringer-Mannheim Kit, were compared for the sake of accuracy. Both techniques were tested with standard ethanol and showed ca. 10% deviation.

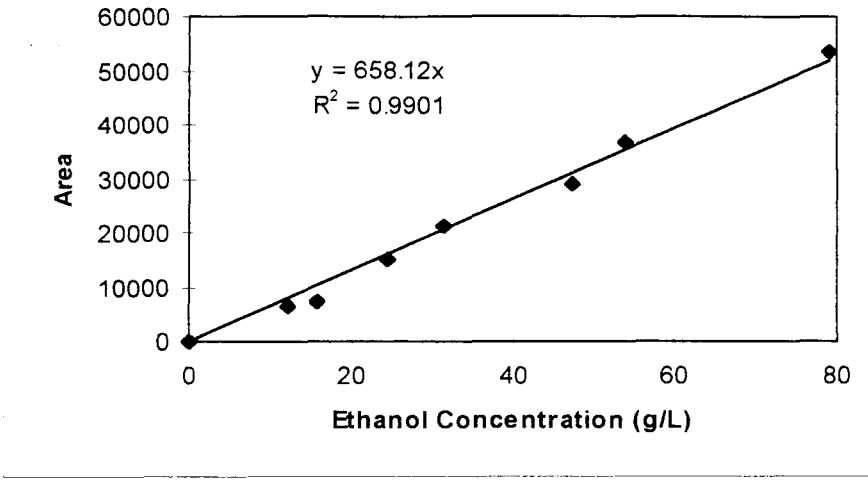


FIGURE 3.7. Calibration of Porapak Q column with EtOH

3.2.5.6 Determination of Extracellular α-amylase Activity. Extracellular α-amylase activity of the samples at different phases of growth was assayed as described by de Moraes et al. (1995). 0.2 g of soluble starch was added to 100 mL boiling 50 mM Na-acetate buffer (pH 5.9). The solution was cooled to 40°C. Iodine reagent was prepared by diluting 1 mL of stock solution (0.5% I_2 in 5% KI) in 500 mL deionized water containing 5 mL of 5 M HCl. 200 µl of the enzyme solution with 1 mL of starch solution was incubated

at 40°C in a water bath for 10 minutes. To stop the reaction, 200 µl of reaction mixture was added to 5 mL of iodine solution, and the degradation of starch was measured at 620 nm against 200 µl water in 5 mL iodine solution as blank. One unit of α-amylase activity was defined as the quantity of enzyme required to hydrolyze 0.1 mg starch in 10 minutes at 40°C when 2 mg starch was present at the start of the reaction.

3.2.5.7 Determination of Extracellular Glucoamylase Activity. Extracellular glucoamylase activity of the cells was assayed as described by de Moraes et al. (1995). 0.5 g soluble starch was added to 100 mL boiling 50 mM Na-acetate (pH 4.5) buffer. 200 µl of the enzyme solution was added to 1 mL aliquots of this starch solution and the reaction was incubated at the same temperature. At intervals of 10 minutes, 200 µl samples were taken and boiled for 10 minutes to denature the enzyme prior to determining the concentration of the glucose using a glucose oxidase assay kit. One unit of glucoamylase was defined as the amount of enzyme required to release 1 µmole glucose/min from starch.

3.2.5.8 Determination of Extracellular Protein Concentration. The method was performed by the kit provided by Bio-Rad based on the Bradford method (Bradford, 1976) following the recommended conditions by the manufacturer. Bovine serum albumin was used as standard.

Bradford dye reagent was diluted with dH_2O (1:4) and 250 µL of this reagent was pipetted into cuvettes. A certain volume of the samples to be tested was also added into the dye reagent. dH_2O was added to a total volume of 1250 µL. The cuvettes were mixed several times by gentle inversion. After a period of time ranging from 5 minutes to 1 hour at room temperature, the absorbancy of each sample was read at 595 nm. against blank containing 250 µL of dye reagent and 1000 µL of dH_2O.

A standard curve was also prepared at the same conditions described above with known amounts of bovine serum albumine (**Figure 3.8**).

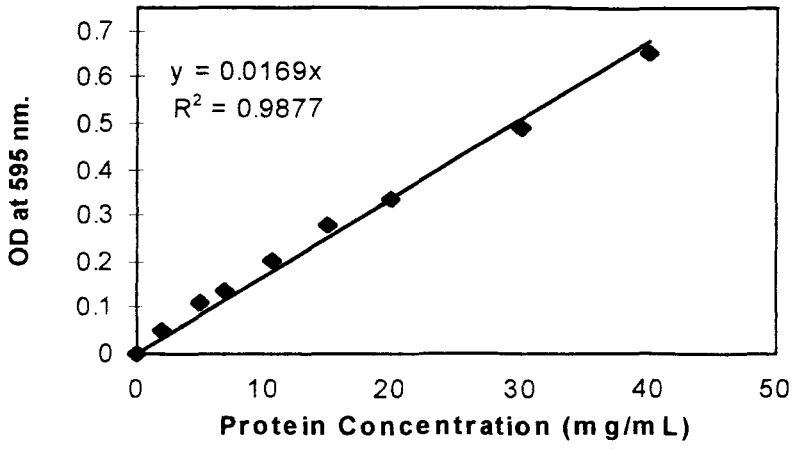


FIGURE 3.8. Calibration of Protein Analysis

3.2.5.9 Determination of Intracellular RNA Concentration. The colorimetric assay involving the reaction of aldopentoses with acidified orcinol to produce a green chromogen was used to measure the RNA content. The purine-ribose links of RNA are easily hydrolyzed by hot acid and the purine-bound ribose of RNA can be determined colorimetrically. Deoxyribose gives 20% of the color of ribose (Yıldır, 1997).

Each sample with known volumes of yeast culture was centrifuged at 10000 rpm for 10 minutes. The cell pellets were acidified by addition of 1.5 mL of ice-cold 0.25N perchloric acid and allowed to stand on ice for at least 30 minutes. The samples were then centrifuged at 10000 rpm for 15 minutes and the precipitates were resuspended in 4 mL of 0.5N perchloric acid. After 15 minutes of incubation at 70°C with occasional shaking, suspensions were centrifuged at 5000 rpm for 10 minutes at room temperature. The cell hydrosylates were diluted with 0.1N HCL so that they contain 10-100 μg of RNA per mL. Onto this solution, two volumes of orcinol reagent which was freshly prepared by mixing equal volumes of ferric chloride reagent with ethanolic orcinol solution. After 30 minutes of incubation at 90°C with occasional shaking, the samples were cooled down to room temperature and the absorbancy of each sample was determined at 665 nm. against blank containing one volume of 0.1N HCL and two volumes of orcinol reagent.

Using the calibration chart prepared by known amounts yeast RNA absorbencies were converted to mg/L of RNA concentration. **Figure 3.9** shows a sample calibration chart correlating absorbencies and mg/L of RNA.

3.2.5.10 Determination of Intracellular Plasmid DNA Concentration. A yeast plasmid isolation protocol was applied for the determination of plasmid DNA concentration in the cell (de Moraes, 1994).

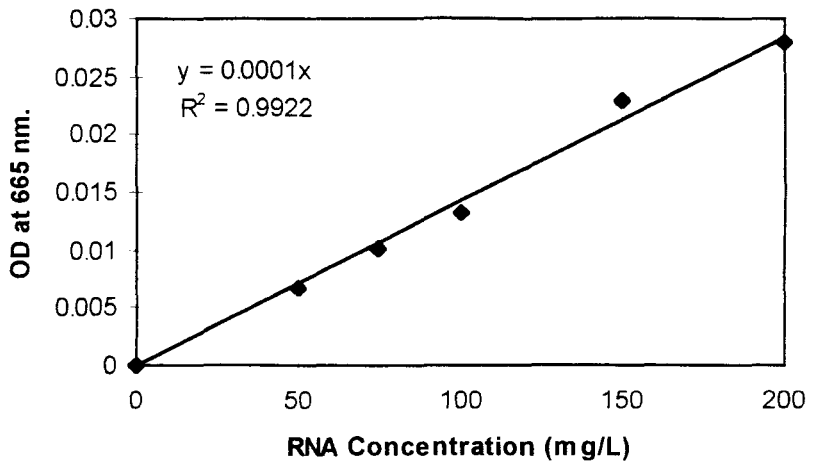


FIGURE 3.9. Calibration of RNA Analysis

10 to 15 mL of yeast culture pellets at its different phases of growth were collected by centrifugation at 5000 rpm at 4°C for 5 minutes. The precipitate was resuspended in 1 mL of sterile dH_2O and collected in a 1.5 mL eppendorf tube. After spinning it down for 30 seconds, 0.3 mL of yeast recovery buffer (as prepared in section 3.1.5.4) was added and the precipitate was solved and mixed by vortexing. It was followed by the addition of 0.9 volume of glass beads (150-212 μ in diameter) and vortexing again for 5 minutes. Into this suspension, 40 μL of 10 μg/mL RNAse which is free of DNAse was added and was incubated at 37°C for at least 30 minutes. After the incubation, one volume of TE saturated phenol was pipetted onto the sample and mixed following a centrifugation period of 5 minutes at 12000 rpm. The clear upper phase was taken very gently to avoid back mixing and one volume of upperphase chloroform:isoamylalcohol (24:1) was added and mixed by

vortexing. Again the upperphase was collected by centrifugation at 12000 rpm for 5 minutes into a new eppendorf. Three volumes of icecold pure ethanol was added and shaken by inverting the tube while observing the bubble formation. This sample was kept at -80°C for about 30 minutes or at -20°C overnight. Ethanol precipitated samples were centrifuged for 25 to 30 minutes at 14000 rpm. Ethanol was then poured out and the tubes were blot dried and the pellet was incubated at 37°C for 25-35 minutes for drying. Then, the final dried DNA pellets were dissolved in 10 µL TE buffer. The amount of DNA was measured spectrophotometrically at 260 nm. against TE buffer. 50 µg/mL DNA was taken as the unit absorbancy value at 260 nm. In addition to that, the absorbencies were also read at 280 nm. to follow the contamination of RNA and protein level in the isolated plasmid DNA.

3.2.5.11 Determination of Plasmid Stability. The plasmid stability of the cell culture was determined by the method of replica plating. Two techniques were applied: plate to plate transfer by using a velvet pad and transfer of single colonies by toothpick (Rehm et al., 1991).

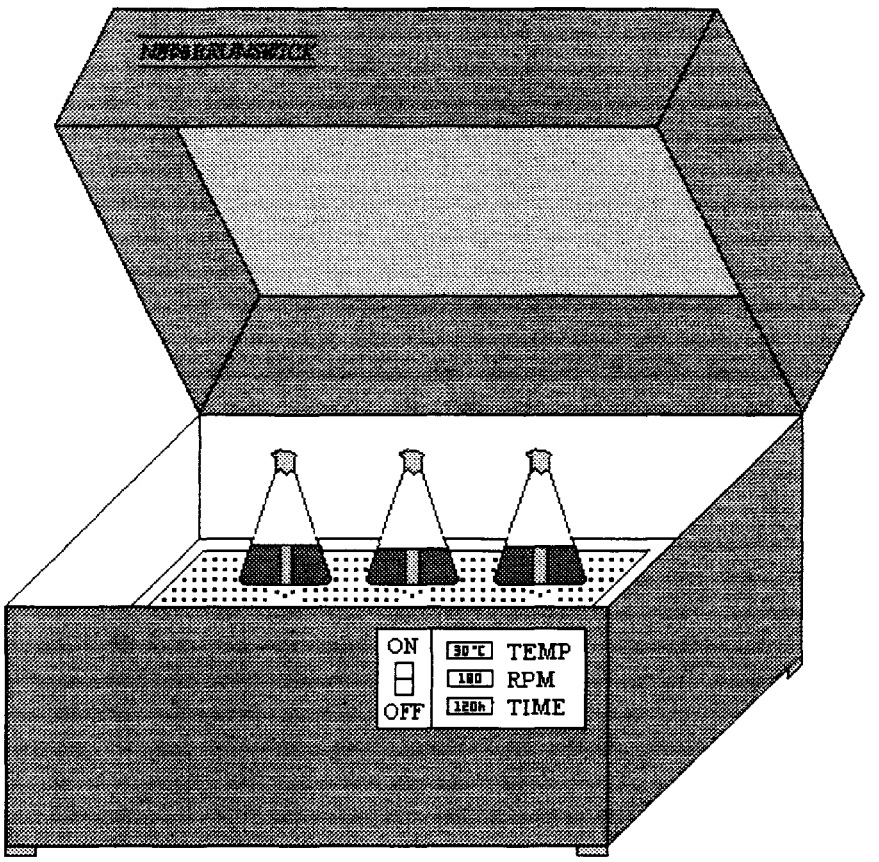
In the former technique, a sterile velvet pad was pressed over the YPG agar plate containing well separated colonies, the colonies picked by the velvet were transferred on a YNB-G agar plate. In the latter technique, the transfer of colonies were done by using a sterile toothpick. Colonies were gently toughed with the tips of the sterile toothpick and transferred to YNB-G agar plates.

In both of the techniques used, the culture samples were appropriately diluted giving rise to growth as single colonies. And these were spread on the YPG agar plates to allow the growth of both plasmid containing and plasmid free cells. After the single colonies were grown on YPG agar plates at 30°C, they were transferred to YNB-G agar plates which allows the growth of plasmid containing cells only. The cell colonies were counted on each plate.

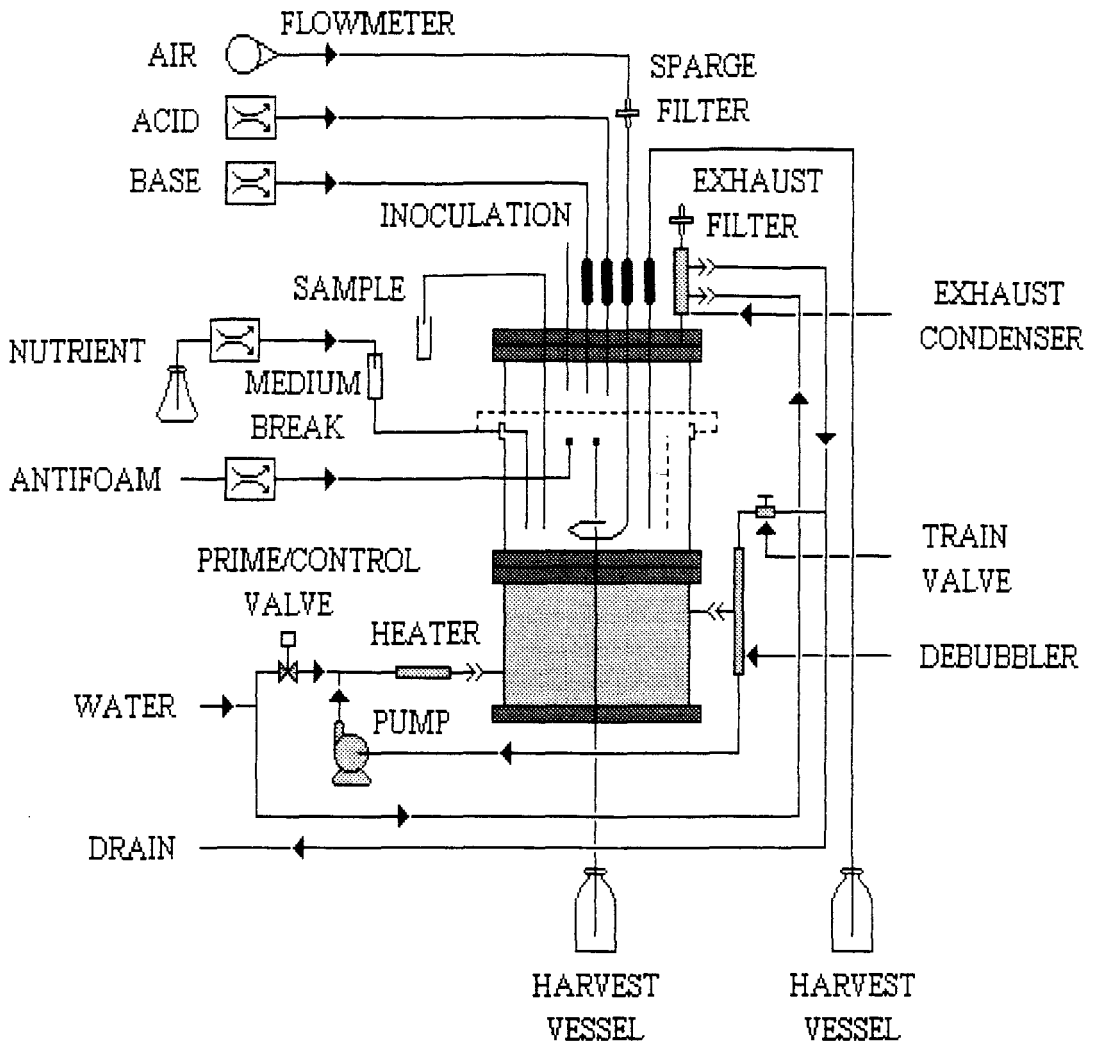
The number of total cells (plasmid containing and plasmid free) minus plasmid containing cells divided by total cell number gave the percent of plasmid free cells.

3.2.6. Experimental Set-Up

Fermentation experiments were performed either in shake flasks or in New Brunswick Bioflo III batch/continuous fermenter. **Figure 3.10 (a)** and **3.10 (b)** show the experimental set-ups.



(a)



(b)

FIGURE 3.10. Experimental Set-Up (a) Shake Flask, (b) Fermenter.

3.2.6.1 Experiments in Shake Flasks. Different sets of experiments were performed in shake flasks. These experiments were in general conducted at 180 rpm and 30°C in orbital shakers either in GFL-3032 or in Innova 4340. The pH was allowed to follow its natural course.

The culture volumes were changed depending on the aim of the experiment, but the flasks were filled with nutrient medium to one-third of their total volume to allow good aeration, and the samples were withdrawn from the flasks under the Laminar Flow Cabinet

(Holten-Lamin Air) to avoid contamination. The total volume of all the samples withdrawn was limited to 10% of the culture medium to eliminate the change in total volume with time.

A series of experiments were conducted for the determination of the growth characteristics of the different recombinants. In these experiments, the time course of cell growth, substrate consumption, accumulation of sugars if any, α-amylase and glucoamylase secretion, extracellular protein and ethanol production and plasmid stability were determined in YNB-G and YEP-Sg media.

Furthermore, in another set of experiments, fermentation characteristics of the recombinants were investigated in these shake flasks. In these experiments, the time course of cell growth, substrate consumption, accumulation of sugars if any, α-amylase and glucoamylase secretion and extracellular protein production, ethanol production, and plasmid stability were determined in YEP-S, YEP-G, YPS and YPG media. In particular experiments, the intracellular RNA concentration and plasmid DNA concentration were also determined.

The total operation time was around 120 - 150 hours for starch fermentation while it was around 24 hours for glucose fermentation.

3.2.6.2 Fermenter Experiments. An inoculum of recombinant *Saccharomyces cerevisiae* cells (200 mL), grown in a shake flask at 180 rpm and 30°C in YNB-G medium until its late exponential phase, was added into the sterile fermenter containing 1500 mL nutrient medium. Depending on the particular experiment, the medium was either YEP-S or YPS or YPG. The total volume of all the samples withdrawn was limited to 10% of the culture volume to eliminate volume change with time.

In the case of starch fermentation, the time course of cell growth, substrate consumption, glucose and reducing sugar accumulation, ethanol production, α-amylase and glucoamylase secretion, extracellular protein production, intracellular RNA concentration and intracellular plasmid DNA concentration were followed by the

analytical techniques described in Section 3.2.4. The total operation time was around 50 - 150 hours in fermenter experiments.

In the case of glucose fermentation, the time course of cell growth, glucose consumption, ethanol production, α-amylase and glucoamylase secretion, extracellular protein production, intracellular RNA concentration and intracellular plasmid DNA concentration were followed by the analytical techniques described in Section 3.2.4. The total operation time was around 24 hours for glucose fermentation.

3.2.6.3 Instrumentation and Control of the Fermenter. *Description of Vessel.* The vessel parts consist of a stainless steel head plate, a flanged glass tube (thick walled) vessel (2.5 L) body which is detachable from the bottom-dished head. The dished head is jacketed for circulation of temperature controlled water. Four sterilizable polypropylene compression ports are provided in the glass wall for the addition of antifoam and nutrients, as well as for the vessel overflow in continuous culture studies. Ports are provided in the headplate for: inoculation, base and acid addition; a thermowell for a resistance temperature detector; a temperature sensor; a sparger; a harvest port; a sampling port; an exhaust condenser; dissolved oxygen and pH electrodes. The drive bearing housing is allocated on the headplate.

Agitation System. A removable agitation servo motor located on top of the bearing housing is connected to the agitation shift with a multi-jaw coupling. It can be easily disconnected while autoclaving the vessel and replaced after sterilization. The motor provides agitation speed range of 12-1000 (±1) rpm.

Temperature Control. The culture temperature may be selected in the range from 20°C to 60°C (±1°C) and is controlled by a microprocessor based PI (proportional and integral) controller. The medium temperature is sensed by an RTD (resistance temperature detector) submerged in the thermowell.

Aeration. Sterile air is introduced into the medium through the ring sparger, and is controlled by the needle valve of the flowmeter. It is able to provide 1.5 (working volume)

of sterile air through 0.2 μm replaceable cartridge filter. The filter is sterilazable with the vessel. With the system, oxygen transfer rates of 350 mM O_2/L/hr may be obtained.

pH Control. pH is controlled in the range of 2.00-12.00 (±0.01).The pH is sensed by a glass electrode. Control is maintained by PID controller which operates two peristaltic pumps connected to acid and base addition ports.

Dissolved Oxygen Control. DO is controlled in the range of 5-95% (± 1%). It is sensed by a polarographic DO electrode and control is maintained by the PID controller which changes the speed of agitation. The polarographic dissolved oxygen electrode consists of polarized platinum and silver electrodes, with the electrolyte separated from the sample by teflon/silicone gas permeable membrane. Oxygen diffuses across the electrode membrane and is reduced to hydroxyl ions at the platinum cathode according to the reaction;

$$O_2 + 2H_2O + 4e^- \longrightarrow 4OH^-$$

The electrons necessary for this process are produced by a reaction at the silver anode. Because the electrolyte contains chloride ions, this reaction occurs as;

$$4Ag + 4Cl^- \longrightarrow 4AgCl + 4e^-$$

At any given temperature, the current flow between cathode and anode is directly proportional to the level of oxygen outside the membrane.

Foam Control. Foam is controlled during batch fermentation by the antifoam probe which is located in the headplate. The controller operates the antifoam addition pump that adds chemical defoamer through the port in the wall of the vessel. Sensitivity and time adjustments are made on potentiometers located on a small printed circuit board mounted on the underside of the electronic shelf, easily accessible when the rear door is open.

Medium Flow Control. A nutrient feed peristaltic pump is provided for use during continuous culture fermentation. The maximum flow rate which can be attained using this pump is 10 mL/min. with the proper tubing.

Exhaust System. The exhaust gases pass into the exhaust condenser where moisture is removed and returned to the vessel. The remaining air passes into the 0.2 μm exhaust filter.

Sampling System. The hooded sampler is attached to a sampling tube that extends to the bottom of the vessel. The sampler has a rubber suction bulb to facilitate collection of representative samples without contamination. A 25 mL screw cap container serves as a reservoir.

Flowmeter. The flowmeter is a simple, precise means of indicating flow rates in fluid systems. Their design is based on the variable area principle.

3.2.6.4 Calibration of the pH Probe. After the electrode was connected to the probe cable, the power was switched to ON position when the agitation switch was OFF. The selector switch was set to pH and the mode switch to ZERO. pH probe was immersed into an external pH 7.00 buffer solution. After waiting for a certain time interval, the display was set to read the pH value of the buffer solution by INC/DEC switch. Then the probe was immersed into a second external buffer solution which is several pH units above or below the pH selected in the previous step and the Mode switch was turned to SPAN. Again by using INC/DEC switch, the display was set to the value of the second buffer solution.

After autoclaving, the pH of a sterile solution at known pH was measured with the probe to check the calibration.

3.2.6.5 Calibration of Dissolved Oxygen Probe. Dissolved oxygen probe was calibrated by following the dynamic gassing out method. After the electrode was connected to probe cable, the power was switched to ON position when the agitation switch was OFF. The selector switch was set to DO and the mode switch to ZERO. DO probe was immersed into

6%(w/v) sodium sulfite solution at 30°C. After waiting for a certain time interval, the display was set to read DO value of 000 by INC/DEC switch. Then the probe was immersed in a one liter Erlenmeyer flask located in a water bath at 30°C which was filled with distilled water and aerated for 100% calibration. Mode switch was turned to SPAN. Again by using INC/DEC switch, the display was set to 100. After adjusting the controller to 100%, the probe was immersed in a defined medium aerated at under same conditions as above. A reading of still 100% revealed that the medium components did not affect the solubility and diffusivity of oxygen and consequently the response of the probe.

4. RESULTS AND DISCUSSION

4.1. Choice of Microorganism

The optimization of the expression and secretion of the functional starch hydrolyzing enzymes, namely α-amylase and glucoamylase in genetically modified *Saccharomyces cerevisiae* was considered to be very important for the one step bioconversion of starch rich materials to ethanol or single cell protein.

In this work, four different recombinant yeast strains which were recently constructed by de Moraes (1994) were used. The cells were investigated in many aspects, including medium composition, fermentation characteristics, behavior under bioreactor conditions, secretion of α-amylase and glucoamylase, and plasmid stabilities under different conditions.

Saccharomyces cerevisiae host strain GRF18 (MATa, his3-11, his3-15, leu2-3, leu2-112, can1, mal) and four recombinant *Saccharomyces cerevisiae* strains, (i) YPB-G which expresses the *Bacillus subtilis* α-amylase - *Aspergillus awamori* glucoamylase (BsAAase/GAase) as a fusion protein, (ii) YPG/AB bearing a double cassette plasmid to produce separately *Bacillus subtilis* α-amylase (BsAAase) and *Aspergillus awamori* glucoamylase (GAase), (iii) YPG/MM that expresses mouse pancreatic α-amylase - *Aspergillus awamori* glucoamylase (MAAase/GAase) as separate proteins and also (iv) YA7 bearing another double cassette plasmid to produce separately *Bacillus subtilis* α-amylase (BsAAase) and *Aspergillus awamori* glucoamylase (GAase), were used in this study.

4.2. Growth Characteristics of Recombinant Yeast Strains

A set of preliminary experiments were conducted in shake flasks in order to understand the growth behavior of the recombinant yeast strains and the host (YPG/AB, YPG/MM, YA7 and GRF-18 respectively) in different media. A homogeneous dispersed culture formation was observed for all media.

4.2.1. Growth on YNB-G Medium

Investigation of the growth characteristics of the recombinant cells in Yeast Minimal Medium supplemented with histidine (YNB-G) was very important, since the precultures were grown on this medium throughout the study. All the recombinant strains were grown into single colonies on YNB-G agar plates at 30°C for 48 hours.

In the first set of experiments, a colony from YNB-G agar plate was directly inoculated into a 250 mL liquid medium and grown at 30°C and 180 rpm. The growth pattern was followed spectrophotometrically. The samples were appropriately diluted in order to avoid divergence from linearity of the absorbancies (see **Figure 4.1**).

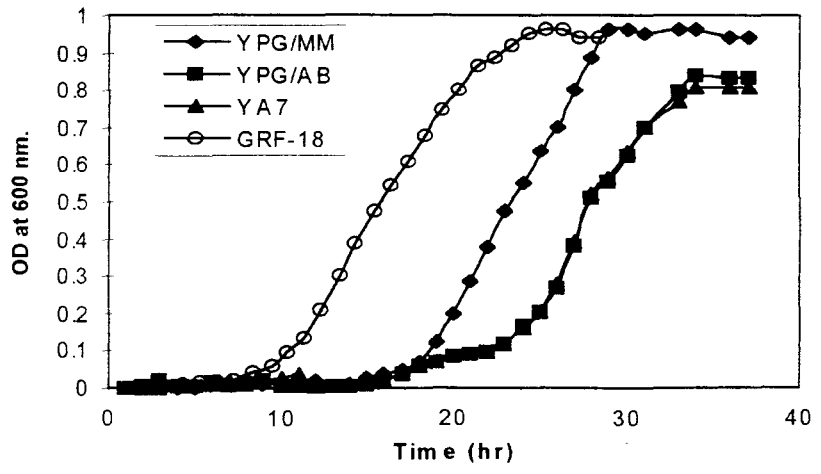


FIGURE 4.1. Growth Patterns of Yeast Strains on YNB-G Medium Starting with a Single Colony (OD readings taken at 1:5 dilution).

The adaptation times to the medium which is called the *lag phase* were around 17, 22 and 22 hours for YPG/MM, YPG/AB and YA7 respectively when a single colony was inoculated into YNB-G liquid medium. The GRF-18 host strain had a relatively shorter adaptation period of 8 hours in the YNB-G medium supplemented with both histidine and leucine. The *exponential phase* at which the cells were growing exponentially lasted for 10-12 hours until the 29, 32 and 32nd hours for YPG/MM, YPG/AB and YA7 respectively. The recombinant strains entered their *stationary phase* after this period, while, GRF-18 entered its stationary phase of growth at the end of 23 hours. When the volume of the culture medium was reduced to 50 mL, the cells were able to reach their stationary phase to an OD value of approximately 1.00 (at 1:5 dilution) at the end of around 24 hours of fermentation. The maximum specific growth rates, μ_{max}, of the recombinant strains were calculated from the slopes of lnx versus time plots by linear regression, as 0.180 ± 0.004 h^{-1} and as 0.265 ± 0.001 h^{-1} for the host.

In the second set of experiments, each culture medium was inoculated with cells grown for 24-hours (OD of approximately 1.00 at 1:5 dilution) with 2%(v/v) inoculum, which could be treated as a dilute inoculation (see **Figure 4.2**).

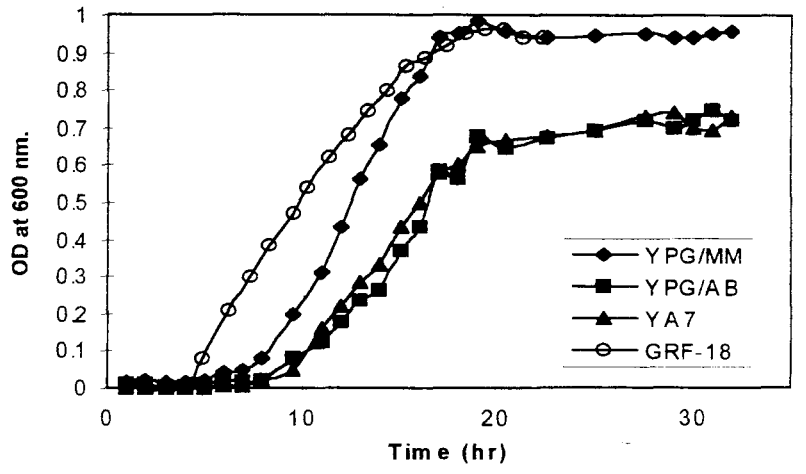


FIGURE 4.2. Growth Patterns of Yeast Strains on YNB-G Medium with 2%(v/v) Vegetative Inoculum (OD readings taken at 1:5 dilution).

The *lag phase* periods were around 8,10 and 10 hours for YPG/MM, YPG/AB and YA7 respectively when vegetative inoculation was made. Host cells had again a shorter adaptation period of 4 hours. The *exponential phase* lasted until the 17, 19, 19 and 19th hours for YPG/MM, YPG/AB, YA7 and GRF18 respectively followed by the *stationary phase*. The maximum specific growth rates, μ_{max}, of the recombinant strains calculated from the slopes of lnx versus time plots by linear regression, were $0.180 \pm 0.004\ h^{-1}$ again. The host strain had also the same maximum specific growth rate ($0.264 \pm 0.001\ h^{-1}$). All the recombinants have shown similar growth patterns in YNB-G medium.

Table 4.1 shows the maximum specific growth rates and the final biomass formations of the strains obtained when single colony inoculation and vegetative inoculation were made. In addition, the pH of the culture medium was also monitored for all the strains. The natural course of pH changes with respect to time from a pH value of 6.50 to approximately 3.00 (**Figure 4.3**).

TABLE 4.1. Summary of the Growth on YNB-G Medium

	Single Colony Inoculation			Vegetative Inoculation		
Strain	$\mu_{max} \pm 0.01$ (h^{-1})	Final Biomass Concentration (g/L)	t_{lag} (hr)	$\mu_{max} \pm 0.01$ (h^{-1})	Final Biomass Concentration (g/L)	t_{lag} (hr)
YPG/MM	0.179	1.69	17	0.180	1.70	8
YPG/AB	0.182	2.02	19	0.184	2.00	10
YA7	0.183	2.10	19	0.184	1.98	10
GRF-18	0.265	4.10	7	0.264	4.11	4

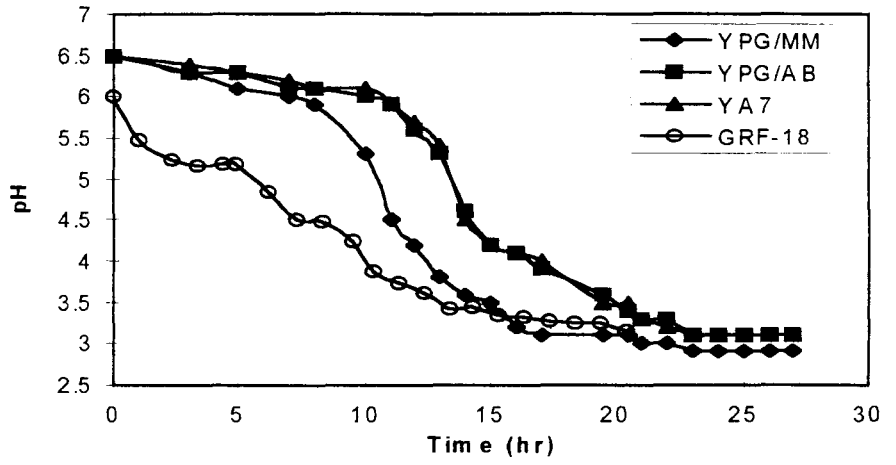


FIGURE 4.3. Variation of the pH of YNB-G Medium with 2%(v/v) Vegetative Inoculum.

These experiments have shown that the only apparent difference between a single colony inoculation and vegetative inoculation was in the adaptation period of the cells. When the cells were grown starting with a single colony, the lag phase lasted for a period which was two times longer than in the case of vegetative inoculation. No significant changes were observed in the final biomass formations and the maximum specific growth rates of the strains. Since the constructions of YPG/AB and YA7 strains were similar to each other, only difference being the orientation of the coding sequences, both have shown very similar growth characteristics.

In all these experiments, the extracellular α-amylase and glucoamylase activities were also assayed but no activity could be detected. Additional experiments were carried out for the activity detection with the following questions in mind: (1) Could something be wrong with the enzyme assays? (2) Might the activities of the secreted enzymes be inhibited due to the pH of the medium? (3) Were the plasmids unstable? (4) Could any mutations in the constructs be possible? (5) Were the α-amylase and glucoamylase not secreted into the medium somehow?

The following tests were conducted to find out the answers to these questions:

4.2.1.1 Enzyme Assay Test. The enzyme assays were performed with standard α-amylase and glucoamylase provided from Boehringer Mannheim to check the assay performance. Different dilutions of the standard enzymes were assayed, and the activities calculated from the measurements were in good agreement with the commercial activities labeled on the bottles of the enzymes being within the 95% confidence limits.

4.2.1.2 Buffering the System. Possible inhibition of the enzyme activity due to the pH changes in the medium with time was checked by buffering the medium with different buffer systems (Rossini et al., 1993). The pH of the culture medium was kept at 5.00-6.00 at which *Bacillus subtilis* and mouse pancreatic α-amylase, and *Aspergillus awamori* glucoamylase show high activities (Bezbaruah et al., 1991; de Moraes et al., 1995).

Since the most appropriate buffer for the enzyme assay was 50 mM Na-Acetate in which the reaction of the enzyme with the substrate takes place, the first buffer system used was Na-Acetate (pH=5.85). One of the recombinant strains (YA7) was grown on YNB-G medium (**Figure 4.4**), and the pH of the medium was kept constant at 5.00 ± 0.05 by the addition of Na-Acetate. The growth was also followed spectrophotometrically. Intracellular and extracellular α-amylase activities were tested. The cells were disrupted by vortexing the yeast cells in the presence of glass beads as described (Rossini et al., 1993) for the intracellular activity measurements. Although intracellular α-amylase activity (in the cytoplasm) was detected, no extracellular α-amylase activity was present in the medium. Whereas, the activity in the periplasm was not measured.

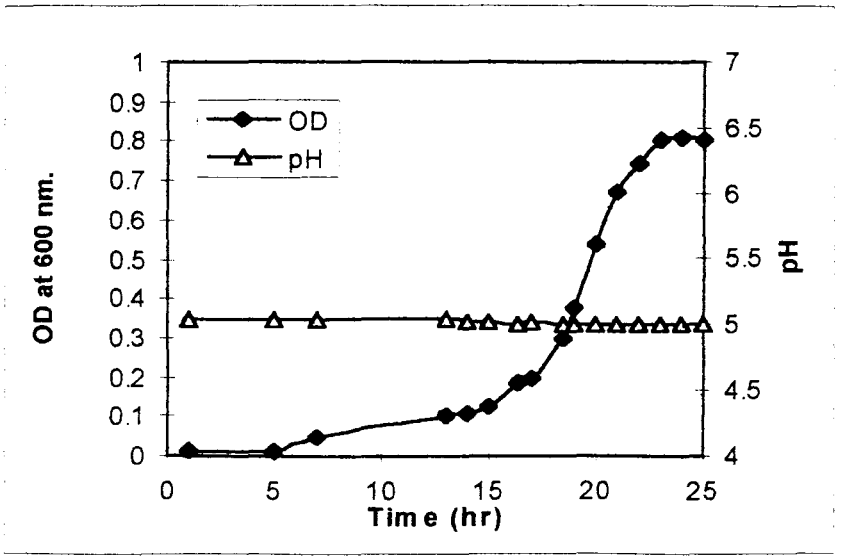


FIGURE 4.4. Growth Pattern of YA7 on YNB-G Medium Buffered with 50mM Na-Acetate.

In the second experiment, the pH of the medium was kept constant between 5.50 and 5.00 by the addition of 3% (w/v) succinic acid (pH=6.80) (De Nobel and Barnett, 1991). All three of the recombinant strains were grown in YNB-G medium buffered with 3% succinic acid (**Figure 4.5**).

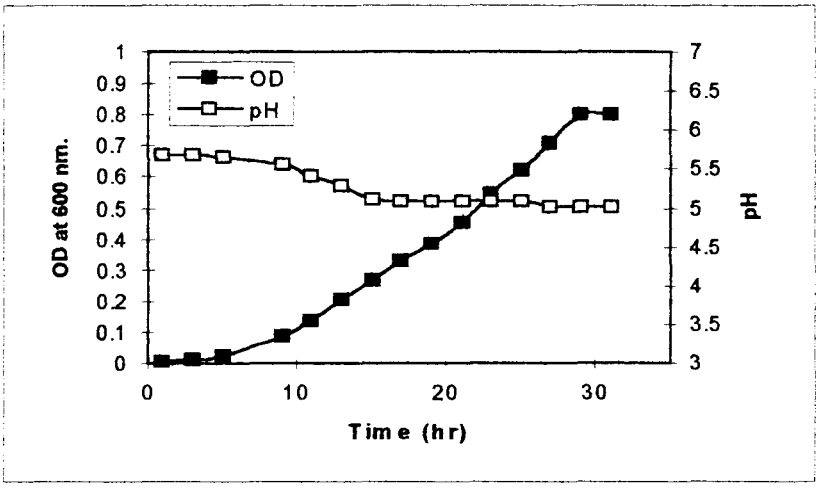


FIGURE 4.5. Growth Pattern of YA7 on YNB-G Medium Buffered with 3%(w/v) Succinic Acid.

Thirdly, 50 mM $CaCl_2$ was also added to the medium buffered with 3% succinic acid to stabilize the α-amylase; the pH was kept around 5.50 - 5.00 (**Figure 4.6**) (De Nobel and Barnett, 1991).

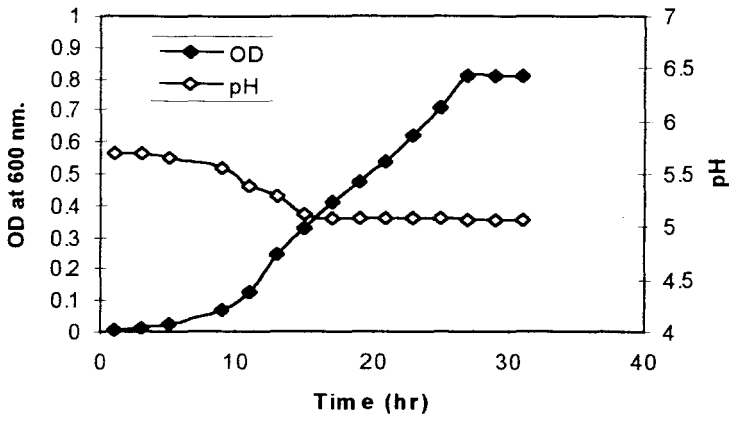


FIGURE 4.6. Growth Pattern of YA7 on YNB-G Medium Buffered with 3%(w/v) Succinic Acid containing 50mM $CaCl_2$.

In all cases, some α-amylase activity was observed within the cell (in the cytoplasm), but there was no detectable activity in the culture medium. These observations have shown that buffering the system did not play a negative role on the growth pattern of the cells. The intracellular α-amylase activity was measured as ca. 12 U/mL in all the buffer systems used.

4.2.1.3 Plasmid Stability. The stability of the plasmids was also investigated by experiments explained in detail in Section 3.2.5.11. The plasmids have shown high stability (ca. 100%) when the recombinant strains were grown on YNB-G medium.

4.2.1.4 Plasmid DNA. The plasmids of the recombinant cells grown on YNB-G medium were isolated as described in Section 3.2.5.10 and were exposed to agarose gel electrophoresis for comparing the lengths of the plasmid DNA with the original samples kindly provided by Dr. Stephen Oliver (UMIST, UK). The presence of the plasmids were confirmed in all cases.

Periodical tests were conducted by growing the cells on YPS agar plates for 72 hours at 30°C in an incubator. These plates were exposed to Iodine vapor, and the white or pink halo formation was observed as described in Astolfi-filho et al. (1986). The halo formation has indicated the presence of the extracellular α-amylase and glucoamylase activities on starch containing medium (**Figure 4.7**).

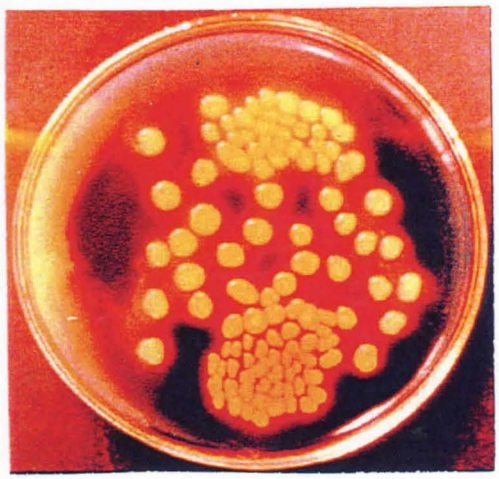


FIGURE 4.7. Colonies Showing Amylolytic Activity - I_2 Staining Test

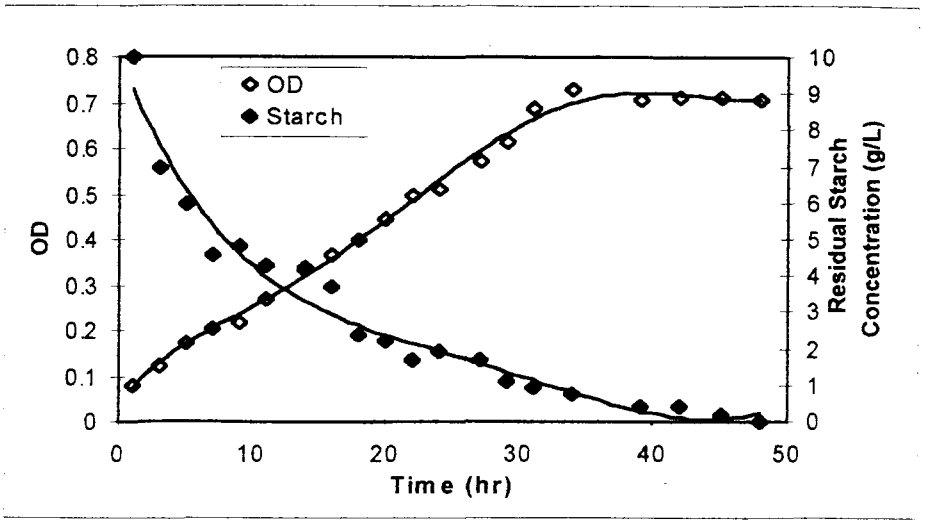
4.2.2. Growth on YEP-Sg Medium

In order to investigate the abilities of the recombinant yeast strains to grow on starch containing media, three strains, namely YPG/MM, YPG/AB and YA7 were used in a series of preliminary experiments. Since the efficiencies of these strains to degrade soluble starch and to use its products to grow were not known, the starch medium was supplemented with glycerol. In these experiments, the concentration of starch in the YEP-Sg medium was kept at 1 and 2% (w/v) at which the starch is completely soluble.

The experiments were carried out at 30°C and 180 rpm in shake flasks in an orbital shaker. The pH was allowed to follow its natural course. The cell growth was followed spectrophotometrically. Starch degradation, glucose accumulation and the change in pH with respect to time were investigated for YPG/MM, YPG/AB and YA7 strains during 48 hours of operation.

YPG/MM strain degraded 50% of the 1%(w/v) and 2%(w/v) initial soluble starch concentration in 9 hours and 12 hours respectively, during its growth at the early stages of the exponential phase in YEP-Sg medium. The maximum accumulation of glucose in the medium was 0.07 g/L on 1%(w/v) soluble starch, and this value increased to 0.18 g/L on 2%(w/v) soluble starch (**Figure 4.8-4.9**).

In the case of the YPG/AB strain, degradation of 50% of the 1%(w/v) and 2% (w/v) initial soluble starch concentration was completed after 18 hours and 20 hours respectively during its growth at the mid-exponential phase on YEP-Sg medium. Simultaneous production and consumption of glucose by the recombinant yeast cells were observed. The accumulation of glucose was higher than that of YPG/MM strain, were measured as 0.24 g/L and 1.64 g/L on 1%(w/v) soluble starch and on 2%(w/v) soluble starch respectively (**Figure 4.10-4.11**).



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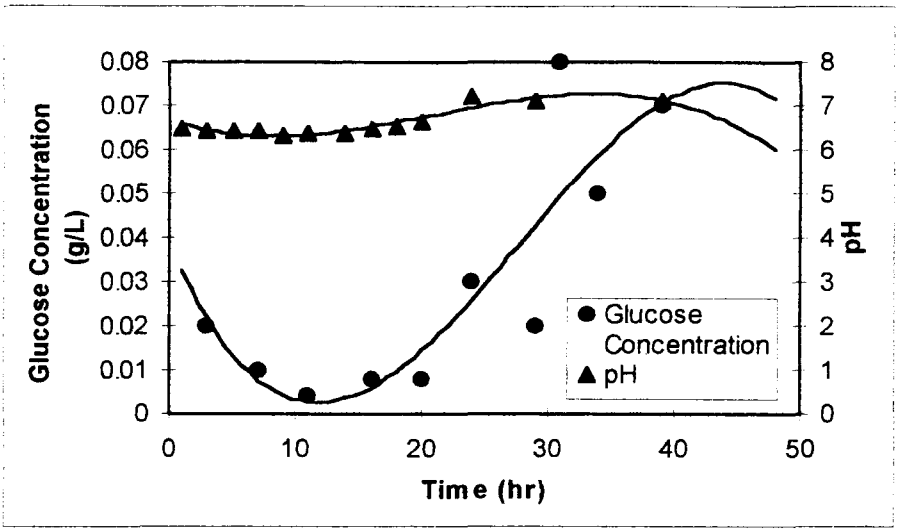
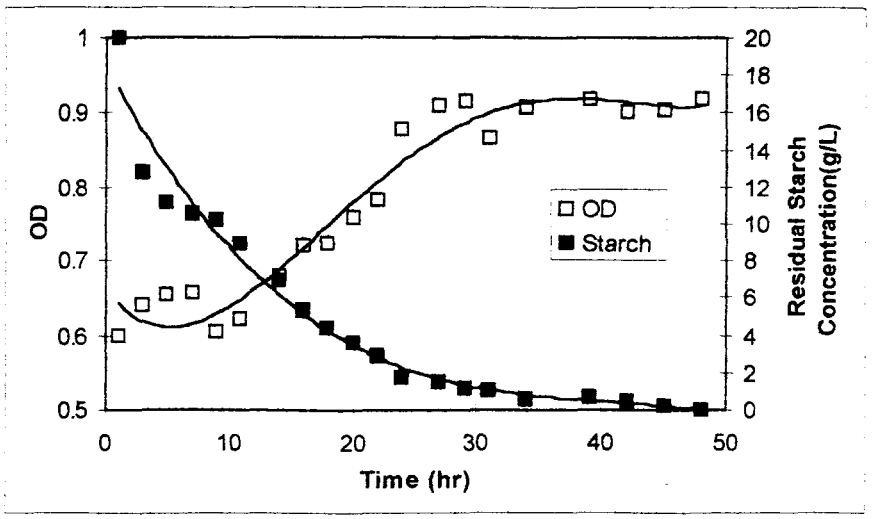


(b)

FIGURE 4.8. (a) Growth Pattern (b) Glucose Accumulation and pH changes of the YPG/MM Strain in YEP-Sg Containing 1% (w/v) Starch.



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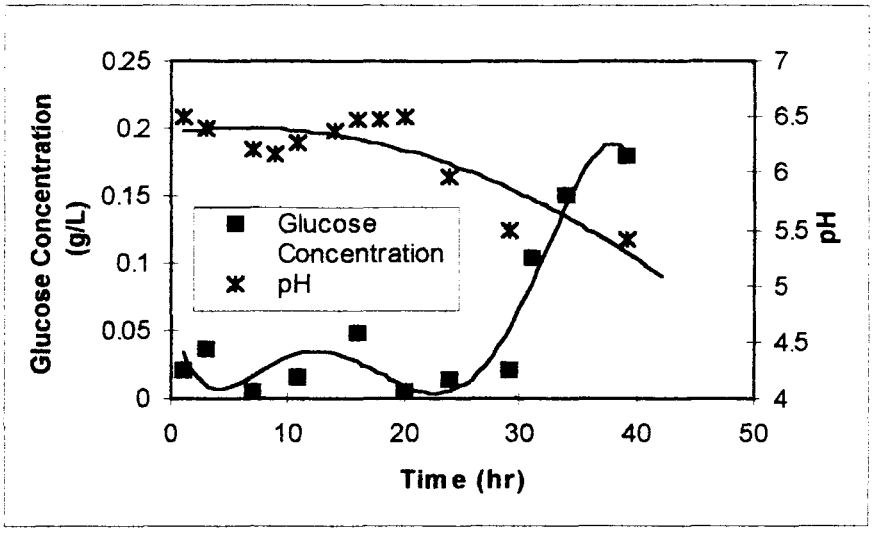
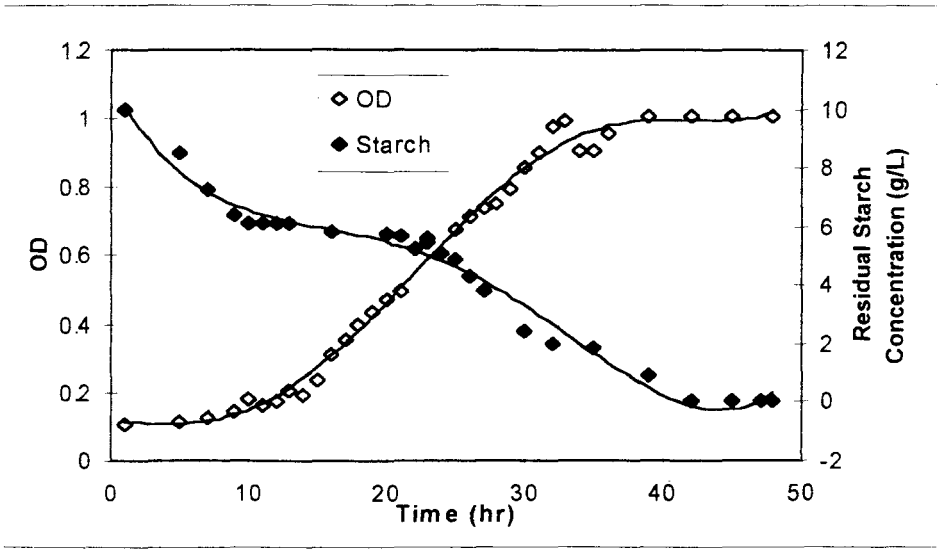


(b)

FIGURE 4.9. (a) Growth Pattern (b) Glucose Accumulation and pH Changes of the YPG/MM Strain in YEP-Sg Containing 2% (w/v) Starch.



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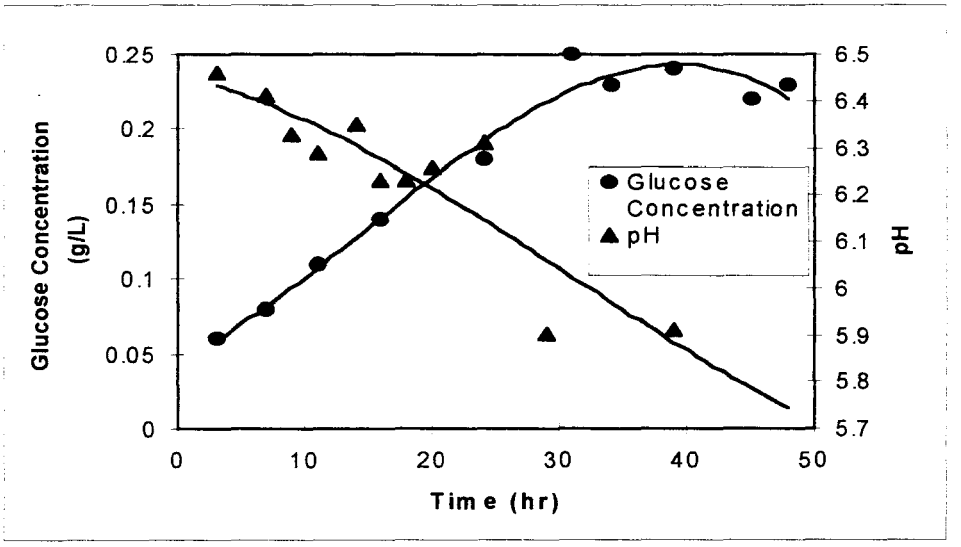
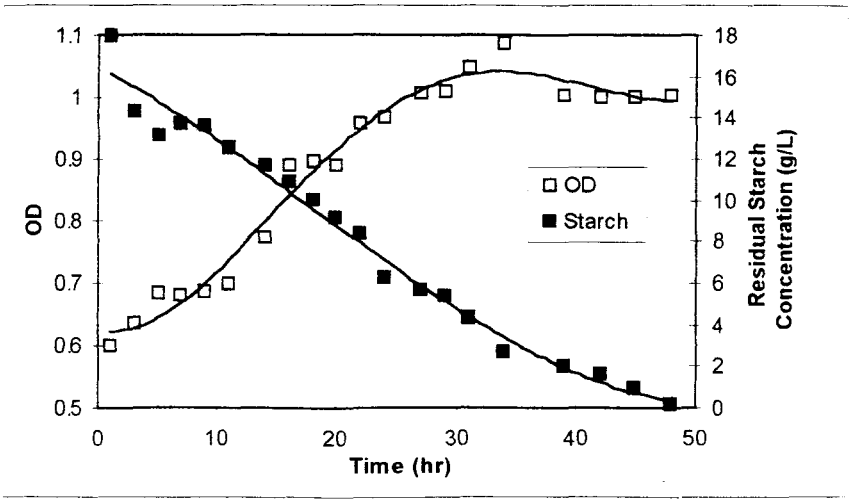


(b)

FIGURE 4.10. (a) Growth Pattern (b) Glucose Accumulation and pH Changes of the YPG/AB Strain in YEP-Sg Containing 1% (w/v) Starch.



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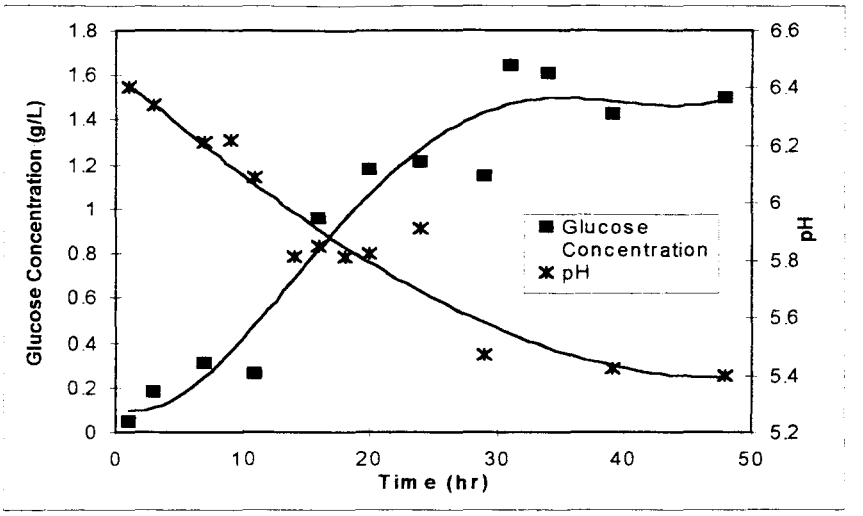

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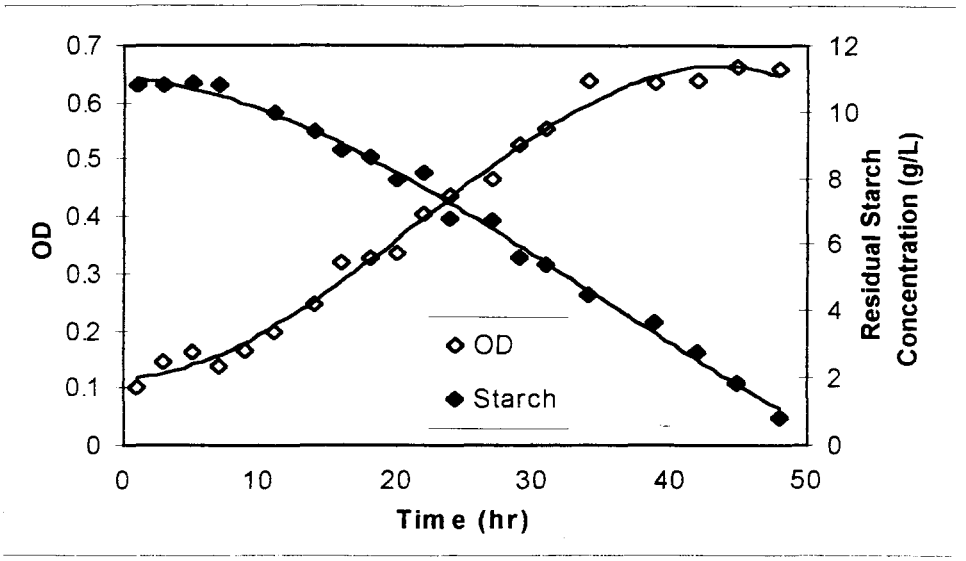
FIGURE 4.11. (a) Growth Pattern (b) Glucose Accumulation and pH Changes of the YPG/AB Strain in YEP-Sg Containing 2% (w/v) Starch.

YA7 strain was the slowest in terms of starch degradation. It could degrade 50% of the 1%(w/v) and 2% (w/v) initial soluble starch in 27 hours and 29 hours respectively. The cell growth was at its late-exponential phase at this time. The maximum accumulation of glucose was 1.14 g/L and 1.41 g/L on 1%(w/v) and 2% (w/v) soluble starch respectively similar to YPG/AB strain (**Figure 4.12-4.13**).



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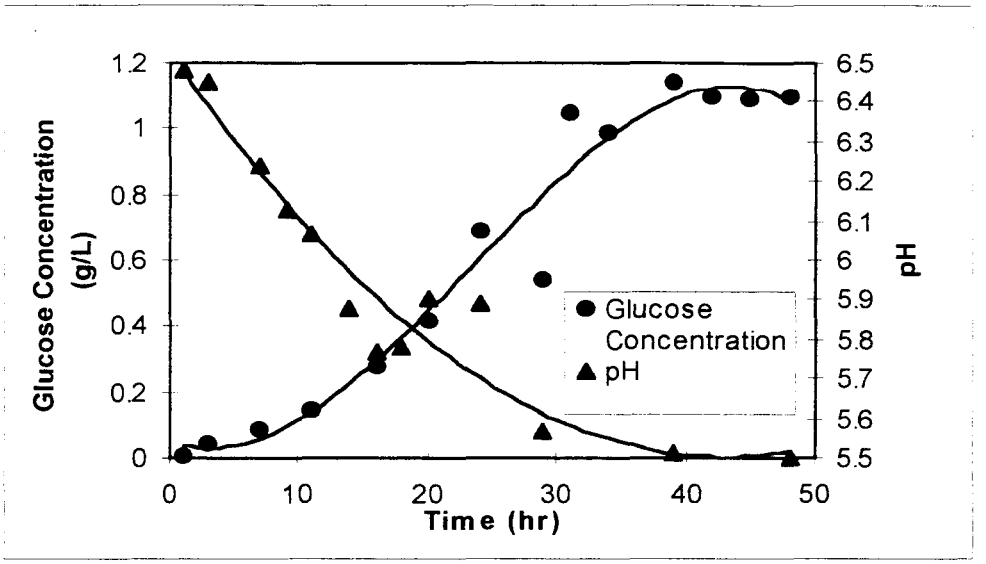

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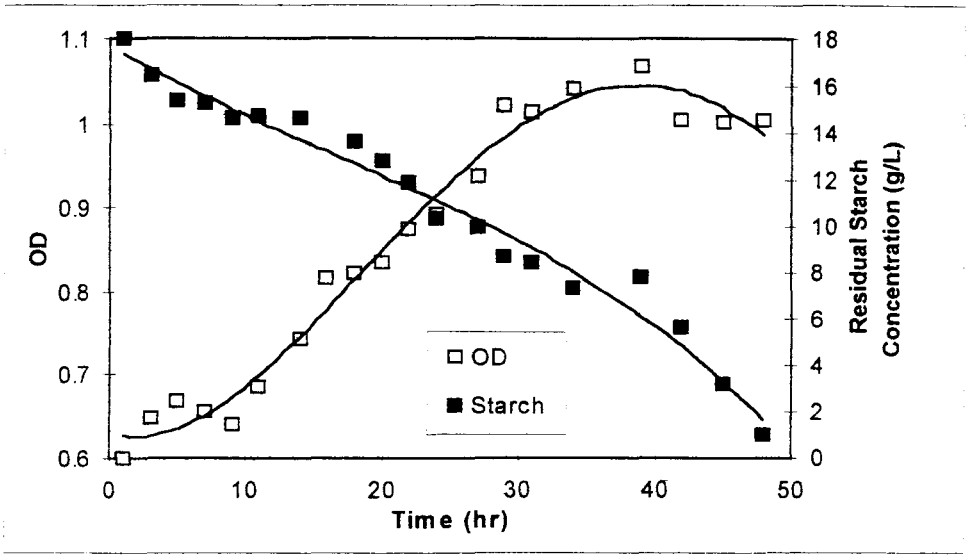
FIGURE 4.12. (a) Growth Pattern (b) Glucose Accumulation and pH Changes of the YA7 Strain in YEP-Sg Containing 1% (w/v) Starch.



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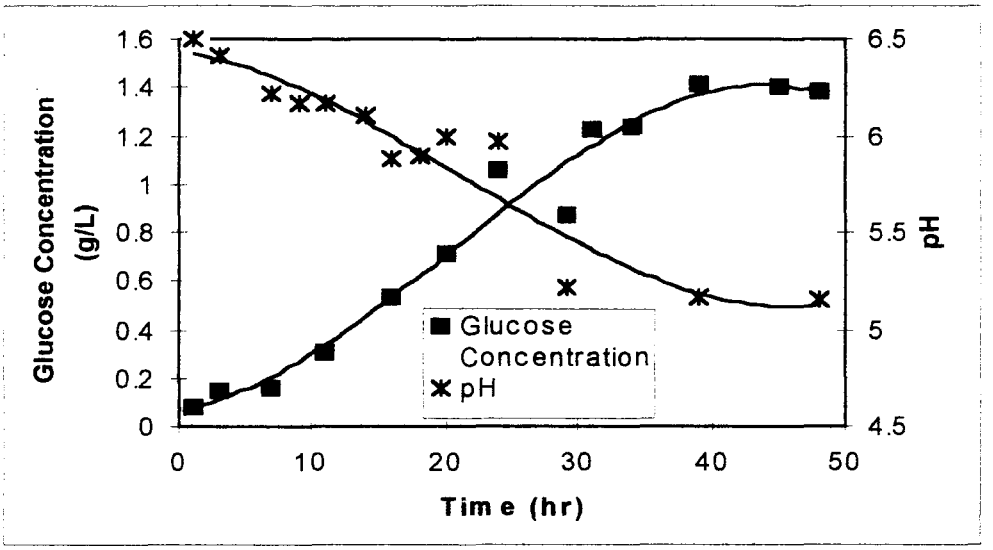


(b)

FIGURE 4.13. (a) Growth Pattern (b) Glucose Accumulation and pH Changes of the YA7 Strain in YEP-Sg Containing 2% (w/v) Starch.

The maximum specific growth rates of the recombinant strains in YEP-Sg medium are tabulated in **Table 4.2**. The growth rates of the strains calculated at the same initial

starch concentration were similar. When the initial starch concentration in the medium was doubled, the growth rates were halved.

TABLE 4.2. Maximum Specific Growth Rates on YEP-Sg Medium

	Maximum Specific Growth Rate (hr^{-1})	
Strain	at 1% Initial Starch Concentration	at 2% Initial Starch Concentration
YPG/MM	0.044	0.022
YPG/AB	0.050	0.019
YA7	0.048	0.019

In these experiments, ethanol, extracellular α-amylase and extracellular glucoamylase activities could not be detected in the medium. But, the experiments have shown that there was enough enzymatic activity in the medium, since the degradation of starch was proper and rapid. The growth curves of the strains were determined by the dry weight method in the later experiments due to the difficulty in the optical density measurements of the culture suspension when the starch concentration was increased.

4.2.3. Growth on YEP-S Medium

From the experiments in YEP-Sg medium, the ability of the recombinant strains to degrade starch molecules into glucose and use it for their growth was tested and proven. As the next step, experiments were conducted on YEP-S medium without glycerol supplementation at 1% and 2% (w/v) initial starch concentrations. A new recombinant yeast strain, namely YPB-G, was selected for further experiments along with YPG/AB. The YPB-G strain expresses the *B.subtilis* α-amylase - *A.awamori* glucoamylase (BsAAse/GAase) as a fusion protein. Since YPG/AB and YA7 harbour a double casette plasmid to produce separately *A.awamori* glucoamylase (GAase) and *B.subtilis* α-amylase

(BsAAase) in different orientations, and display completely similar fermentation characteristics, YPG/AB was selected for further studies.

YPB-G strain degraded 50% of the 1% and 2% (w/v) initial starch in 22 hours and 28 hours respectively. The maximum accumulation of glucose was 2.2 g/L and 0.85 g/L, while biomass formations of 5.8 g/L and 4.6 g/L were recorded on 1% and 2% (w/v) initial starch respectively (**Figure 4.14-4.15**). The maximum specific growth rates of 0.074 and 0.070 hr^{-1} were calculated respectively.

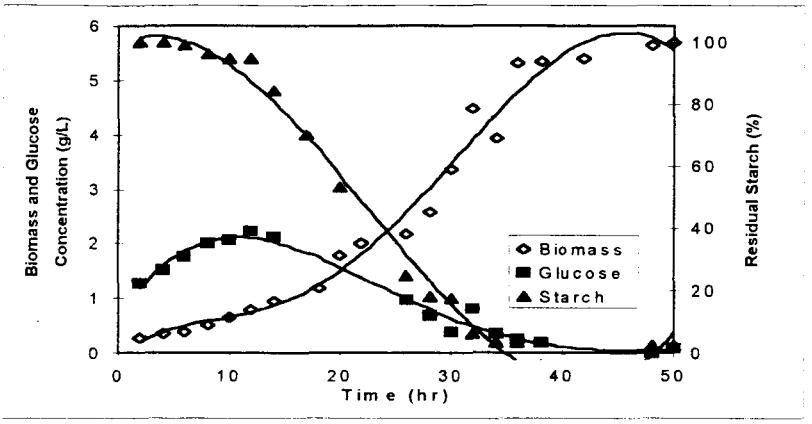


FIGURE 4.14. Growth Characteristics of YPB-G Strain on YEP-S Containing 1% (w/v) Starch.

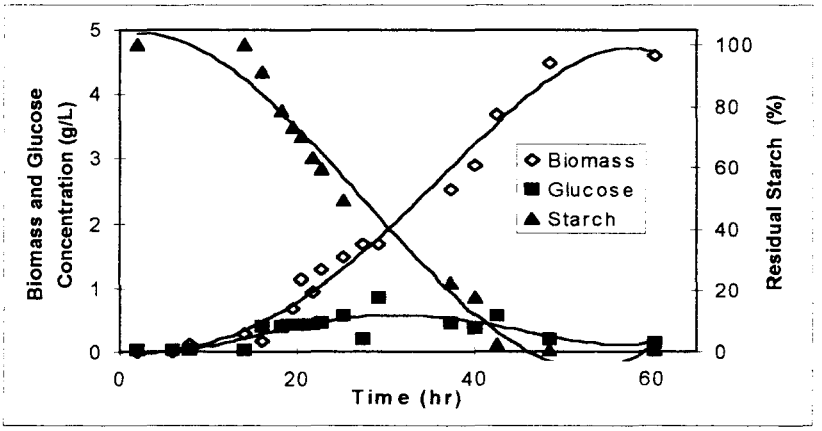


FIGURE 4.15. Growth Characteristics of YPB-G Strain on YEP-S Containing 2% (w/v) Starch.

On the other hand, 50% degradation of 1% and 2% (w/v) initial starch by the YPG/AB strain was completed in 22 hours and 32 hours respectively. 1.6 g/L and 0.74 g/L of glucose accumulation and 5.5 g/L and 4.6 g/L of biomass formations were measured on 1% and 2% (w/v) initial starch respectively (**Figure 4.16-4.17**). The maximum specific growth rates were 0.076 and 0.071 hr^{-1} respectively (see **Table 4.3**).

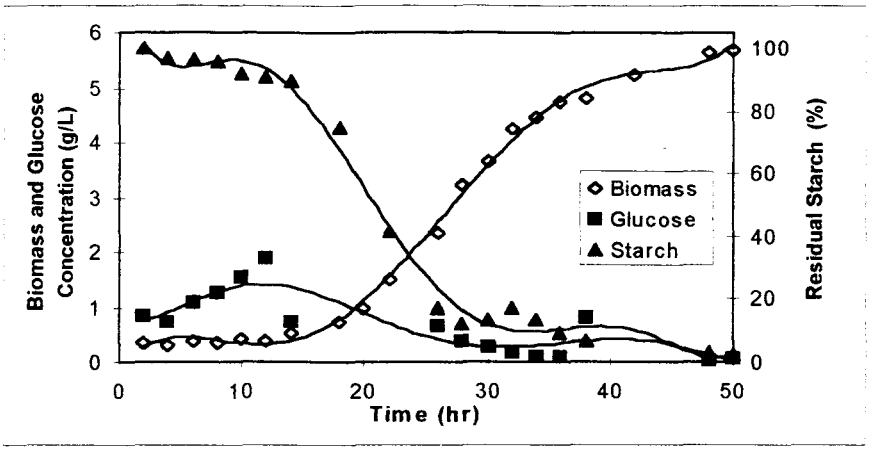


FIGURE 4.16. Growth Characteristics of YPG/AB Strain on YEP-S Containing 1% (w/v) Starch.

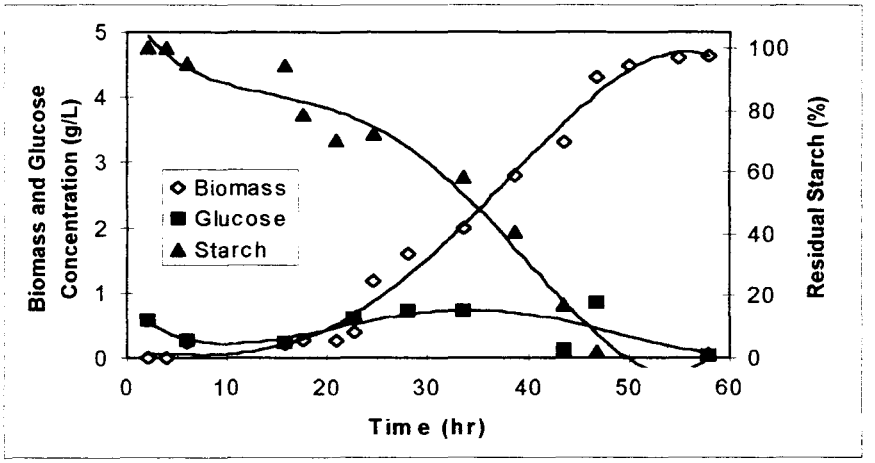


FIGURE 4.17. Growth Characteristics of YPG/AB Strain on YEP-S Containing 2% (w/v) Starch.

TABLE 4.3. Maximum Specific Growth Rates

	Maximum Specific Growth Rate ± 0.01 (hr^{-1})	
Strain	at 1% Initial Starch Concentration	at 2% Initial Starch Concentration
YPB-G	0.074	0.070
YPG/AB	0.076	0.071

When comparing the growth characteristics of YPG/AB strain on YEP-Sg and YEP-S, the time for 50% degradation of initial starch was longer on YEP-S medium than YEP-Sg medium. Similarly, this strain has entered the exponential phase of its growth after a 10 hour lag period on YEP-Sg, while it took about 20 hours to finish its lag phase on YEP-S.

The adaptation time of the strains to the starch containing medium was decreased to one half by the presence of glycerol in the medium. The cells have also shown their S shaped growth behavior on YEP-S. On the other hand, the maximum specific growth rates were more than doubled in YEP-S without glycerol. Therefore, glycerol was omitted from the medium composition to avoid complications which may affect the interpretation of data on substrate utilization.

4.3. Effect of Initial Glucose Concentration

The growth of microorganisms is an unusually complex phenomenon (Thatipamala et al., 1992). During balanced growth, only a single parameter μ (or the population doubling time) is required to characterize population growth kinetics. For this reason, the magnitude of the specific growth rate is widely used to describe the influence of the cells' environment on the cells' performance (Bailey and Ollis, 1986). The general goal in making a medium is to support good growth and/or high rates of product synthesis. Contrary to intuitive expectations, this does not necessarily mean that all nutrients should be supplied in great excess. In fact, that might inhibit or even poison cell growth. Moreover, if the cells grow too extensively, their accumulated metabolic end products will often disrupt the normal biochemical processes of the cells. Consequently, it is common to practice limit total growth by limiting the amount of one nutrient in the medium. If the concentration of one essential medium constituent is varied while the concentrations of all other medium components are kept constant, the growth rate typically changes in a hyperbolic fashion (Bailey and Ollis, 1986).

The growth characteristics in a batch culture of microorganisms include several growth phases, each of them being of potential importance in microbial processing. Not only active cell growth but also the activities of resting and dying cells are of interest, since many bioprocesses are of commercial and environmental importance where growth has stopped (Bailey and Ollis, 1986).

In this part of the study, the effect of the change in initial glucose concentration on cell growth, protein production, substrate utilization and plasmid stability of the recombinant yeast strains were investigated in yeast minimal medium supplemented with histidine, YNB-G. The initial glucose concentrations were 5, 10, 20 and 50 g/L. Shake flasks containing 1L YNB-G each with different initial glucose concentrations were inoculated with precultures grown until the late exponential phase with an inoculum size of 2% (v/v) and incubated in an orbital shaker. YPB-G and YA7 strains were used.

Aliquots of the cell suspension were taken at certain time intervals and cell growth was followed spectrophotometrically. Residual glucose and protein concentrations in the medium were also analyzed using the methods described in the Materials and Methods Chapter, to follow substrate utilization and protein production.

The variations in the dry cell weight, residual glucose concentration and protein concentration of each cell culture are plotted against time in **Figures 4.18-4.25**. A lag phase ranging between 2 - 4 hours for YPB-G and 6 - 8 hours for YA7 was observed for each fermentation broth. During the lag phase, glucose consumption was almost constant, then a rapid decrease was observed as the cells entered their exponential phase.

The glucose was completely exhausted in the media containing 5 and 10 g/L initial glucose for both strains. When the glucose concentration was increased to 20 g/L and 50 g/L, 5.6% and 50% of the initial glucose remained unconsumed in the YPB-G culture media respectively. Similar trends were observed in the YA7 culture media, 7.3% and 36% of the initial glucose was unconsumed in YNB-G containing 20 g/L and 50 g/L of initial glucose respectively. This might be due to a limitation in the nutrient components other than carbon such as nitrogen which also prevents the utilization of glucose by the cells in the medium.

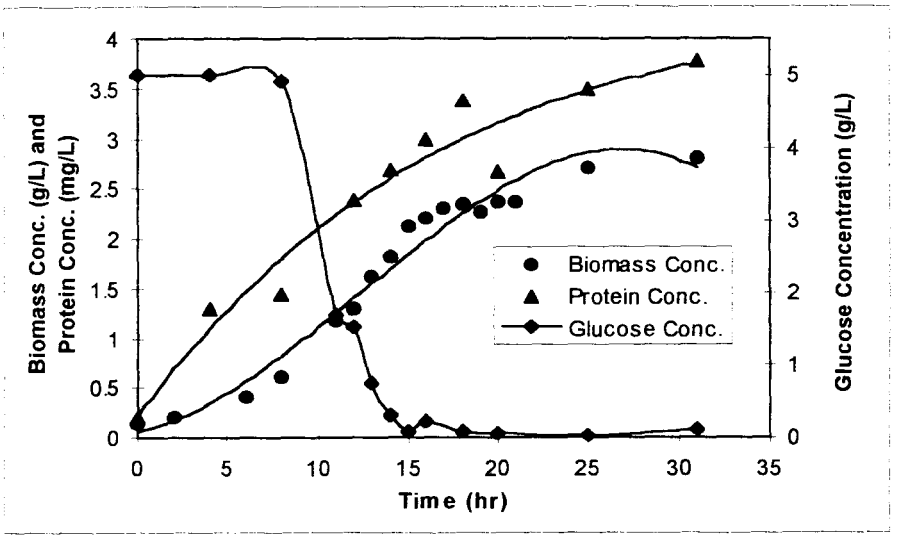


FIGURE 4.18. Fermentation Characteristics of YPB-G in YNB-G containing 5 g/L Initial Glucose

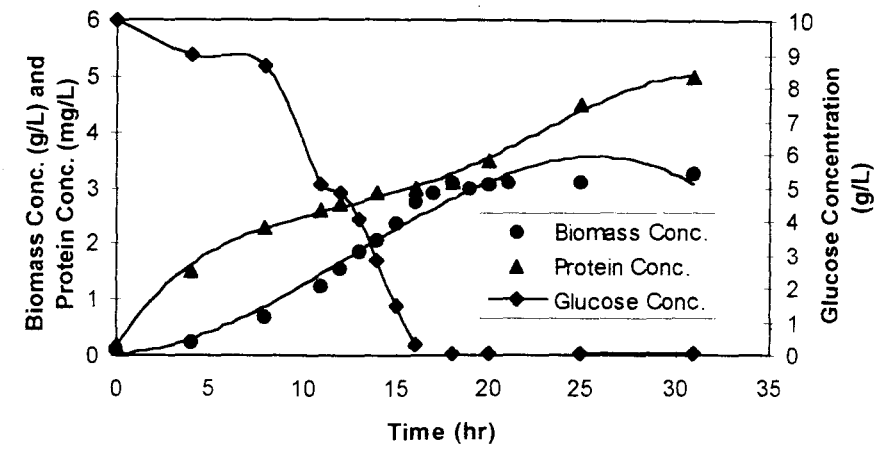


FIGURE 4.19. Fermentation Characteristics of YPB-G in YNB-G containing 10 g/L Initial Glucose

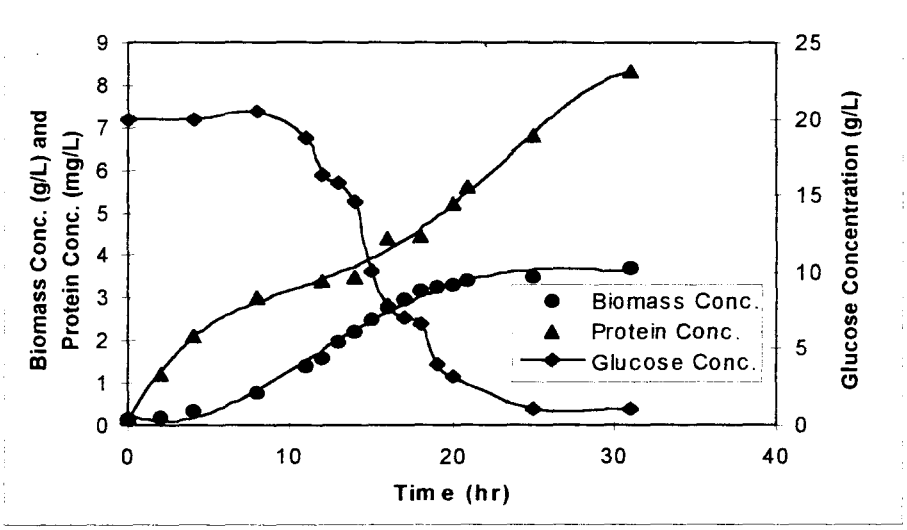


FIGURE 4.20. Fermentation Characteristics of YPB-G in YNB-G containing 20 g/L Initial Glucose

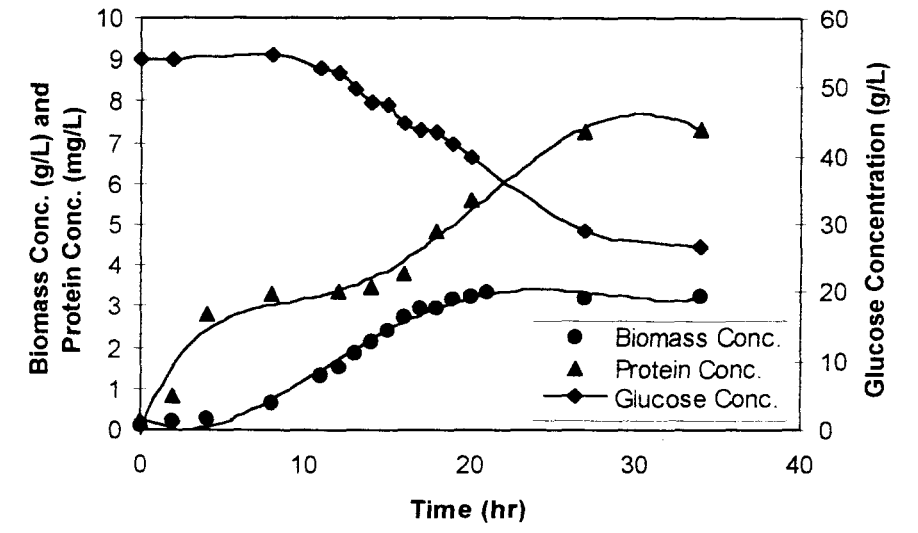


FIGURE 4.21. Fermentation Characteristics of YPB-G in YNB-G containing 50 g/L Initial Glucose

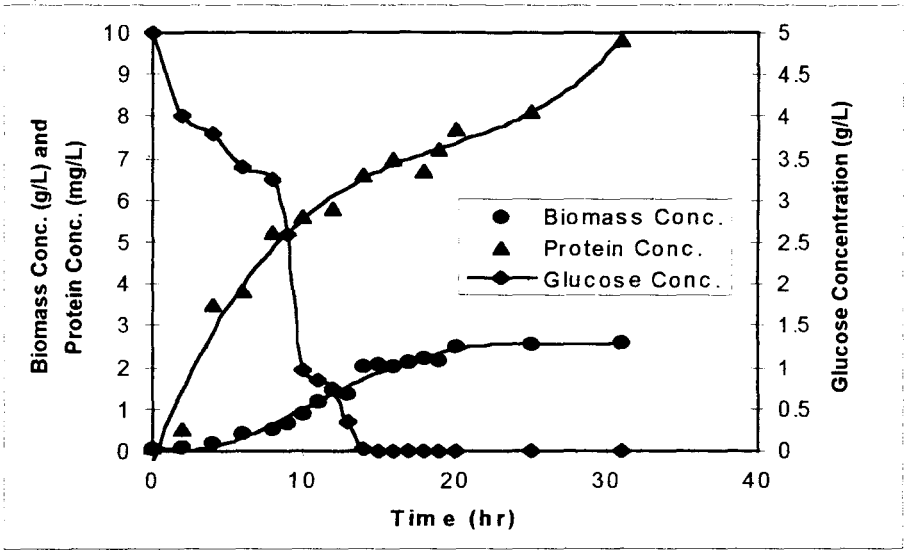


FIGURE 4.22. Fermentation Characteristics of YA7 in YNB-G containing 5 g/L Initial Glucose

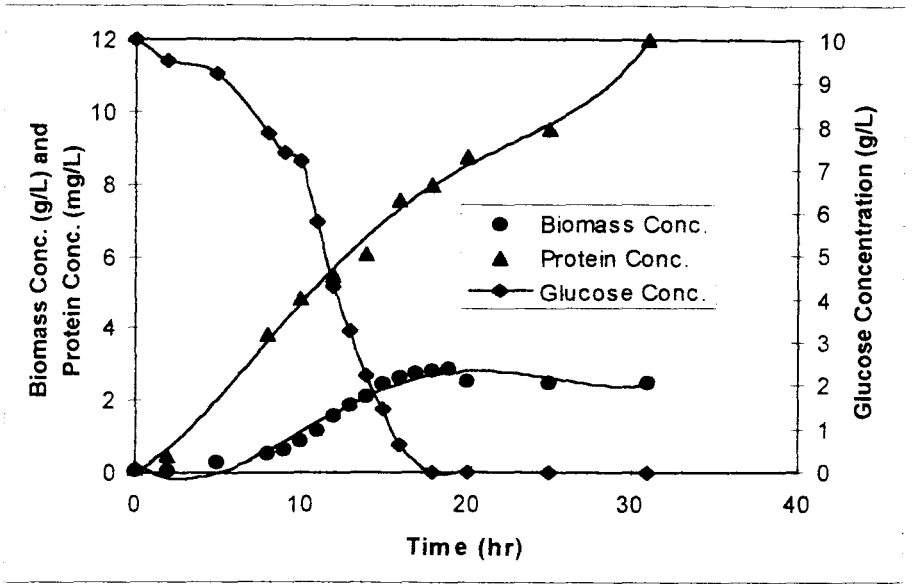


FIGURE 4.23. Fermentation Characteristics of YA7 in YNB-G containing 10 g/L Initial Glucose

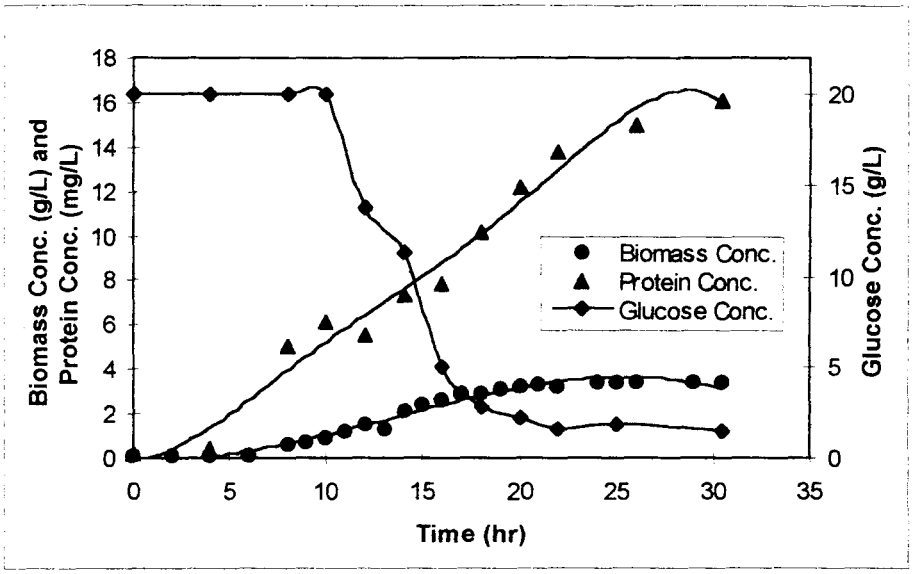


FIGURE 4.24. Fermentation Characteristics of YA7 in YNB-G containing 20 g/L Initial Glucose

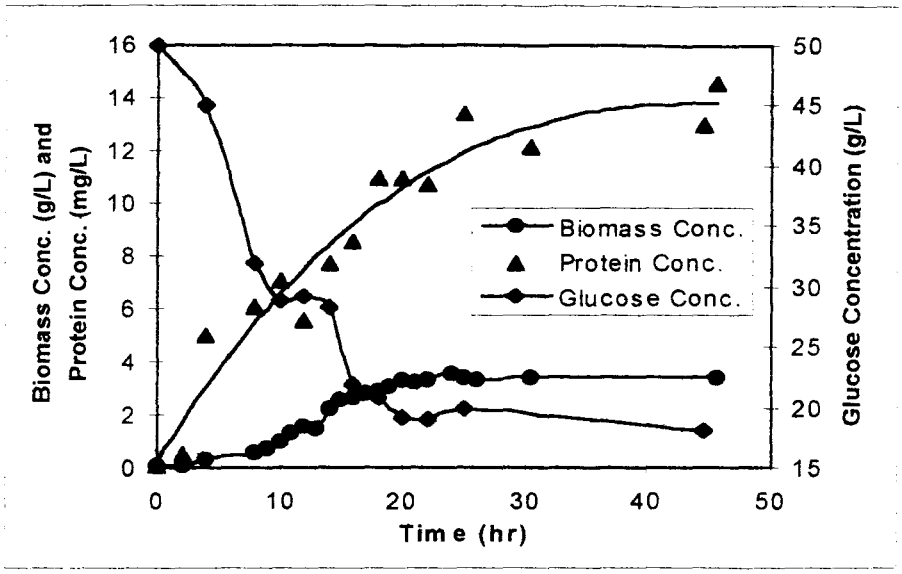


FIGURE 4.25. Fermentation Characteristics of YA7 in YNB-G containing 50 g/L Initial Glucose

Similar trends were observed in the time profiles of dry cell weights. Dry cell weights determined at the stationary phase were found to increase with increasing initial glucose concentrations up to 20 g/L, then it leveled off for both strains. The cell mass increased by a factor of 1.31 in both YPB-G and in YA7 when the initial glucose concentration was changed from 5 to 20 g/L. The use of a higher glucose concentration (50 g/L) did not result in any remarkable increase in the final cell mass which reached 3.23 g/L and 3.40 g/L in YPB-G and YA7 respectively. **Table 4.4** shows the results obtained from the fermentation of glucose. There might be a treshold value for glucose between 10 g/L - 20 g/L for these strains above which the cells can not grow and utilize glucose further. In addition the maximum biomass formation reaches a value of ca. 3.5 g/L. This suggests that above this treshold value of glucose, limitation by the other components of the nutrient might begin.

TABLE 4.4. Comparison of the Fermentation Results of Both Recombinant Yeast Strains

Strain	Initial Glucose Concentration (g/L)	Final Glucose Concentration (g/L)	Final Biomass Concentration (g/L)	Final Protein Concentration (mg/L)	$\mu_{max} \pm 0.01$ (h^{-1})
YPB-G	5	0	2.80	3.78	0.133
	10	0	3.24	5.00	0.149
	20	1.12	3.67	8.34	0.150
	50	26.85	3.23	7.30	0.138
YA7	5	0	2.60	9.81	0.148
	10	0	2.48	12.00	0.171
	20	1.46	3.40	16.11	0.184
	50	18.14	3.40	14.59	0.178

The specific growth rates were determined from the slopes of the lnx versus time plots in the exponential phase using linear regression (**Table 4.4**). These specific growth rates were also plotted versus initial glucose concentration for both recombinant yeast strains (**Figure 4.26**). The maximum specific growth rates at 5g/L of initial glucose concentration were lower, while those between 10 g/L and 50 g/L were similar within experimental error, showing a simple saturation type kinetics (Bailey and Ollis, 1986).

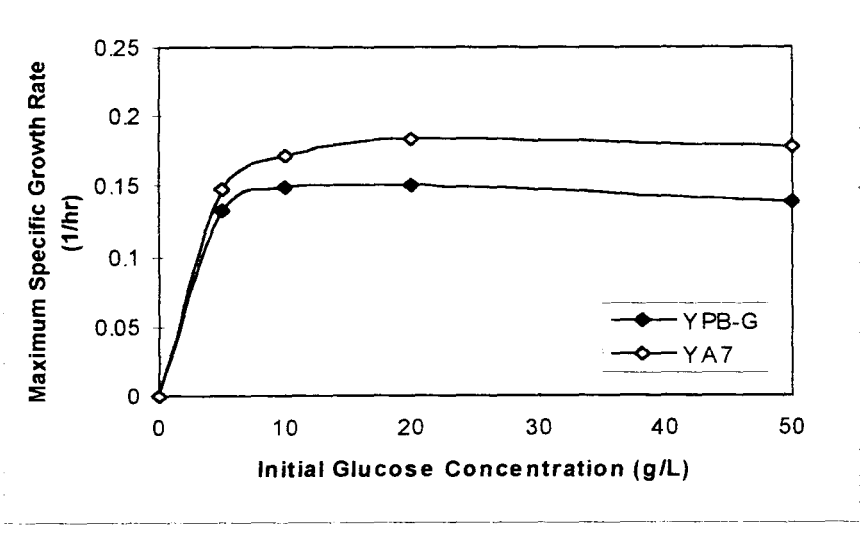


FIGURE 4.26. Maximum Specific Growth Rates at Different Glucose Concentrations for the Recombinant Strains.

The results have shown that both recombinant strains have similar growth behavior with respect to different initial glucose concentrations under the same fermentation conditions.

Samples taken from the fermentation media at regular time intervals were also analyzed for extracellular protein concentration in the supernatant. Time profiles of protein concentrations are also shown in **Figures 4.18-4.25**. The behavior of protein production has displayed a parallelism to the biomass formation. As the biomass increased with increase in the initial glucose concentration, the protein concentrations also increased. 3.78, 5.00, 8.33 and 7.30 mg/L of protein were recorded for YPB-G strain at 5, 10, 20 and 50 g/L initial glucose respectively. 9.81, 12.00, 16.10 and 14.59 mg/L of protein concentrations were obtained for YA7 strain at 5, 10, 20 and 50 g/L initial glucose respectively. YA7 strain showed a superiority over YPB-G strain in terms of secretion efficiencies of the proteins to the medium.

4.3.1. The Overall Yield Factors

It has been observed frequently that the total amount of cell mass formed by cell growth is proportional to the mass of substrate (typically carbon sources, energy source and oxygen) utilized. The yield factors are sometimes used to characterize system performance. The overall yield factors for biomass formation per substrate consumption, Yx/s (g cells formed per g glucose consumed), the product formation per biomass production, Yp/x (g protein formed per g cells formed) and product formation per substrate consumption, Yp/s (g protein formed per g glucose consumed) are given in **Table 4.5** for both strains.

TABLE 4.5. The Overall Yield Factors for Both Recombinant Yeast Strains

Strain	Initial Glucose Concentration (g/L)	Yx/s (g cells/g glucose)	Yp/s (g protein/g glucose) $*10^{3}$	Yp/x (g protein/g cells) $*10^{3}$
YPB-G	5	0.560	0.756	1.350
	10	0.320	0.500	1.540
	20	0.194	0.440	2.269
	50	0.139	0.315	2.267
YA7	5	0.520	1.960	3.760
	10	0.247	1.200	4.840
	20	0.183	0.868	4.740
	50	0.106	0.457	4.280

The Yx/s and Yp/s values decreased with increasing initial glucose concentration for both strains. On the other hand, the Yp/x values showed that protein formation increased up to 20 g/L and 10 g/L for YPB-G and YA7 respectively, and it leveled off for YPB-G while it has decreased for YA7 (**Figure 4.27**). It is common to obtain a Yx/s value of 0.5 g/g for *S. cerevisiae* strains under aerobic conditions (Bailey and Ollis, 1986). It may be concluded that 5 g/L of initial glucose concentration is a good choice for both strains.

The stability of the plasmids were also measured by the method of replica plating as described in Section 3.2.5.11. The plasmids have displayed a very high stability (≥ 95%) in YNB-G medium regardless of the initial glucose concentration.

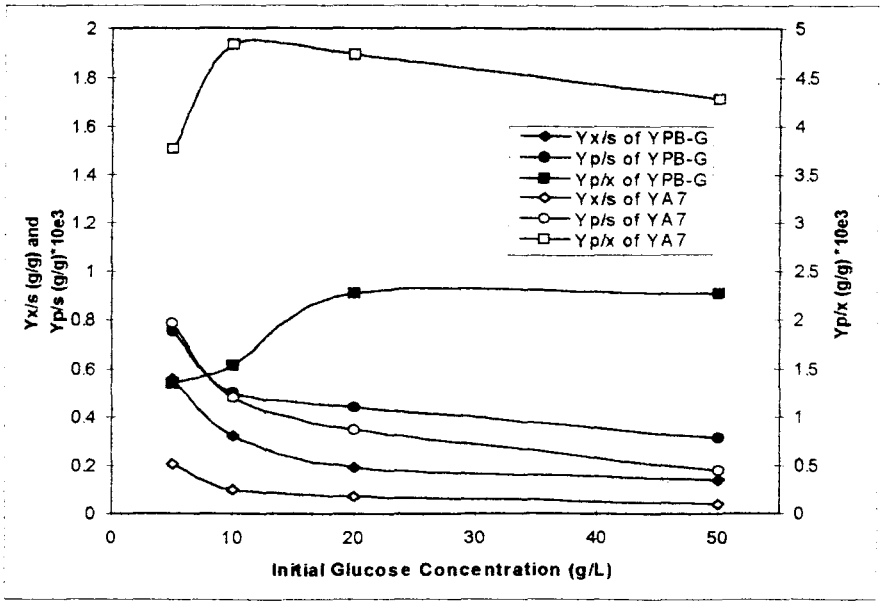


FIGURE 4.27. The Variation of Overall Yield Factors with Initial Glucose Concentration.

4.4. Kinetic Studies on Protein and Biomass Production from Glucose by Recombinant Yeast Strains

Production of α-amylase by microorganisms has been practiced for many years in small and large scale operations and the literature on this enzyme is voluminous (Kekos and Macris, 1987). *Aspergillus niger* and *Aspergillus oryzae* have been reported as the main fungal species used for commercial production of the enzyme. Similarly, the genus *Bacillus* produces a large variety of extracellular enzymes, some of which such as the amylases and proteases are of significant industrial importance (Roychoudhury et al., 1989).

The α-amylases produced by different *Bacillus* species vary not only in their types (saccharifying or liquefying) but also in the range of pH and temperature for their optimal

activity. The strain-specific nature of the synthesis of α-amylase is further evidenced by significant variations in

1. initiation of the enzyme synthesis (during the growth phase or at the termination of the growth phase),
2. degree of catabolite repression of the enzyme synthesis by glucose,
3. requirement, if any, of starch for the enzyme synthesis, and,
4. relations among the specific cell growth rate and rates of transcription and translation of messenger RNA specific for α-amylase .

Extracellular enzymes are believed to be secreted because of the inability of substrates they act on, such as starch in the case of α-amylase to enter the cell (Roychoudhury et al., 1989).

In this part of the work, growth and enzyme production characteristics of batch cultures of recombinant *Saccharomyces cerevisiae* strains were studied using glucose as the main carbon source. In all of the models applied, the biomass concentrations were correlated with protein concentrations in the culture supernatant at different initial glucose concentrations. Protein concentrations were used instead of α-amylase and glucoamylase activities which were more difficult to assay for the sake of simplicity. The results reported in previous sections indicated a relationship between the extracellular protein concentrations and, the activities of both enzymes. Also, it has been reported in the literature that *Saccharomyces cerevisiae* normally secretes only a very small number (0.5%) of its own proteins to the medium (Das and Shultz, 1987) which is advantageous for product recovery (Wang and Da Silva, 1993). Therefore, it can be assumed that the protein concentrations measured in the medium were correlated to α-amylase and glucoamylase amounts. Additionally, the fact that YPB-G strain produces fusion proteins allows the assumption that the total proteins are in a 1:3 ratio α-amylase to glucoamylase whereas, this ratio is 1:7 α-amylase to glucoamylase in the case of YA7 strain that produces proteins separately as mentioned in de Moraes (1994).

Three programs were developed for taking a data file as input and writing the result to an output file. The data structure was arranged in such a manner that, smoothing and

interpolation programs could be applicable on raw data files as well as the output data files of smoothing and interpolation programs. A detailed information for the smoothing, interpolation and differentiation can be found in Appendix A. Similarly, the differentiation program could be applied to any data file that did not have differentiation information. The idea can be visualized by the flow chart on **Figure 4.28**.

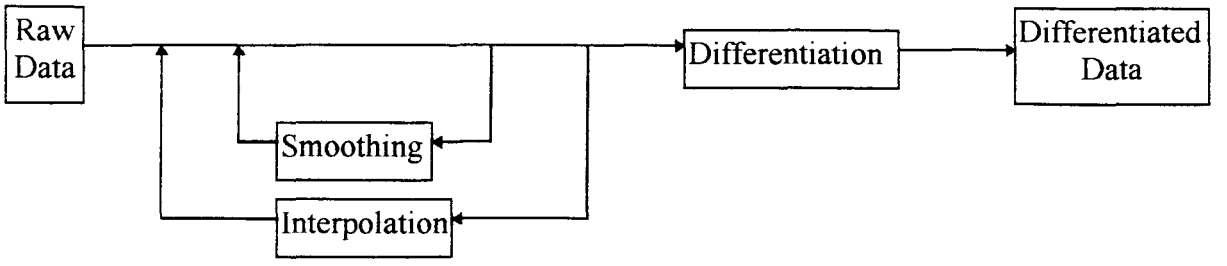


FIGURE 4.28. The Flow Chart of Data Manipulation to Prepare Data for Modeling

4.4.1. Kinetic Models

Several models proposed in literature for the enzyme production are listed in **Table 4.6**. The first four models in **Table 4.6** are linear models, and therefore the model parameters were estimated using least squares methods. The fifth model is a nonlinear one, and the steepest descent method was used to estimate its parameters. The cost function used in the optimization of parameters for the steepest descent method was the sum of the squares of the time derivatives of the experimental data and their estimated derivatives. In the modified Montesinos model, due to the nature of the steepest descent method, the converged results depend on the initial guess for the parameters and the step size for the

iterations. Thus the parameter values were checked for robustness due to neighboring starting guesses and to different step sizes.

TABLE 4.6. Kinetic Models Applied to Microbial Enzyme Production

Model	Formula	Reference
Kono-Asai	$\frac{dP}{dt} = k_2 \frac{dX}{dt}$	(Kekos and Macris, 1987)
Kosaric-Yu-Zajic	$\frac{dP}{dt} = K \frac{dX}{dt} + K_1 X$	(Kekos and Macris, 1987)
Brown-Vass	$\frac{dP}{dt} = k_p \frac{dX_{t-t_m}}{dt}$	(Kekos and Macris, 1987)
Le Duy	$\frac{dP}{dt} = k_2 \frac{dX^*}{dt} + k_1$	(Kekos and Macris, 1987)
Modified Montesinos	$\frac{dP}{dt} = k_m \frac{P}{K_s + P} X$	(Montesinos et al., 1995)

Here, X , $X^*$and $X_{t\text{-}tm}$ are biomass concentrations in g/L at t, $t\text{-}t_s$, and $t\text{-}t_m$ respectively. P is protein concentration in mg/L. k_p, k_1, K are constants in mg/g. k_2, K_1, k_m and K_s are also constants in mg/L/hr, mg/g/hr, 1/g/hr and mg/L respectively.t, t_s and t_m are the growth time, lag time and maturation time in hr respectively..

In the Kono-Asai and Kosaric-Yu-Zajic models, only the exponential phase of the growth was considered. In these models the interactions of product formation and biomass growth rates were assumed to occur spontaneously. In Brown-Vass model, the rate of protein production was related to the delayed rate of biomass formation where the delay for the biomass formation was termed as the maturation time (t_m). In the Le Duy model, similarly, the lag phase (t_s) of the yeast cells was used to delay the rate of biomass formation. Although the functions of the concepts of maturation time and lag phase look similar, they were in fact, two distinct concepts, t_s being able to be determined directly looking at the growth pattern of the cells, while, t_m was a parameter to be optimized first. The Brown-Vass and Le Duy models were applicable starting from their time delays until

the end of the stationary phase of growth. The Montesinos model was simplified by neglecting the effect of incremental change in the substrate concentration.

The model parameters were calculated for YPB-G and YA7 strains grown in YNB-G containing 0.5 - 5% initial glucose concentration as described in Section 4.3. Furthermore, the model parameters of YPB-G strain grown in YPS medium containing 0.5% starch (refer to Section 4.8) were also evaluated to check the applicability of the models. The model parameters are tabulated in **Table 4.7** and protein concentration versus time plots were given in **Figure 4.29**. Here, χ^2 probability distribution tables were also considered. As a rule of thumb, if one encounters a probability value above *5%*, the estimated model parameter(s) is (are) said to be *acceptable* (Bevington and Robinson, 1994).

TABLE 4.7. Parameters of the Models for YPB-G and YA7 Strains

Model	Parameter	Parameter Values		
		YPB-G*	YPB-G**	YA7***
Kono-Asai	k_p	1.36	4.91	1.12
Kosaric-Yu-Zajic	K	1.53	6.42	0.85
	K_1	-0.05	-0.08	0.05
Brown-Vass	t_m	9	9	-
	k_p	0.74	4.61	-
Le Duy	k_1	0.55	-2.12	-0.24
	k_2	0.11	1.04	0.24
Modified Montesinos	k_m	0.17	0.27	3.05
	K_s	3.47	37.68	256.4

* grown on YNB-G containing 2% glucose, ** grown on YPS containing 0.5% Starch

*** grown on YNB-G containing 0.5% glucose, - no correlation

Negative parameter values encountered could be interpreted as a negative correlation between the protein production rate and the respective multiplicand (either the biomass formation rate or the biomass concentration itself). They were obtained only in the Kosaric-Yu-Zajic and Le Duy models for both strains.

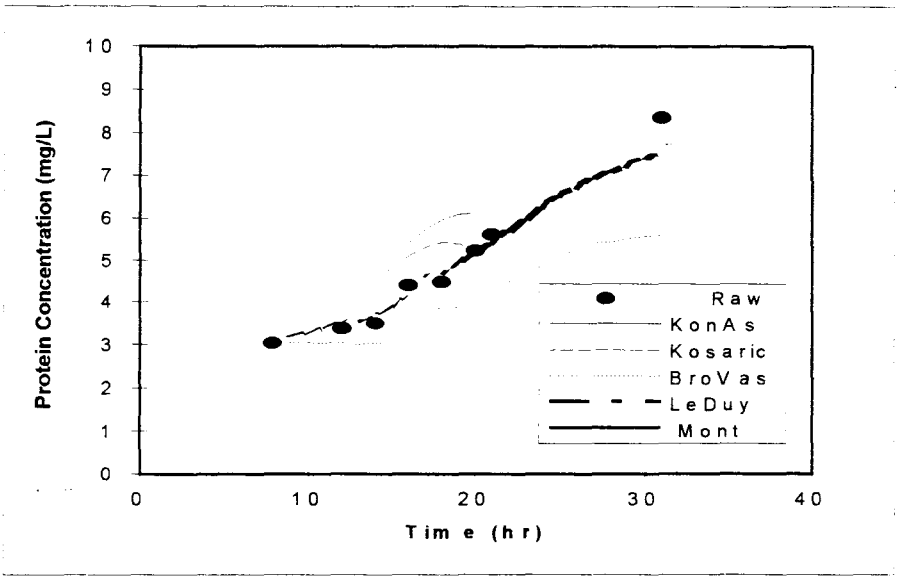
The fit of the kinetic models to the data of the YA7 strain was either poor or there was no correlation in the data sets. However, the fits obtained for the YPB-G strain were quite good. As a general tendency, the models for YPB-G strain resulted in better fits with increasing initial glucose concentration. This might be due to the nature of the experimental data, which displayed the same characteristics for the biomass and protein production rates, having roughly the same average slopes in the time profiles of biomass and protein concentrations.

Kono-Asai and Kosaric-Yu-Zajic models showed peaks during the exponential phase of growth while Le Duy and Brown-Vass models successfully described the S-shape of the growth pattern. Modified Montesinos model, on the other hand, was good in describing the exponential phase of growth, but, it would not bend to form an S-shape at the end of that phase due to the nature of its model equation which predicts a positive trend for P, i.e. $\frac{dP}{dt} > 0$.

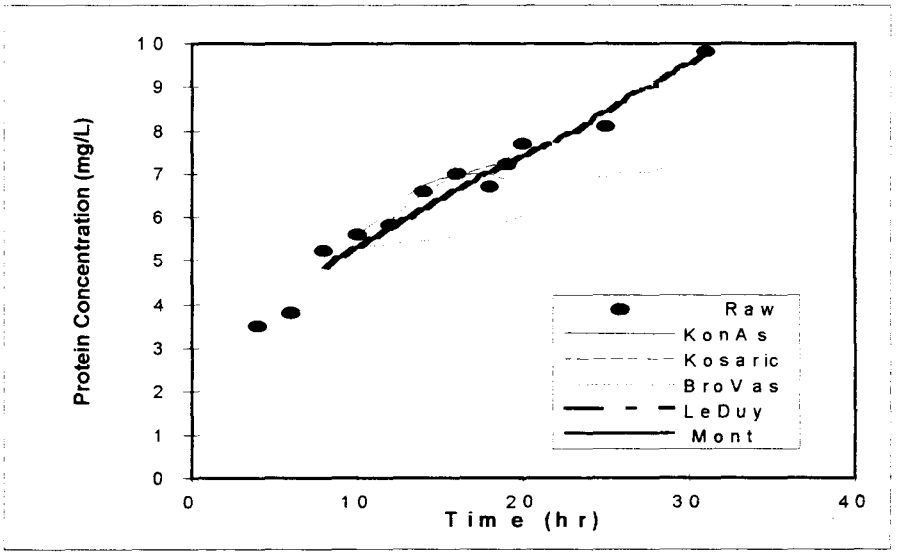
The results of fitting the experimental data of biomass growth pattern to the above models showed a high degree of correlation with certain of the examined kinetic models. The correlation coefficients for the following cases were above 90%:

1. Kono-Asai model in 0.5% and 2% initial glucose concentrations with YA7 strain and 2% initial glucose concentrations with YPB-G strain;
2. Kosaric-Yu-Zajic model in 0.5%, 1% and 5% initial glucose concentrations with YA7 strain and 0.5% and 5% initial glucose concentrations with YPB-G strain;
3. Brown-Vass model in 5% initial glucose concentrations with YA7 strain and 1%, 2% and 5% initial glucose concentrations with YPB-G strain;
4. Le Duy model in 0.5% initial glucose concentrations with YA7 strain and in all initial glucose concentrations with YPB-G strain;
5. Montesinos model in all fits with both strains;

Among these, the correlation coefficients for Kosaric-Yu-Zajic and Brown-Vass models in 5% initial glucose concentration with YPB-G strain; for Le Duy model in 0.5% initial glucose concentration with YA7 strain and 2% initial glucose concentration with YPB-G strain; for Montesinos model in 1% and 2% initial glucose concentrations with YPB-G strain were particularly high (above 99%).



(a)



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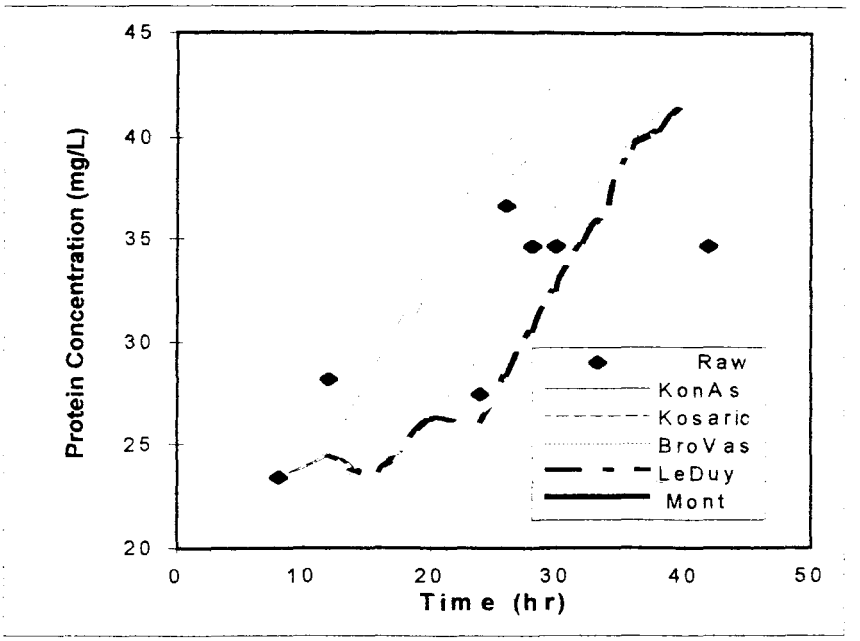


(c)

FIGURE 4.29. Model Predictions of Protein Concentrations
(a) for YPB-G on 2% YNB-G, (b) for YPB-G on 0.5% YPS
(c) for YA7 on 0.5%YNB-G

4.5. Effect of Inoculum

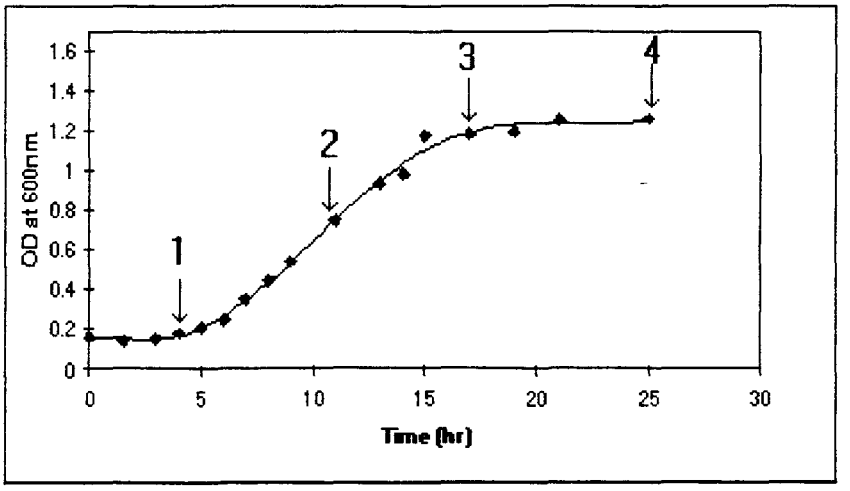
The inoculum consistency in terms of size and quality is an important parameter in improving fermentation efficiency and/or the yield of the process. Biochemical factors have long been recognized as the most effective in determining the yield and productivity of industrial fermentations (Birol and Özergin-Ülgen, 1995). Despite its importance, only very few researchers have investigated the effect of inoculum on cell yield and productivity (Su and Lei, 1993; Smith and Calam, 1980; Beal and Corrieu, 1991). Although there is a substantial amount of work on *Saccharomyces cerevisiae* fermentations in the literature, it is difficult to find publications studying the effects of inoculum quality and size on its growth. In a previous work, the optimum size and quality of the inoculum to be used with a flocculating strain of *Saccharomyces cerevisiae* in both freely suspended and immobilized forms was investigated to optimize growth characteristics and ethanol productivity (Birol and Özergin-Ülgen, 1995).

In this part of the study, the optimum time and size of inoculum to be used in the fermentation of starch by recombinant *S.cerevisiae* strains for the production of ethanol was determined. Such an optimization was needed, since these recently constructed recombinant strains were going to be exposed to different media containing higher concentrations of starch.

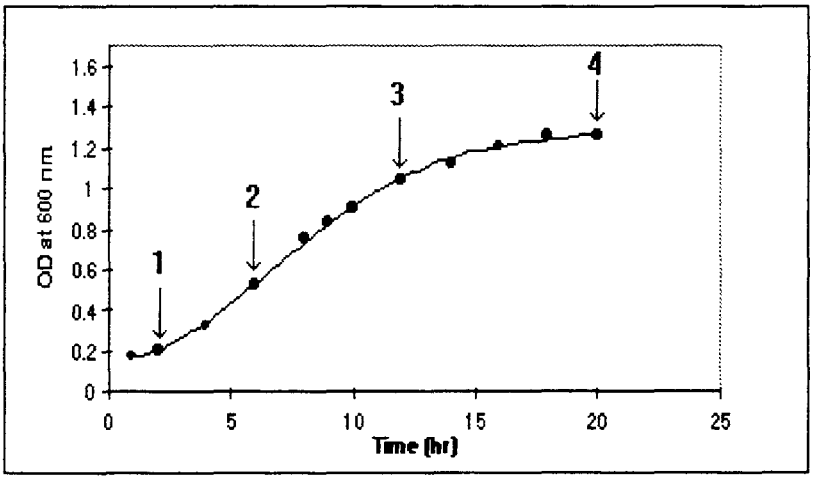
4.5.1. Optimization of Inoculum Time

Recombinant yeast strains of YPG/AB and YPB-G were grown on YEP-S medium containing 2% (w/v) soluble starch in these experiments. The total volume of the culture medium was 2L in a 6L flask. A liquid preculture with a single colony taken from YNB-G

agar plates was prepared in YNB-G medium to avoid plasmid instability and to shorten the lag phase of the recombinant strains in YEP-S medium. The same amount of inoculum (10% (v/v) preculture/nutrient medium) was transferred into each shake flask (**Figures 4.30 (a)-4.30 (b)**).



(a)



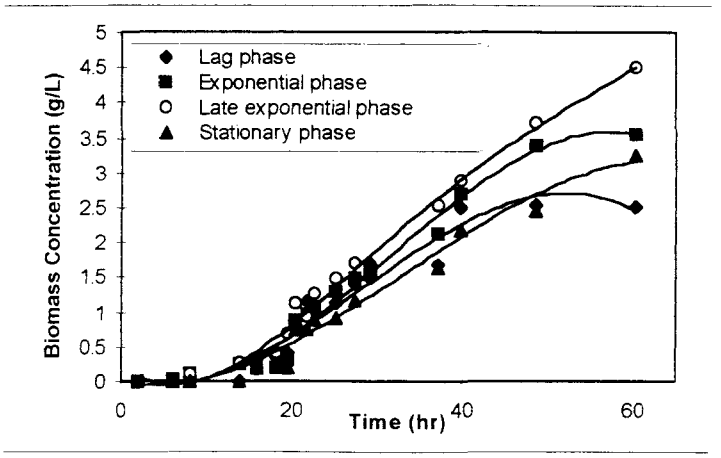
(b)

FIGURE 4.30. Growth Pattern of Preculture (a) YPB-G Strain, (b) YPG/AB Strain in YEP-S. arrows indicate the time when samples were taken from preculture to inoculate shake flasks 1-4

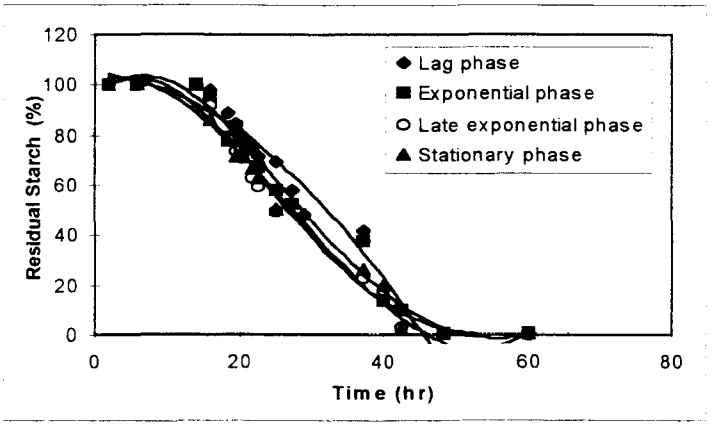
Although the same amount of inoculum (10% (v/v)) was transferred into each shake flask, the initial cell mass was observed to increase with growth phase from 0.05 to 0.28 g of cells for YPB-G, and from 0.06 to 0.37 g of cells for YPG/AB. The starch utilization, glucose accumulation, biomass formation and ethanol production of the strains were monitored.

The apparent lag phases differed in the four shake flasks and was found to be longer in flasks 1 and 4, while shorter lag phases were observed with both strains for flasks 2 and 3 (**Figures 4.31 (a) and 4.32 (a)**).

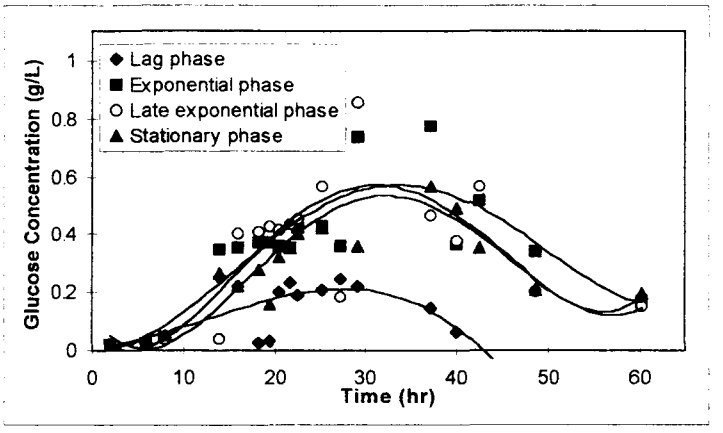
Similar maximum specific growth rates were observed in shake flasks 1-4, i.e. $0.070 \pm 0.009\ h^{-1}$, for both YPB-G and YPG/AB (**Table 4.8 and 4.9**). The maximum biomass formation of 4.50 g/L and 4.65 g/L were obtained when the inoculation was made with the preculture taken at its late exponential phase for YPB-G and YPG/AB strains respectively. Although the initial cell mass concentration was highest with the preculture taken at its stationary phase, the final biomass concentrations reached were 3.24 g/L and 3.45 g/L for the YPB-G and YPG/AB strains respectively (**Table 4.8 and 4.9**), and their maximum specific growth rates were also relatively lower.



(a)

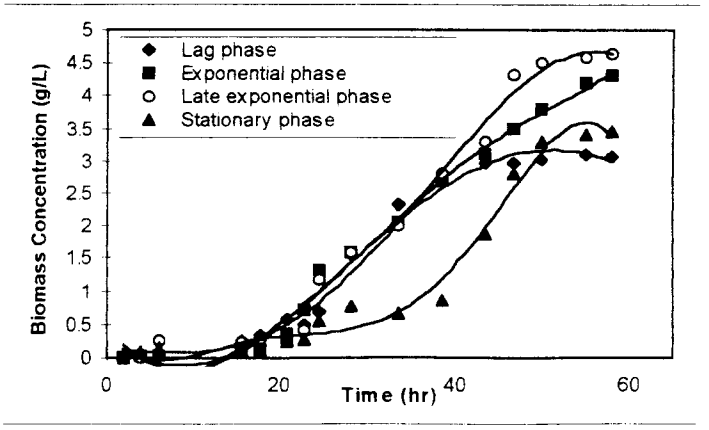


(b)

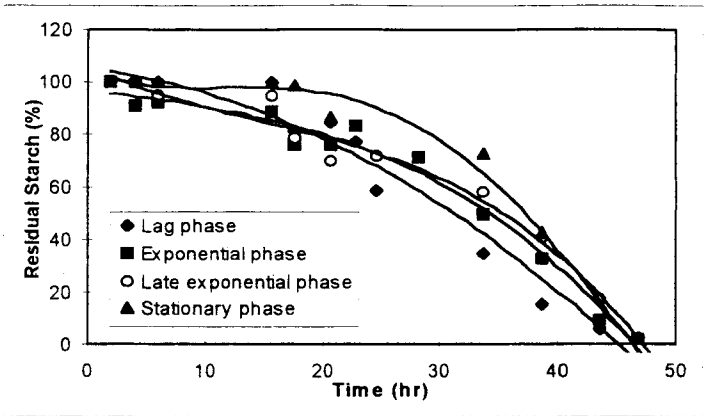


(c)

FIGURE 4.31. Time Course of (a) Biomass Formation, (b) Residual Starch and (c) Glucose Accumulation by YPB-G Strain for the Optimization of Inoculum Time



(a)



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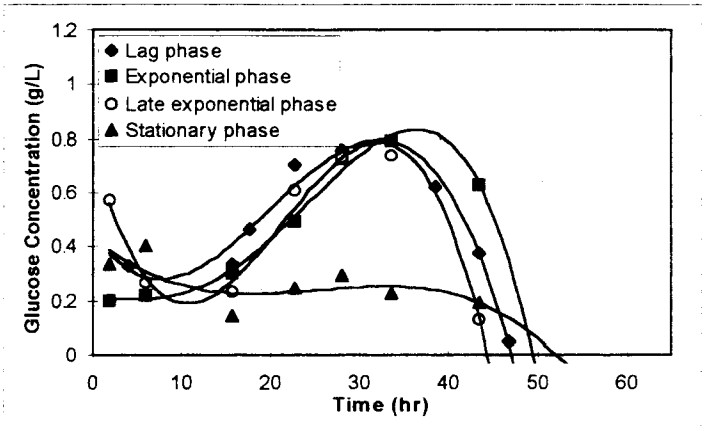


(c)

FIGURE 4.32. Time Course of (a) Biomass Formation, (b) Residual Starch and (c) Glucose Accumulation by YPG/AB Strain for the Optimization of Inoculum Time

TABLE 4.8. The Effect of Inoculum Time on Cell Growth for YPB-G Strain

Shake Flask No:	Inoculum Time (hr)	Total $X_{initial}$ (g)	X_{final} (g/L)	μ_{max} ($\pm$0.001)(h^{-1})
1	4 (Lag phase)	0.05	2.50	0.069
2	11 (Exponential phase)	0.17	3.54	0.073
3	16 (Late Exponential phase)	0.27	4.50	0.070
4	25 (Stationary phase)	0.28	3.24	0.062

TABLE 4.9. The Effect of Inoculum Time on Cell Growth for YPG/AB Strain

Shake Flask No.	Inoculation Time (hr)	Total $X_{initial}$ (g)	X_{final} (g/L)	μ_{max} ($\pm$0.001)(h^{-1})
1	2 (Lag phase)	0.06	3.10	0.061
2	6 (Exponential phase)	0.16	4.30	0.076
3	12 (Late Exponential phase)	0.31	4.65	0.071
4	20 (Stationary phase)	0.37	3.45	0.067

Figures 4.31 (b) and **4.32 (b)** show that the rates of starch utilization of both strains are similar, since their maximum specific growth rates at different inoculum times were very close to each other. Starch utilization rate increased when the cells entered their exponential phase of the growth and the starch in the medium was completely consumed when the cells reached their stationary phase.

The maximum accumulation of glucose observed was 0.88 g/L and 0.78 g/L for YPB-G and YPG/AB respectively, both inoculated with precultures taken at their late exponential phase (**Figures 4.31 (c)** and **4.32 (c)**).

Ethanol concentration was also measured in the experiments. No ethanol could be detected when YPB-G was grown on YEP-S medium, while an ethanol concentration of around 5 g/L was measured when YPG/AB was grown on YEP-S. The ethanol was

detected in the medium just after the glucose accumulation passed through its maximum value (around 32 hours later) when the cultures were inoculated with precultures taken at lag, exponential and late exponential phases of their growth. In the case of the stationary phase inoculum, no ethanol was detected, because the accumulation of glucose in the medium was not at a satisfactory level for the production of ethanol.

The less favorable growth of YPG/AB cells inoculated with a preculture taken at its stationary phase might be due to plasmid loss of the strain that also resulted in the lower accumulation of glucose and the lower production of biomass. On the contrary, it was not surprising to have less biomass formation and a longer adaptation period in the culture inoculated with a preculture taken at its lag phase. The net biomass formation (Δx) was 2.6 $\pm$ 0.5 g/L and 2.6 $\pm$ 1.0 g/L for YPB-G and YPG/AB strains respectively at each flask, although the initial amounts of inoculum were different.

Keeping other parameters constant, the optimum time of inoculum was found to be the late exponential phase in terms of biomass formation for these recombinant yeast cells which is in agreement with the results of Birol and Özergin-Ülgen (1995).

4.5.2. Optimization of Inoculum Size

In order to determine the optimum inoculum size, increasing sizes of the inoculum (from 0.5% to 20%) taken from a single preculture at its late exponential phase were added to 2L volumes of YEP-S medium. To avoid the difficulty of dealing with 2% (w/v) starch in the medium, these experiments were carried out on YEP-S containing 1% (w/v) starch.

A set of shake flasks containing 180 mL YNB-G nutrient medium were inoculated with 20 mL of preculture (OD_{600} of 1.00) grown until the late exponential phase. To avoid variations which might come from different batches of precultures, they were combined in

a 1L flask aseptically and then transferred into YEP-S medium in quantities ranging from 0.5 - 20% (v/v). The growth of biomass, starch utilization, glucose accumulation and ethanol production were measured.

With increasing percentages of the inoculum, it was expected that the initial cell mass would also be increased. This increase in the initial cell mass was reflected in the final cell concentrations. An inoculum size of 10% was the threshold value for the maximum amount of biomass achieved as in Birol and Özergin-Ülgen (1995). After passing through a maximum of 10% of inoculum size, the final cell mass remained constant. Any value below the inoculum size of 10% (v/v) caused a decrease in the biomass yield (**Table 4.10 and 4.11**). The maximum specific growth rates did not change with the change in the inoculum size. Furthermore, the net biomass formation (Δx) was 3.5 ± 1.0 g/L for both YPB-G and YPG/AB strains at each flask, although the initial amounts of inoculum were different.

TABLE 4.10. The Effect of Inoculum Size on Final Cell Concentration for YPB-G

Inoculum Size (%)	X_{final} (g/L)	μ_{max} (±0.001)(h^{-1})
0.5	2.56	0.071
1	3.27	0.073
5	3.45	0.072
10	5.69	0.072
20	5.88	0.074

TABLE 4.11. The Effect of Inoculum Size on Final Cell Concentration for YPG/AB

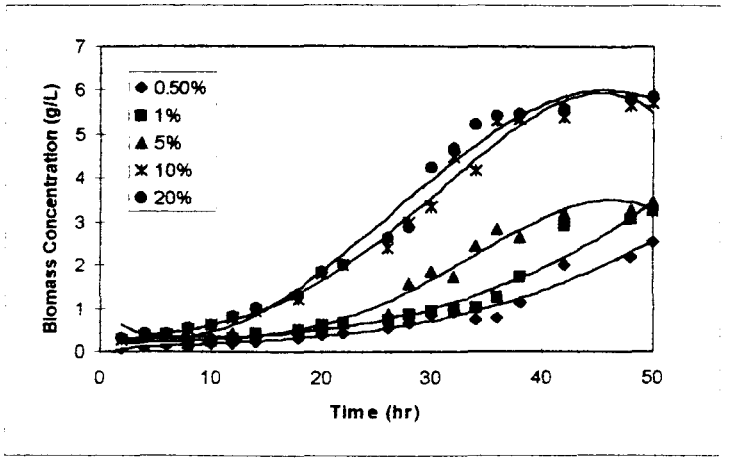
Inoculum Size (%)	X_{final} (g/L)	μ_{max} (±0.001)(h^{-1})
0.5	2.54	0.060
1	3.16	0.055
5	5.42	0.077
10	5.80	0.076
20	5.86	0.072

Apparent lag phase periods were found to be different in the five shake flasks corresponding to each inoculum size. The lag phase was longer in the culture up to 5% (v/v) inoculum. It took about 28 hours for both strains to enter their exponential phase when the cultures were inoculated with 0.5 and 1% (v/v) inoculum. This was reduced to 22 and 26 hours for YPB-G and YPG/AB respectively at 5% (v/v) inoculum size. In the case of 10 and 20% (v/v) inoculum, the cells entered their exponential phase after 14 and 18 hours of operation for YPB-G and YPG/AB respectively (**Figures 4.33 (a)** and **4.34 (a)**).

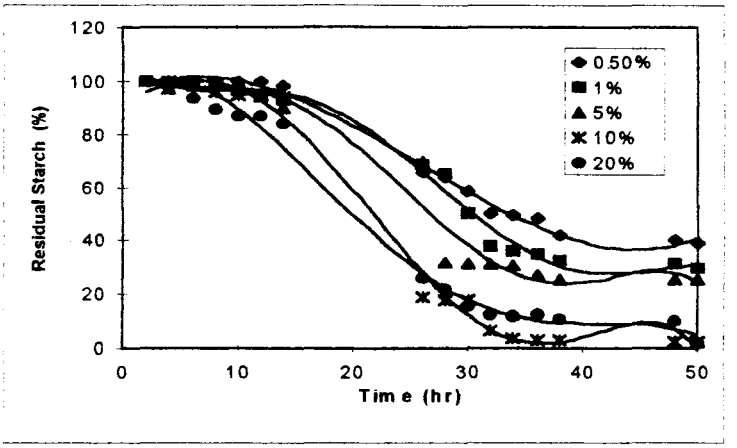
25 - 45% of the initial starch remained unconsumed in the medium in a fermentation period of 50 hours when the inoculum size was below or equal to 5% for both strains. The culture with 10% inoculum showed the highest consumption of the substrate using all the initial starch in 50 hours. Around 10% of initial starch remained unconsumed in the medium when an inoculum of 20% (v/v) was used for both strains (**Figures 4.33 (a)** and **4.34 (a)**).

Glucose accumulation reflected the cell growth behavior very clearly. The glucose concentration reached its maximum value just after the cells entered their exponential phase of growth in all cases. A proper accumulation followed by a regular consumption of glucose was observed at inoculum sizes of 5, 10 and 20% (v/v) for both strains. The glucose was accumulated in the medium to a lesser extent at relatively later times for the 0.5 and 1% (v/v) of inocula.

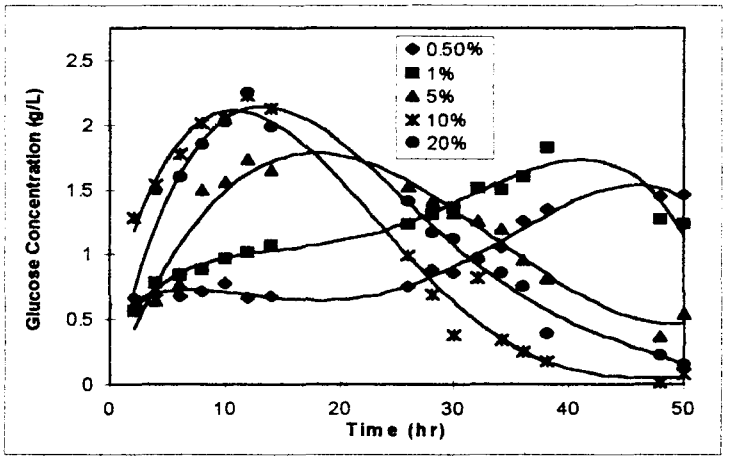
Variation in the inoculum size did not have any effect on the ethanol production, and no ethanol could be detected for both strains in YEP-S containing 1% (w/v) starch.



(a)

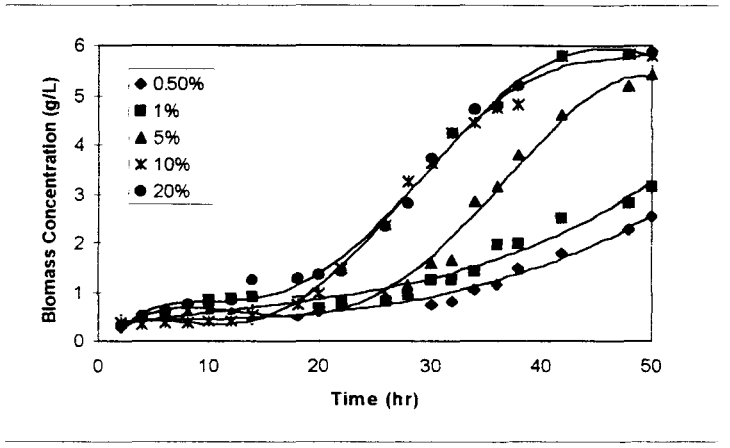


(b)

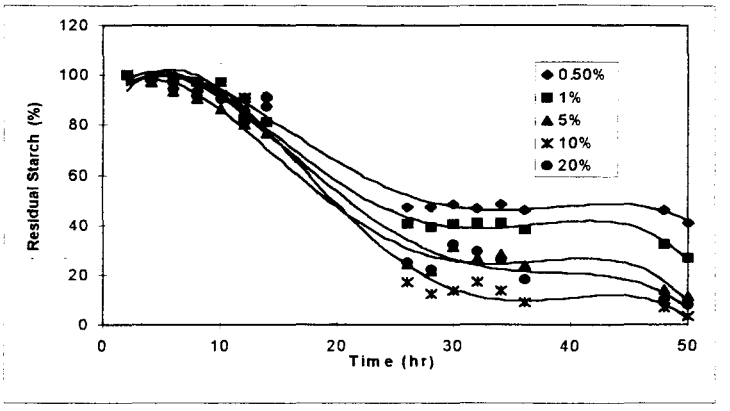


(c)

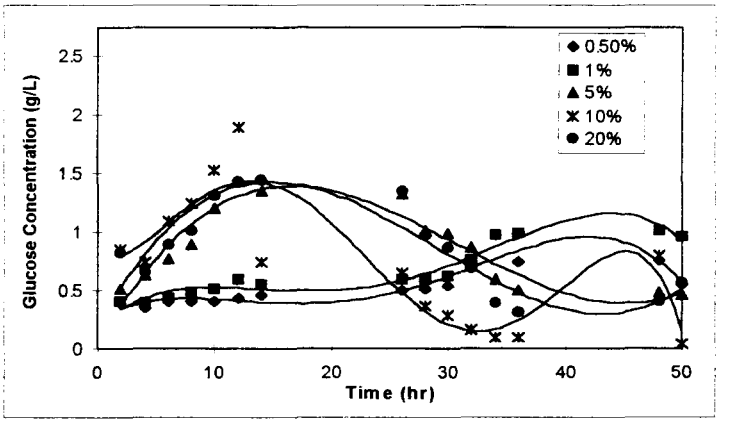
FIGURE 4.33. Time Course of (a) Biomass Formation, (b) Residual Starch and (c) Glucose Accumulation by YPB-G Strain for the Optimization of Inoculum Size



(a)



(b)



(c)

FIGURE 4.34. Time Course of (a) Biomass Formation, (b) Residual Starch and (c) Glucose Accumulation by YPG/AB Strain for the Optimization of Inoculum Size

Keeping parameters like medium formulation, medium sterilization and culture growth conditions constant, the initial inoculum size was found to influence the apparent lag time and the amount of biomass in the subsequent culture. An inoculum size of 10% was found to be the optimum for maximum biomass production and starch utilization.

4.6. Optimum Conditions for Enzyme Activities

The optimum conditions for the activity of the secreted amylolitic enzymes (α-amylase and glucoamylase) were studied to optimize the fermentation conditions of the recombinant yeast strains for the production of ethanol from starch.

α-amylase is one of the oldest enzymes to be extensively studied (Bezbaruah 1991). Generally, the optimum activity of α-amylase was recorded at a pH range of 4.0-7.0 and a temperature range of 30-70°C (Bezbaruah et al., 1991; Simones-Mendes, 1984; Thomsen, 1983; Gasperik et al., 1991; Moseley and Keay, 1970; Paquet et al., 1991). *Clostridium thermosaccharolyticum* and *Aspergillus niger* Glucoamylases, on the other hand, had an optimum pH range of between 4.0-6.0 and a temperature range of again 40-70°C (Specka et al., 1991; Stoffer et al., 1993). It has also been reported that, *Saccharomyces cerevisiae* normally secretes only a very small number (0.5%) of its own proteins to the medium (Das and Shultz, 1987), which is advantageous for product recovery.

In this part of the study, two of the recombinant strains were chosen, namely YPG/AB which secretes *B.subtilis* α-amylase and *A.awamori* glucoamylase and YPG/MM which secretes mouse pancreatic α-amylase and *A.awamori* glucoamylase. These were grown in YNB-G liquid medium until their stationary phase in an orbital shaker. Enzymes in the supernatant of the samples collected by centrifugation of the culture at 10000 rpm for 15 minutes were partially purified as described in Section 3.2.4, and the optimum pH and temperature of these enzymes were determined.

4.6.1. Optimum pH

A series of 50mM Na-Acetate buffers ranging from a pH of 2.70 to 9.25 were freshly prepared. 10 mL cell culture was grown until its late exponential phase and was centrifuged to collect the supernatant. α-Amylase and glucoamylase in the supernatant were partially purified as described in Section 3.2.4 and were assayed as described in Sections 3.2.5.6 and 3.2.5.7 respectively at 40°C. Both mouse pancreatic (from YPG/MM) and *B. subtilis* α-amylases (from YBP-G) have shown a wide range of activity. Mouse pancreatic α-amylase displayed a constant activity up to pH 7.00, then its activity decreased rapidly, while *B. subtilis* α-amylase showed remarkable pH stability between pH 2.70 to 9.25 (**Figure 4.35**).

Glucoamylase from *A. awamori* gene (from YBP-G) has displayed a maximum activity between a pH range of 5.00 to 7.00, but no activity was detected when the pH was reduced to 3.00 or below (**Figure 4.35**)

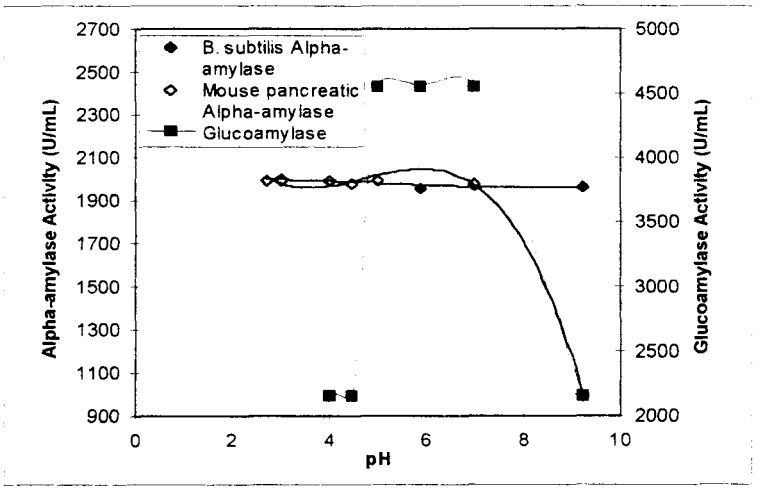


FIGURE 4.35. Effect of pH on the Activity of Extracellular α-amylase and Glucoamylase

4.6.2. Optimum Temperature

Partially purified α-amylase and glucoamylase were assayed in a temperature range of 20 - 80°C at the pH values of 5.9 and 4.5 respectively. The α-amylase from *B. subtilis* (YPG/AB) has shown considerable activity over the 20 - 40°C , then the activity decreased. Glucoamylase had a stable and high activity at 40°C and a decrease in the activity of the enzyme was observed at higher temperatures (**Figure 4.36**).

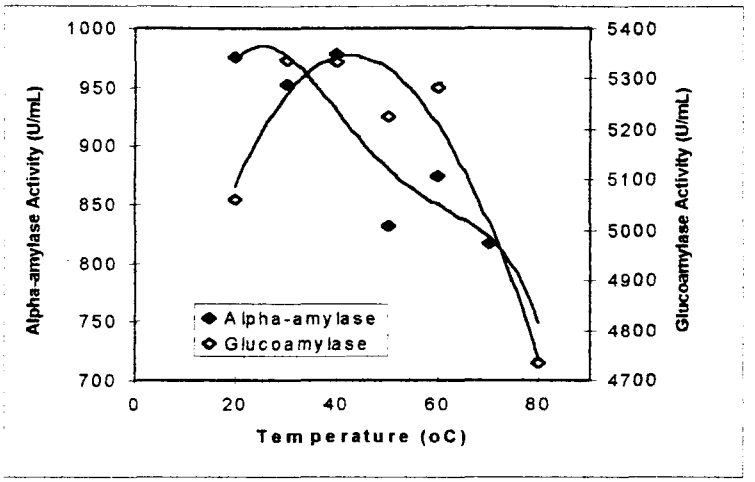


FIGURE 4.36. Effect of Temperature on the Activity of Extracellular α-amylase and Glucoamylase

4.7. Characteristics of Recombinant Yeast Strains

The optimization of the expression and secretion of the functional starch hydrolyzing enzymes, namely α-amylase and glucoamylase in genetically modified *Saccharomyces cerevisiae* was considered to be very important for the one step bioconversion of starch rich materials to ethanol or single cell protein. In most of these constructs heterologous α-amylase (AMY) or glucoamylase (STA2) genes from various organisms have been expressed and excreted. Co-expression of AMY and STA2 genes in recombinant *Saccharomyces cerevisiae* has resulted in the production of higher amylolitic activity (Steyn et al., 1992). The expression of a bifunctional protein from the fusion of the complete reading frames of both the α-amylase and the glucoamylase cDNAs from the filamentaous fungus, *Aspergillus shirousamii* (Shibuya et al., 1992a) in *S.cerevisiae* was a more effective approach, and this construct displayed a higher level of activity with raw starch as substrate than a mixture of the two native enzymes (Shibuya et al., 1992b). Finally, de Moraes et al. (1995) prepared eight different constructions, these include strains that produce *Bacillus subtilis* α-amylase (BsAAase), mouse pancreatic α-amylase (MAAase) or *Aspergillus awamori* glucoamylase (GAase), either singly or in combination, as well as strains that produce either BsAAase/GAase or MAAase/GAase fusion enzymes. They have observed good performances not only in digesting soluble and corn starch but also in using all of the hydrolysis products for growth.

In the present work, three strains of *Saccharomyces cerevisiae* namely YPG/MM, YPG/AB and YPB-G constructed by de Moraes et al. (1995) were examined. Two different growth media containing glucose or starch as the main carbon source were used to grow the recombinant yeast strains. YEP-G contained 5% (w/v) glucose, 0.5% (w/v) yeast extract, 1% (w/v) peptone, 0.002% (w/v) histidine. YEP-S media (YEP-S1 and YEP-S2) contained 0.5% (w/v) yeast extract, 1% (w/v) peptone, 0.002% (w/v) histidine, and 10% or 5% (w/v) soluble starch respectively. Experiments were performed at 30 °C and 180 rpm in an orbital shaker (GFL-3032). The 6 L- flasks were filled with medium to one-third of their volume (2 L) to allow good aeration.

To eliminate structural instability, a selection procedure described by Wei et al. (1989) was applied. The cells from frozen stocks were grown on YNB-G agar plates, they were transferred to YNB-GS agar plates. After allowing growth into single colonies, the plates were I_2 stained. The colony which shows a white amylolitic halo was chosen for further growth in YNB-G medium until mid-exponential phase. This culture was used as an inoculum on a 10% (v/v) basis.

Growth characteristics, ethanol production, starch utilization, glucose and reducing sugar accumulation properties, protein secretion and plasmid stability of the recombinants were investigated in starch containing media (YEP-S1 and YEP-S2). Furthermore, these strains were grown in glucose containing media (YEP-G) in order to compare their characteristics and investigate the effects of medium enrichment.

4.7.1. Growth Characteristics

Three recombinant yeast strains YPB-G, YPG/AB and YPG/MM were grown in batch cultures. The cultures were monitored for biomass formation, plasmid stability and carbon source utilization. The strains investigated showed similar physiological pattern.

When YPG/AB strain was grown in YEP-S1, starch was completely exhausted in the medium after about 70 hours, and maximum amounts of 8.2 g/L of glucose and 14.5 g/L of total reducing sugar were accumulated after approximately 35 - 40 hours (**Figure 4.37**). Similarly, YPB-G strain exhausted the starch in the medium after about 60 hours, and a maximum accumulation of only 2.5 g/L of glucose and 20.5 g/L of total reducing sugar in YEP-S1 medium were observed (**Figure 4.38**). Furthermore, YPG/MM strain exhausted the starch in the medium after about 80 hours with a maximum accumulation of only 1.4 g/L of glucose and 19.8 g/L of total reducing sugar in YEP-S1 medium (**Figure 4.39**).

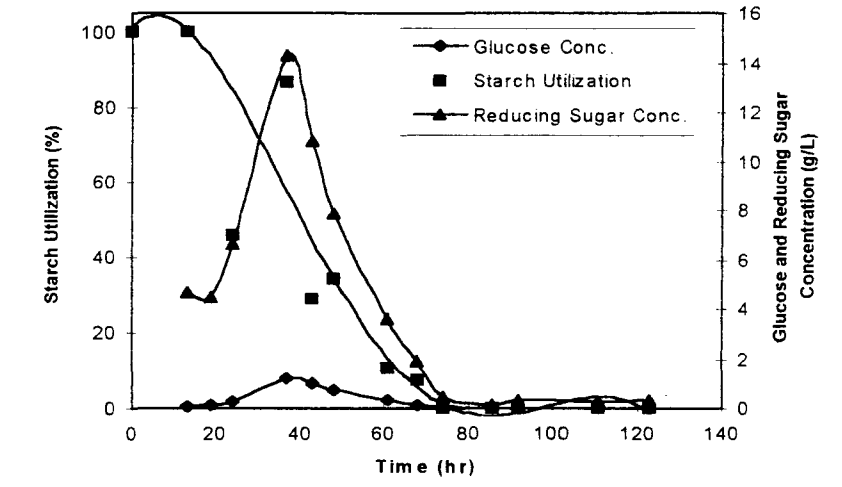


FIGURE 4.37. Carbon Source Utilization of YPG/AB Strain in YEP-S1

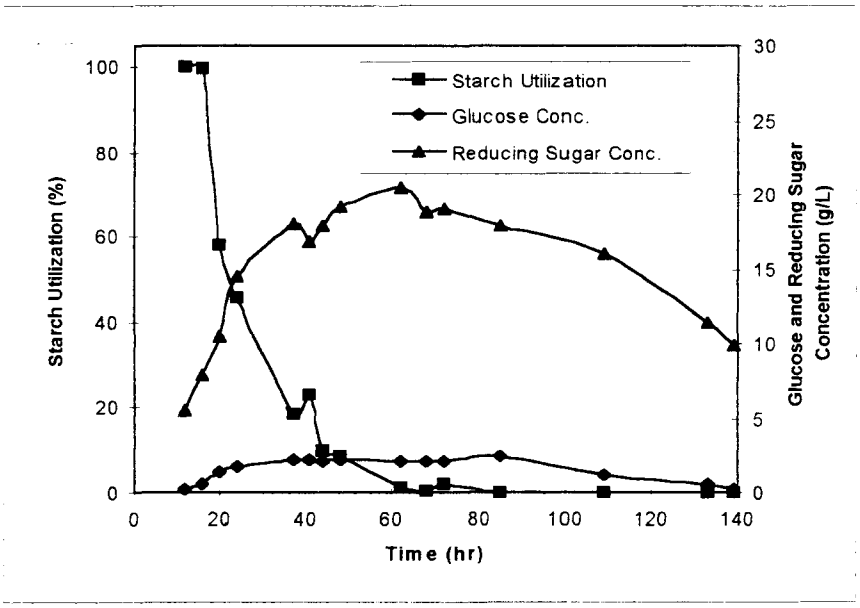


FIGURE 4.38. Carbon Source Utilization of YPB-G Strain in YEP-S1

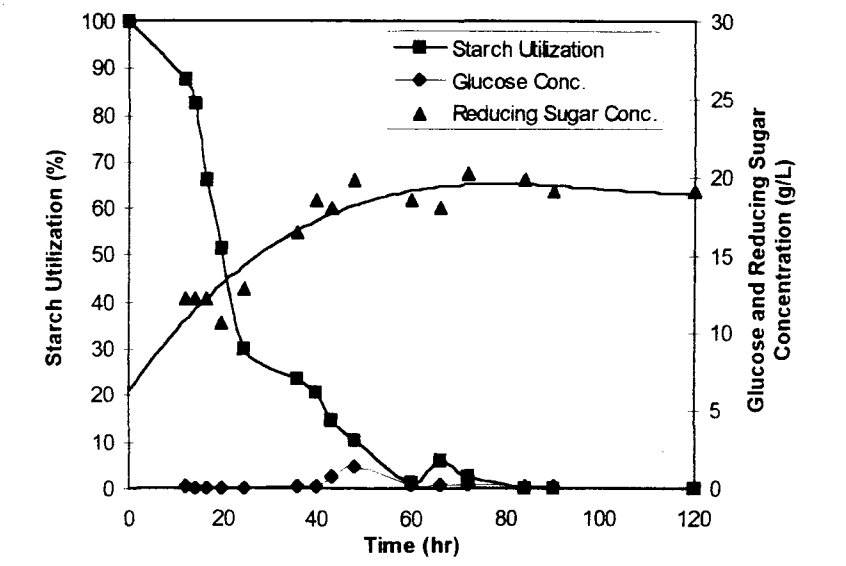


FIGURE 4.39. Carbon Source Utilization of YPG/MM Strain in YEP-S1

In YEP-S2 medium, on the other hand, the starch was found to be completely exhausted after 45 - 50 hours, and a maximum accumulation of 4.7 g/L glucose and 6.8 g/L of total reducing sugar were observed for YPG/AB. When YPB-G was grown in YEP-S2, only 0.5 g/L of glucose and 12 g/L of total reducing sugar could be accumulated at maximum in the medium. Similarly, the starch was exhausted after 80 hours and only 0.8 g/L of glucose and 16.3 g/L of total reducing sugar were accumulated in this medium for YPG/MM (**Figures 4.40-4.42**).

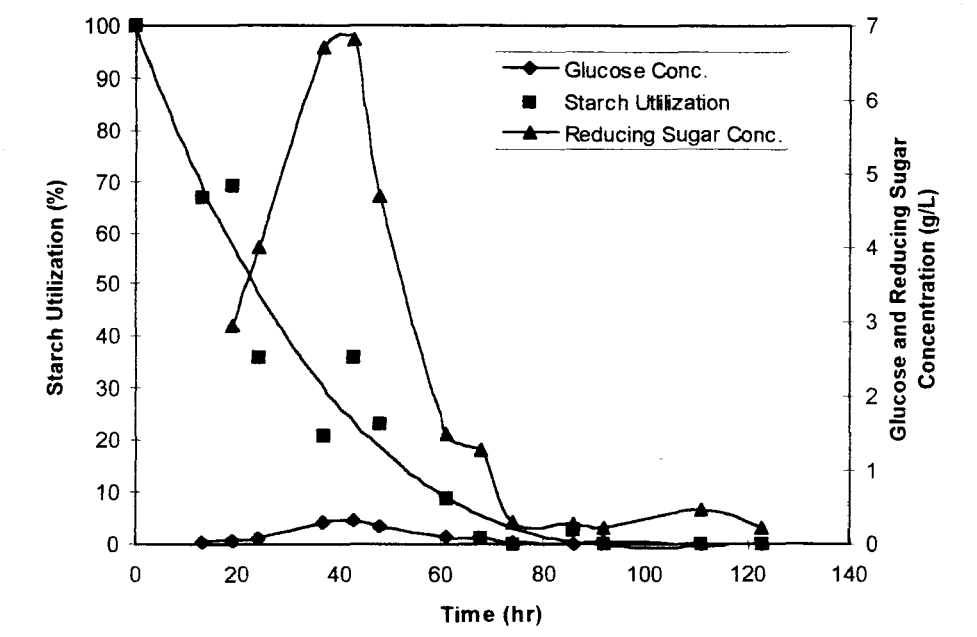


FIGURE 4.40. Carbon Source Utilization of YPG/AB Strain in YEP-S2

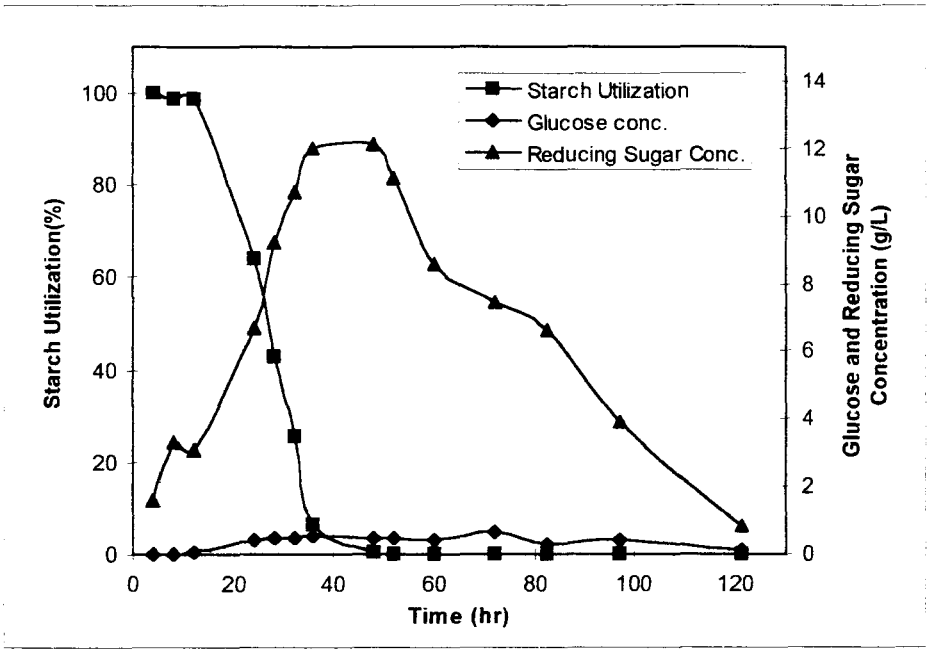


FIGURE 4.41. Carbon Source Utilization of YPB-G Strain in YEP-S2

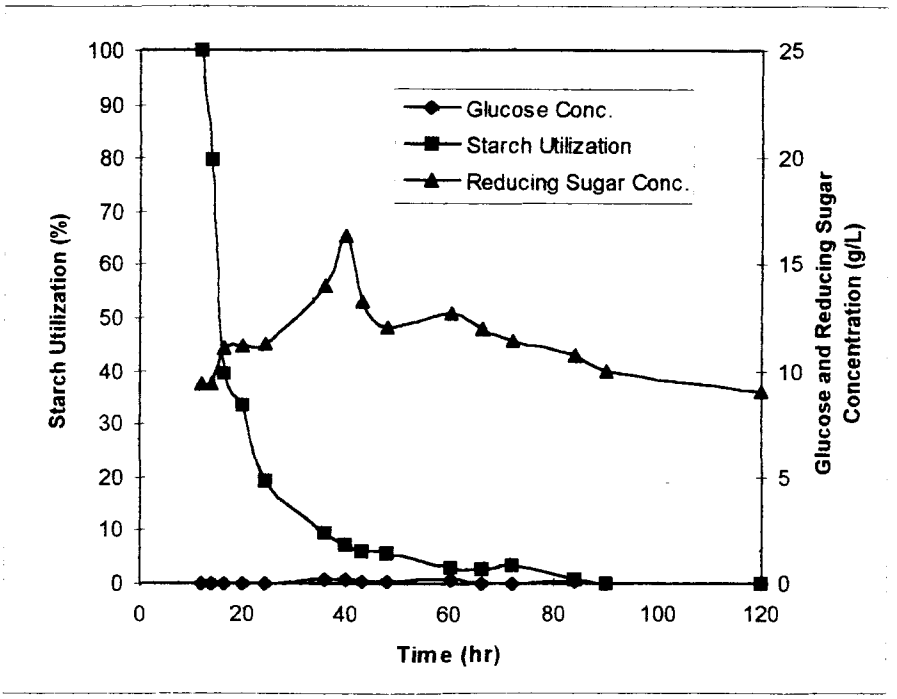


FIGURE 4.42. Carbon Source Utilization of YPG/MM Strain in YEP-S2

Although glucose and reducing sugar concentrations were found to decrease with time during the growth of all three strains, in the case of both YPB-G and YPG/MM strains, a residual level of these sugars could still be detected even after 120 - 130 hours. These results indicate that YPG/AB consumes these substances more rapidly.

The growth patterns of YPG/AB, YPB-G and YPG/MM in two different media, namely YEP-S1 and YEP-S2, containing 10% and 5% (w/v) soluble starch respectively, are presented in **Figures 4.43-4.45**. Although similar maximum specific growth rates (μ_{max} 0.036 h^{-1}) were calculated for YPG/AB and YPB-G strains in YEP-S1 media, 8.2 g/L and 5.9 g/L of biomass were formed respectively. On the other hand, YPG/MM strain had a lower growth rate (μ_{max} 0.027 h^{-1}) and only 2.4 g/L of biomass was formed in YEP-S1.

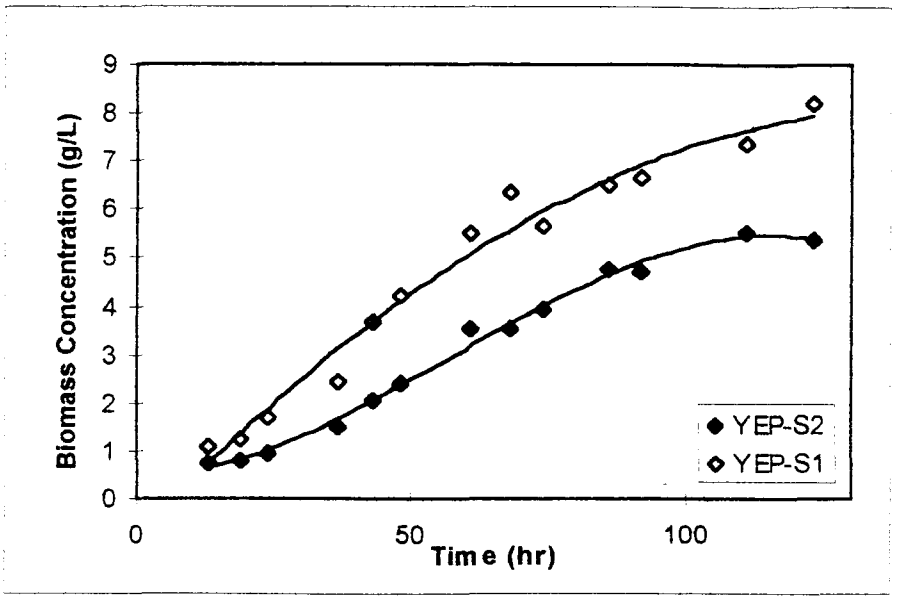


FIGURE 4.43. Growth Pattern of YPG/AB Strain in YEP-S.

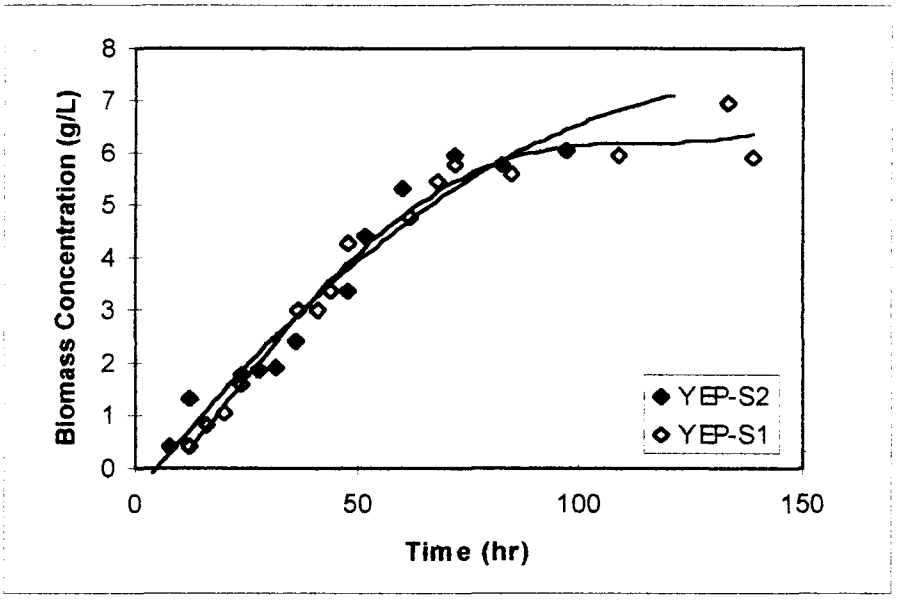


FIGURE 4.44. Growth Pattern of YPB-G Strain in YEP-S.

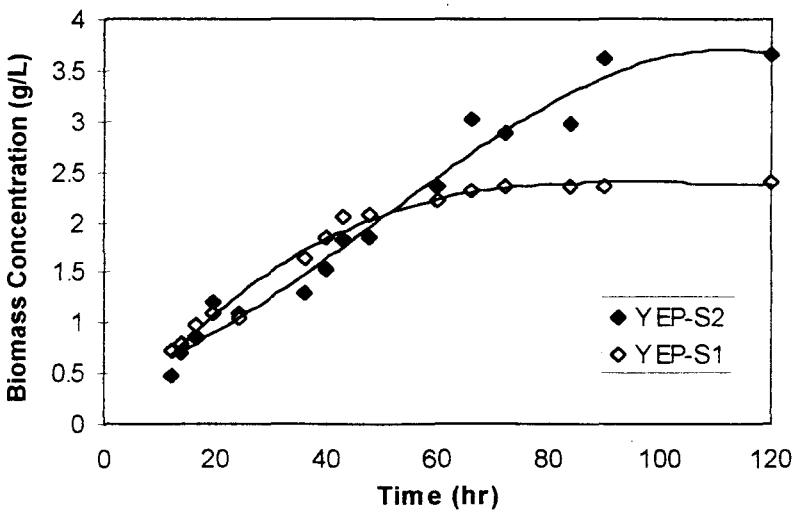


FIGURE 4.45. Growth Pattern of YPG/MM Strain in YEP-S.

Furthermore, in medium containing 5% starch (YEP-S2), biomass formation of 6.05 g/L, 5.37 and 3.00 g/L were obtained with approximately similar maximum specific growth rates for YPB-G , YPG/AB and YPG/MM strains respectively.

When the cells were grown in YEP-G, all the glucose was consumed at the end of 24 hours by the three strains. The biomass formation of 3.07 g/L for YPG/AB, 2.16 g/L for YPB-G and 6.41 g/L for YPG/MM were obtained. Time course of biomass formation and glucose consumption are plotted through **Figures 4.46-4.48**. Maximum specific growth rates were increased approximately 10 fold for all the strains. Although the higher yields of biomass were obtained in starch containing media for both YPG/AB and YPB-G strains, biomass yield increased by about twice in glucose containing media for YPG/MM. The use of higher starch concentrations in the medium reduced the biomass yield in all cases (**Table 4.12**).

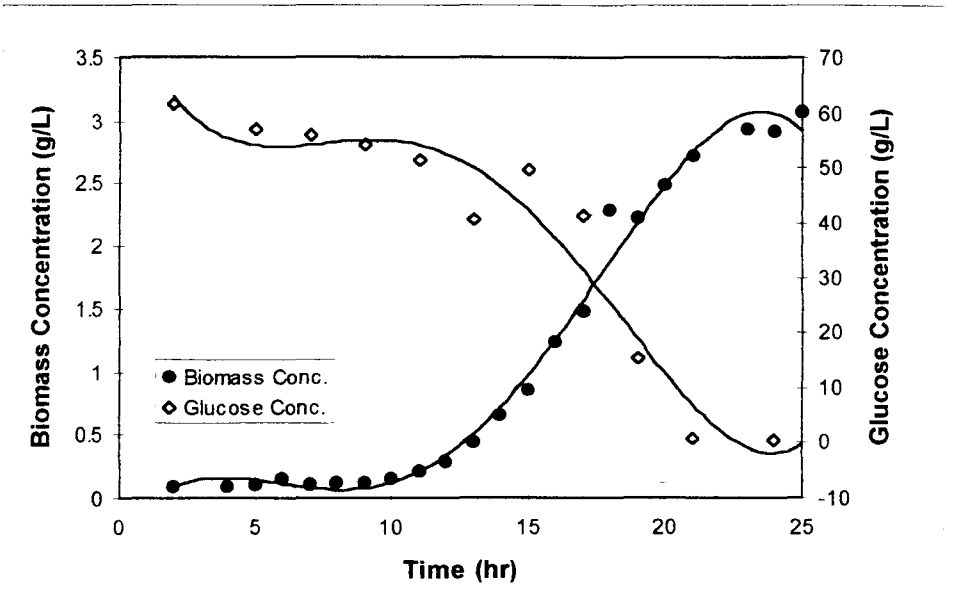


FIGURE 4.46. Growth Pattern and Substrate Utilization of YPG/AB Strain in YEP-G.

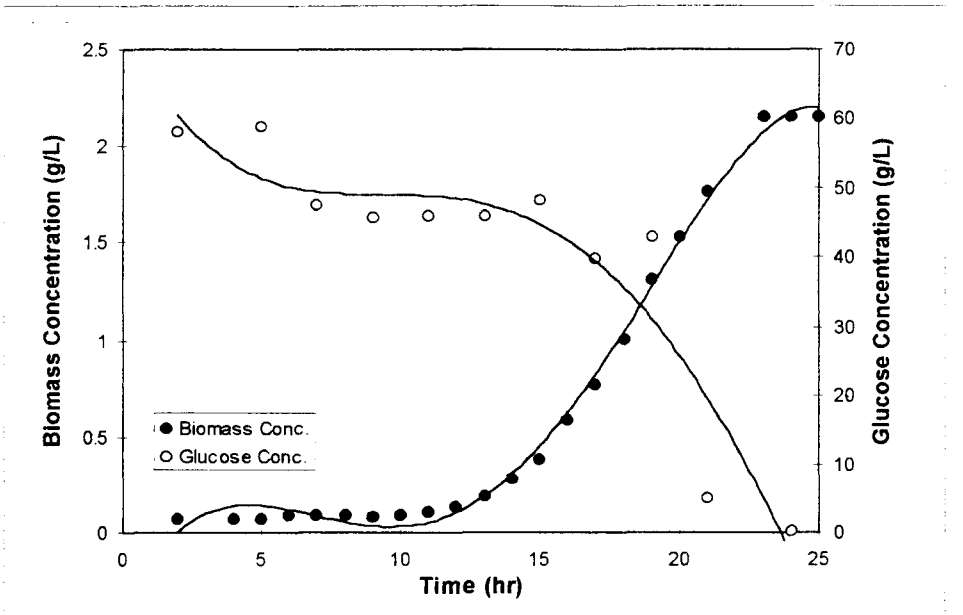


FIGURE 4.47. Growth Pattern and Substrate Utilization of YPB-G Strain in YEP-G

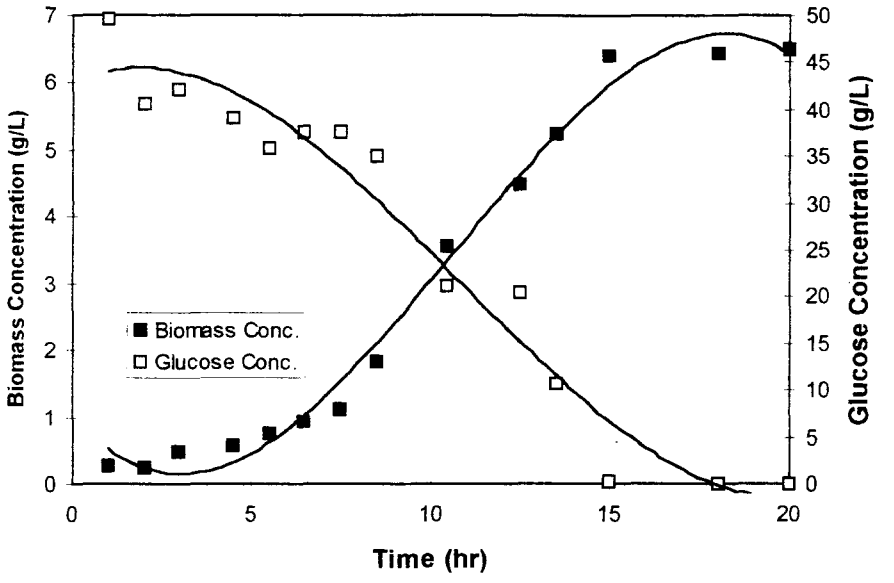


FIGURE 4.48. Growth Pattern and Substrate Utilization of YPG/MM Strain inYEP-G

4.7.2. Ethanol Production

The production of ethanol was 43.8 g/L and 18.8 g/L for YPG/AB strain in YEP-S1 and YEP-S2 media respectively.

YPB-G strain did not produce ethanol when it was grown in YEP-S2 medium which might be due to the very low amount of glucose released from starch. However, when a glucose level of 2.5 g/L was reached in YEP-S1 medium, 35.2 g/L of ethanol production was observed.

YPG/MM strain did not produce any ethanol in YEP-S media. This might be due to the very low activities of glucoamylase detected in the medium which resulted in both very low reduction of starch into glucose and low production of biomass.

21.6 g/L, 24.3 g/L and 24.3 g/L of ethanol production were observed for YPG/AB, YPB-G and YPG/MM respectively in YEP-G. These results show that the ethanol produced by YPG/AB and YPB-G in 10% starch medium, YEP-S1, are more than those obtained in YEP-G. Comparing the yields of biomass and ethanol, these strains tend to produce ethanol rather than biomass especially in YEP-G medium.

TABLE 4.12. Experimental Parameters of Fermentation

Medium	Strain	$\mu_{max}(h^{-1})$ ± 0.01	$Y_{X/S}$ (g biomass / g substrate)	Ethanol Concentration (g Ethanol/L)
YEP-S1	YPG/AB	0.0359	0.082	43.8
YEP-S2		0.0352	0.107	18.8
YEP-G		0.301	0.061	21.6
YEP-S1	YPB-G	0.0360	0.059	35.2
YEP-S2		0.0330	0.121	nd
YEP-G		0.234	0.043	24.3
YEP-S1	YPG/MM	0.027	0.024	nd
YEP-S2		0.024	0.060	nd
YEP-G		0.242	0.128	24.3

nd: not detectable

Table 4.13 shows a comparison of the amount of ethanol produced and the production rate of ethanol from the soluble starch under several incubation systems. Dostalek and Haggstrom (1983) studied the conversion of starch into ethanol in a mixed culture of an amylolitic yeast, *Saccharomycopsis fibuliger*, and an anaerobic bacterium, *Zymomonas mobilis*, and they obtained a maximum ethanol concentration of 9.7 g/L and a production rate of ethanol of 0.54 g/L/h in a fermentation of 30 g/L starch. Tanaka et al. (1986) investigated the production of ethanol from starch by a coimmobilized mixed culture system of aerobic and anaerobic microorganisms, *Aspergillus awamori* and *Zymomonas mobilis*, in Ca-alginate gel beads, and they obtained a maximum ethanol concentration of 22.0 g/L with a production rate of 0.61 g/L/h in the fermentation of 100 g/L starch. Recently, Nakamura et al. (1997) were able to produce 24.9 g/L of ethanol with

a production rate of 0.66 g/L/h in the direct fermentation of 100 g/L starch by using recombinant *Saccharomycess cerevisiae* strains. In this work, we have reached an ethanol concentration of 43.8 g/L. From this result, it was found that the direct fermentation of starch by the recombinant yeast was an effective method because it used only one microorganism and was able to reach even higher levels of ethanol concentrations.

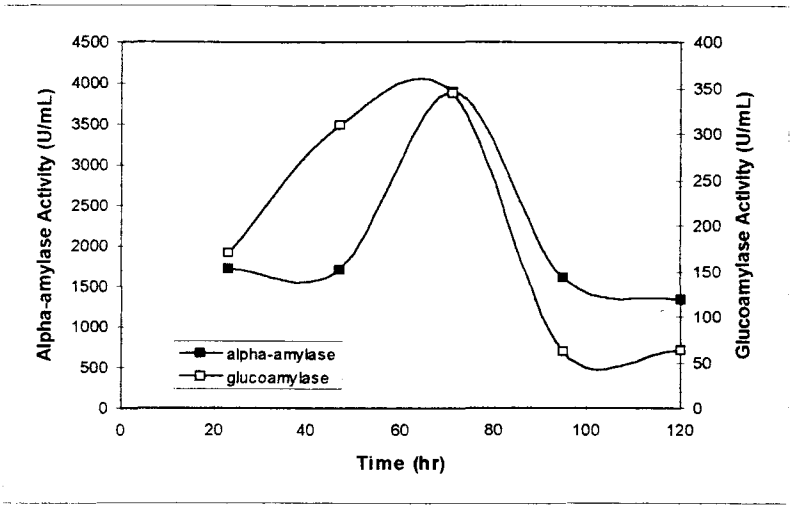
TABLE 4.13. A Comparison of Ethanol Production and Rate of Production of Ethanol under Different Incubation Systems

Microorganism		Initial Soluble Starch Concentration (g/L)	Final Ethanol Concentration (g/L)	Production Rate of Ethanol (g/L/hr)	Ref.
Saccharification	Fermentation				Dostalek and Haggtrom (1986)
S. fibuliger	*Z. mobilis*	30	9.7	0.54	
A. awamori	*Z. mobilis*	100	22.0	0.61	Tanaka et.al. (1986)
S. cerevisiae		100	24.9	0.66	Nakamura et al. (1997)
S. cerevisiae		100	43.8	0.44	This work

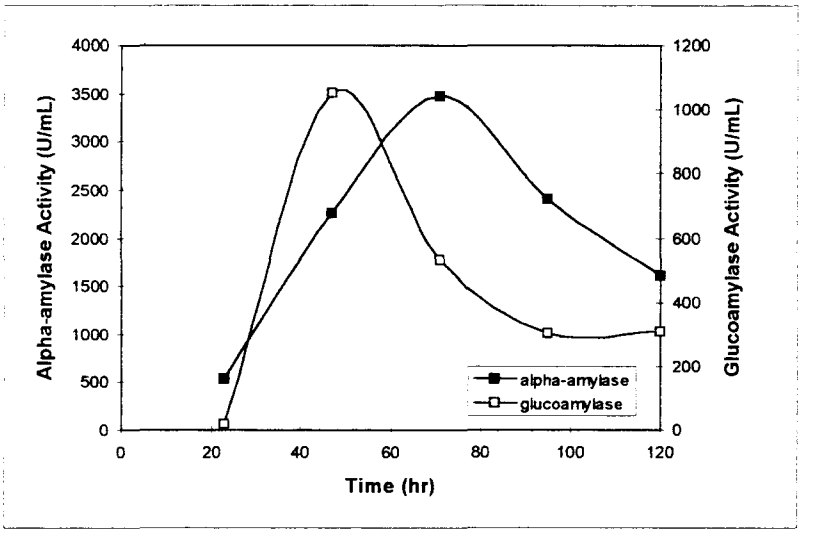
4.7.3. α-Amylase and Glucoamylase Secretion

α-amylase and glucoamylase secretion patterns of the three recombinants in YEP-S1 and YEP-S2 are shown in **Figure 4.49-4.51**. α-amylase and glucoamylase activities reached their maximum levels after around 40 to 80 hours when the starch concentration in the medium decreased rapidly for both YPG/AB and YPB-G strains in YEP-S1. On the

other hand, α-amylase activity showed an increasing trend while almost no glucoamylase activity could be detected for YPG/MM strain in the same medium.

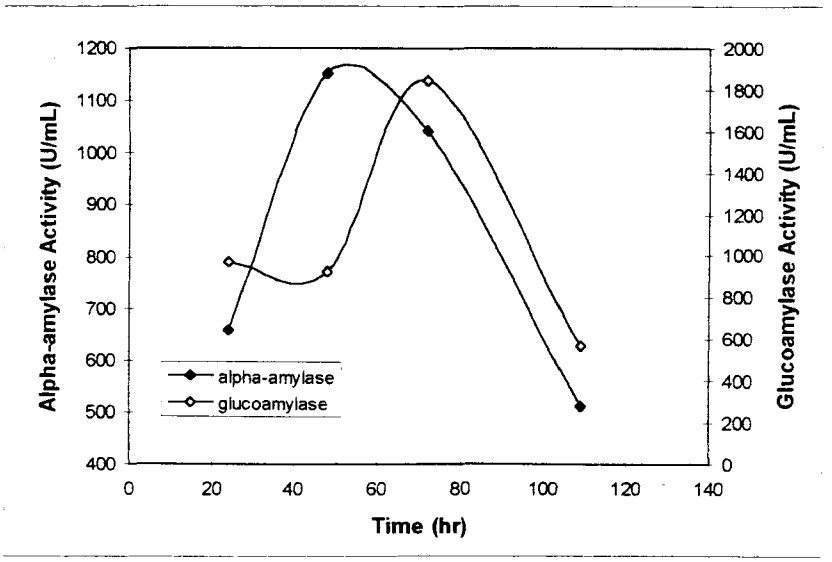


(a)

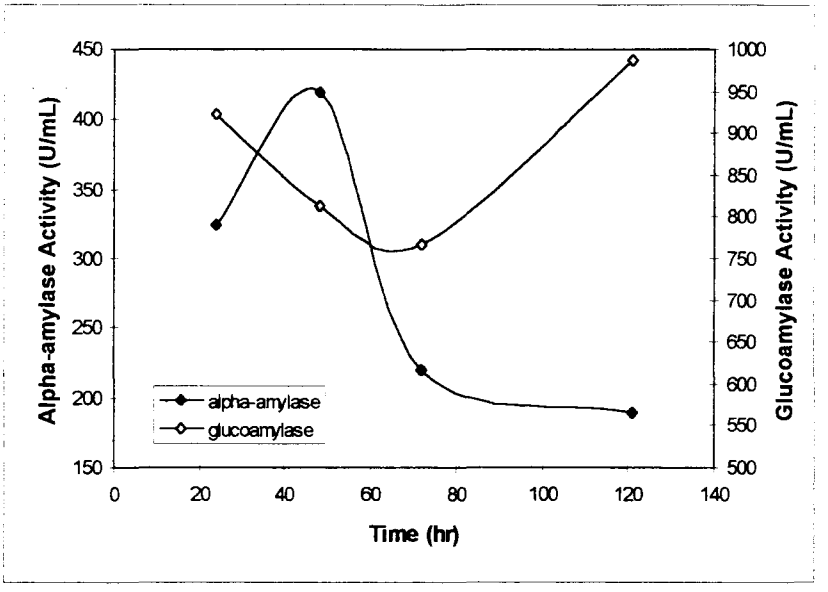


(b)

FIGURE 4.49. Time Profiles of α-amylase and glucoamylase Activities on (a) YEP-S1 and (b) YEP-S2 for YPG/AB Strain

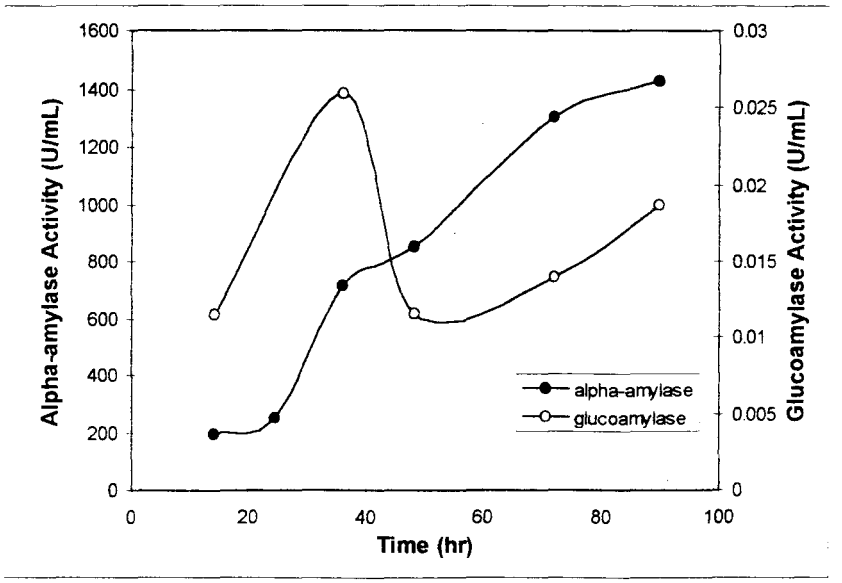


(a)

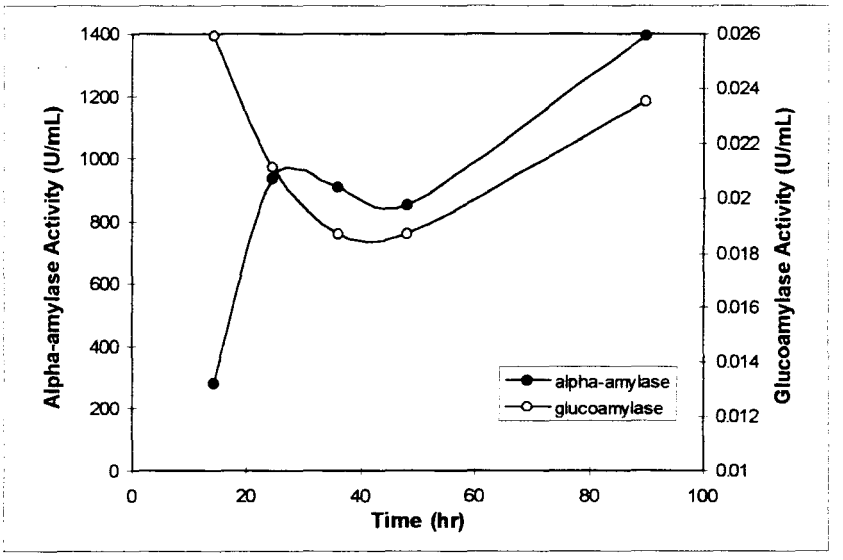


(b)

FIGURE 4.50. Time Profiles of α-amylase and glucoamylase Activities on (a) YEP-S1 and (b) YEP-S2 for YPB-G Strain



(a)



(b)

FIGURE 4.51. Time Profiles of α-amylase and glucoamylase Activities on (a) YEP-S1 and (b) YEP-S2 for YPG/MM Strain

The maximum activity of α-amylase secreted by YPG/AB strain was 4000 U/ml and 3500 U/ml in YEP-S1 and YEP-S2 media respectively, while maximum activities, 1800 U/ml and 900 U/ml, in YEP-S1 and YEP-S2 media respectively were observed for

the YPB-G strain. The α-amylase activity which showed an increasing trend by the secretion of YPG/MM reached a maximum value of 1500 U/ml and 1370 U/ml in YEP-S1 and YEP-S2 respectively towards the end of the operation. When grown in YEP-G medium, approximately a similar α-amylase activity was detected for YPG/MM. However, YPG/AB and YPB-G strains were found to secrete considerably lower amounts of α-amylase (700 U/ml and 300 U/ml respectively) in YEP-G.

The maximum activity of glucoamylase secreted by YPG/AB strain was 1053 U/ml and 345 U/ml in YEP-S1 and YEP-S2 media. Similarly, maximum activities of 1100 U/ml and 450 U/ml were recorded for the YPB-G strain in YEP-S1 and YEP-S2 media . No glucoamylase activity was observed for YPG/MM in both media.

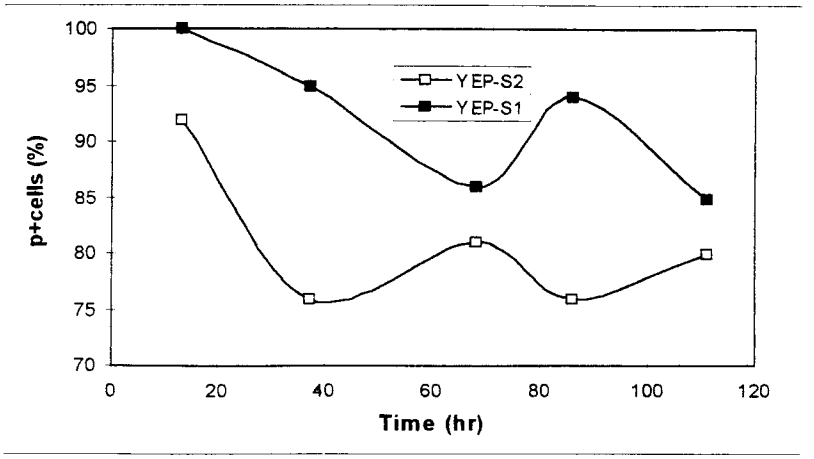
Slightly higher maximum glucoamylase activities, 1297 U/ml and 1376 U/ml for YPG/AB and YPB-G respectively were observed in YEP-G medium. A barely detectable glucoamylase activity (10 U/ml) was observed with YPG/MM.

4.7.4. Plasmid Stability

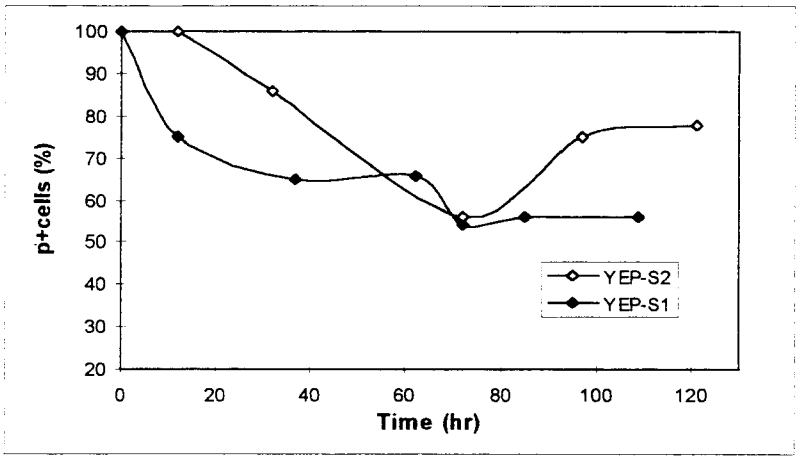
The plasmid stability of all the strains was investigated in YEP-S media for a period of 5 days. The plasmid stability was calculated as the ratio of the number of plasmid containing cells to the number of total (plasmid free plus plasmid containing) cells (**Figure 4.52**). The strain YPG/AB has displayed a plasmid stability of 85% and 80% in YEP-S1 and YEP-S2 respectively. YPB-G was found to lose about 30% - 40% of recombinant plasmids in YEP-S1, and the plasmid stability of this strain was calculated as 80% in YEP-S2. YPG/MM strain had a plasmid stability of 75% and 83% in YEP-S1 and YEP-S2 respectively (**Figure 4.52**). All of the three strains were found to be more stable in YEP- G, presenting a plasmid stability higher than 95%.

These results have indicated the instability of YPB-G construct in the medium containing higher concentrations of starch. The higher plasmid stabilities of both strains in YEP-G might be due to the higher growth rates which are attained in glucose containing media. It has been postulated that plasmid stability would increase with higher growth rates since proper partitioning would be more likely due to larger bud sizes (Hjortso et al., 1985). A similar situation was also observed by Parker and DiBiasio (1987). In addition, since starch is a poor substrate compared to glucose, the yeast cells could grow at relatively low growth rates which results in a decrease in the percent of plasmid containing cells.

Lower growth rates of YPB-G strain may be due to a defect in the ability of the glucoamylase domain of the fusion protein to act on the branched natural substrate indicated by the control experiments. Although it has been reported by Moraes et al. (1995) that the fusion protein displays a de-branching activity. The defect is not in the sugar uptake system of YPB-G. Since, this strain has a high intrinsic glucoamylase activity measured in the cell extracts, the defect is likely to be in the secretion mechanism.



(a)



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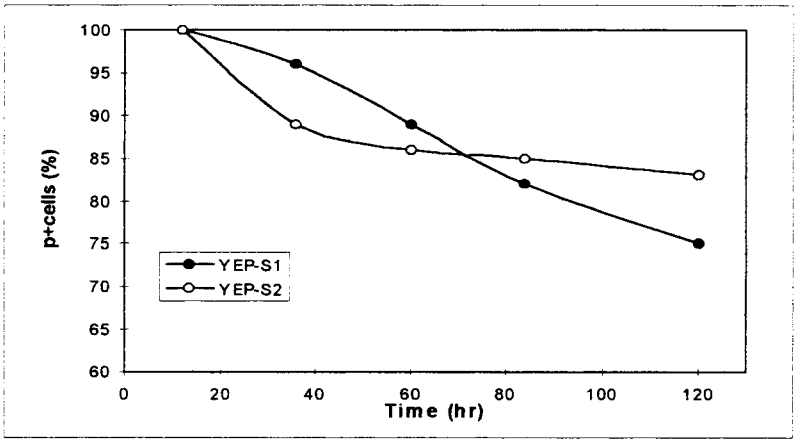


(c)

FIGURE 4.52. Plasmid Stability Profiles of (a) YPG/AB (b) YPB-G and (c) YPG/MM in YEP-S media

4.7.5. Concluding Remarks

A number of conclusions could be drawn from these results:

The effects of medium composition on biomass formation, ethanol production, enzyme secretion and plasmid stability of three genetically engineered yeast strains were studied, and strains secreting the enzymes as separate proteins (YPG/AB and YPG/MM) or as fusion proteins (YPB-G) were compared.

Batch cultures of the three strains demonstrated that in each case superior growth was associated with media containing glucose. The ethanol production of the strains increased with increasing starch concentration in the medium resulting in higher levels of reducing sugar. Furthermore, YPG/AB strain was found to consume the reducing sugars more rapidly, while YPB-G and YPG/MM strains accumulated them even after 120 - 130 hours, thus showing a weak efficiency for the utilization of the hydrolysis products of starch. Comparing the two media, namely YEP-S and YEP-G, the level of ethanol production was higher in YEP-S for both YPG/AB and YPB-G strains. Ethanol was not produced by YPG/MM in starch containing media because of its very poor secretion of glucoamylase into the medium. YPG/AB strain, which secretes the enzymes separately, showed a higher ethanol productivity than YPB-G strain which secretes fusion proteins in YEP-S.

When the enzyme secretion efficiencies of the strains are compared, it is found that the α-amylase activity of the YPG/AB strain is about 2.2 times higher than that of YPB-G, while the glucoamylase activities secreted by YPG/AB and YPB-G are at the same level. This indicates that the more efficient utilization of starch by YPG/AB strain is due to the higher levels of α-amylase activity in the medium. YPG/MM yields an α-amylase activity that is lower than that of YPB-G and almost no glucoamylase activity.

All strains showed higher plasmid stabilities when grown on glucose containing medium. The studies on plasmid stability have indicated the superiority of YPG/AB which

has displayed a plasmid stability of 80 - 85 % in YEP-S. A similar plasmid stability was also observed for the other two strains only in the medium containing low starch concentration. However, lower plasmid stabilities were detected in the medium containing higher concentration of starch.

The use of lower starch concentration in the medium had no detectable effect on the maximum growth rates and/or biomass formation of YPG/AB, YPB-G and YPG/MM. Furthermore, lower glucoamylase activities detected in this type of medium can not be explained by the plasmid instability. In the case of α-amylase, the synthesis and/or the secretion of the enzyme was found to be the same for YPG/AB but was reduced to one half in the other two strains in the medium containing the lower starch concentration. Identification of the molecular basis of this problem needs further investigation.

Parametric studies involving medium composition and fermentation conditions need to be conducted in order to optimize biomass growth and product formation. Since these strains are capable of secreting α-amylase and glucoamylase simultaneously, thereby mediating one-step starch utilization, with a correct strategy they can conveniently be used for ethanol production.

4.8. Studies in the Batch Fermenter

Biotechnological processes have created a need to quantify metabolic processes of microorganisms so that they can be most thoroughly and efficiently exploited. Improved quantification resulted in (*i*) increased yield of microbial products, (*ii*) increased rate of product formation, (*iii*) maintenance of microbial product quality and uniformity and (*iv*) attainment of process uniformity (Sonnleitner and Kappeli, 1986).

Many workers have studied the control of metabolism of *S. cerevisiae* under aerobic and anaerobic conditions (Barford and Hall, 1981). In the last decade, an increasing potential on the recombinant DNA technology resulted in the construction of many yeast strains that are genetically modified and able to hydrolyze starch and use their products in the production of ethanol (Steyn et al., 1992; Shibuya et al., 1992(a) and (b); Steyn and Pretorius, 1990; and de Moraes et al., 1995).

In the present study, the fermentation characteristics and the metabolic processes of the recombinant yeast strains were investigated in a batch fermenter to evaluate their performance for possible usage in large scale production of ethanol.

4.8.1. Preliminary Experiments

Batch fermentation experiments were carried out in the New Brunswick Bioflo III batch/continuous fermenter either in the presence or in the absence of oxygen by changing the initial starch concentrations of the YPS medium. The strain used was YPB-G.

The sterilization of the fermenter, and the calibration of the pH and dissolved oxygen probes were made as described in the Materials and Methods Section.

In the preliminary experiments, several chemicals were tested to keep the pH of YPS medium at 5.60. 10 mM $CaCl_2$ (pH=11.00), 0.1M $CaCl_2$ (pH=8.30), 1M Na-Acetate (pH=12.00) and 2.5 M Na-Acetate (pH=11.00) were the buffers tested. It was observed that the necessary amounts of these buffers were remarkably high causing changes in the concentrations of the components in the medium. Finally, 3% (w/v) succinic acid (pH=2.30) and 12.5 % (v/v) NH_4OH (pH=11.70) buffer system worked well. The addition of NH_4OH also reduced the risk of contamination of the culture since NH_4OH was known to be a strong antiseptic.

The fermenter was first tried to be operated at "DO Active" Mode by setting the dissolved oxygen (DO) to 30% of saturation automatically adjusted with a variable mixing rate from 25 to 500 rpm. However, the system failed to operate at the "DO Active" mode; therefore, the experiments with oxygen supply were carried out at "DO Passive" mode at constant 400 rpm, which presented some difficulty in keeping the dissolved oxygen level constant. The oxygen was supplied with an air tube connected to the rotameter at the fermenter inlet. The air was filter-sterilized by a 0.2 μm replaceable cartridge filter. A silicone antifoaming agent was used at 1:10 dilution with sterile dH_2O.

The medium was sterilized separately in a 6L flask, since the sterilization times of both the fermenter and the medium were different. The medium was cooled down to 50°C and then poured into the fermenter aseptically. This procedure caused a loss of starch in the medium, because it was precipitated and could not be totally withdrawn from the flask into the fermentation vessel. This decreased the reproducibility of the experimental conditions, and caused considerable changes even when the same concentration of starch medium was prepared.

The temperature was kept constant at 30°C, by cooling the system with an external circulation of tap water through the coils. The change in the flowrate of the tap water also failed to keep the temperature constant. This problem was overcome by using a Heto-waterbath having external circulation via a pump from a waterbath at 18°C.

4.8.2. Experiments with and without Oxygen Supply

The experiments were conducted in the Bioflo III fermenter operated in the batch mode either by changing the initial starch concentrations from 5g/L to 26g/L with an air supply of 0.5 L/min or by changing the initial starch concentrations from 5g/L to 50g/L without an air supply.

Time course of biomass formation, starch utilization, glucose and reducing sugar accumulation, α-amylase and glucoamylase excretion, protein concentration, intracellular RNA, plasmid DNA concentrations and ethanol production were followed in all cases.

4.8.2.1 Growth Pattern. **Figures 4.53 (a) - 4.57 (a)** show the growth pattern of YPB-G strain on YPS media with 5, 10, 17, 20 and 26 g/L initial starch concentration under aerobic conditions. Corresponding carbon source utilization graphs are in **Figures 4.53 (b)** through **4.57 (b)**. The experiments have shown that the biomass formation increased with increasing substrate (starch) concentrations. 4.55 g/L and 11.70 g/L of biomass formations were obtained with 5 g/L and 26 g/L initial starch containing media respectively. The maximum specific growth rates (μ_{max}) were calculated as 0.098 ± 0.01 hr^{-1} up to 20 g/L of initial starch but decreased to 0.030 ± 0.01 hr^{-1} at 26 g/L initial starch (**Table 4.14**).

TABLE 4.14. Summary of Aerobic Fermentation

Initial Starch Conc (g/L)	μ_{max}±0.01 (h^{-1})	Final Biomass Concentration (g/L)	Max. Accumulation of Glucose (g/L)	Max. Accumulation of Reducing Sugars (g/L)
5	0.098	4.55	0.16	2.27
10	0.099	6.01	0.17	2.02
17	0.098	6.34	0.23	2.88
20	0.088	7.50	0.22	2.13
26	0.033	11.70	1.72	9.70

Similarly, **Figures 4.58 (a)-4.62 (a)** show the growth pattern of YPB-G strain on YPS media with 5, 20, 26, 36 and 50 g/L initial starch concentrations under anaerobic conditions. The absence of oxygen supply to the medium was termed *anaerobic*. Corresponding carbon source utilization graphs are in **Figures 4.58 (b)** through **4.62 (b)**. In these experiments, final biomass formation increased with increasing substrate concentration from 1.50 g/L to 6.00 g/L in 5 g/L and 50 g/L initial starch containing media

respectively. The maximum specific growth rates (μ_{max}) were calculated as 0.030 ± 0.01 hr^1 in all initial starch concentrations (**Table 4.15**).

TABLE 4.15. Summary of Anaerobic Fermentation

Initial Starch Conc (g/L)	μ_{max}±0.01 (h^{-1})	Final Biomass Concentration (g/L)	Max. Accumulation of Glucose (g/L)	Max. Accumulation of Reducing Sugars (g/L)
5	0.025	1.50	0.25	3.20
20	0.032	2.00	0.14	1.94
26	0.023	3.80	0.66	5.16
36	0.030	3.50	0.48	3.94
50	0.030	6.00	2.49	7.45

In general, starch was completely degraded when the cell growth was approximately at its mid-exponential phase under aerobic conditions. Under anaerobic conditions, complete starch utilization was observed in the late-exponential or even in the stationary phase. The experiments were run about 2 days up to 20 g/L and 5 days for other initial starch concentrations in both aerobic and anaerobic fermentation experiments. Reducing sugars and glucose were still observed in the medium at the end of 2 days of operation in both cases.

4.8.2.2 Dissolved Oxygen. It was also interesting to follow the change of dissolved oxygen concentration with time in the medium. It has been reported that a trace amount of oxygen stimulate yeast fermentation, improves ethanol tolerance of the yeast and is required for yeast growth as a building block for the biosynthesis of polyunsaturated fats and lipids required in mitochondrion and plasma membrane (which are expected to influence the ethanol tolerance of yeast) (Thatipalama et al., 1992).

The dissolved oxygen in the system was measured with a polarographic DO probe which was calibrated by the dynamic gassing out method as described in Section 3.2.6.5 prior to use. The starch concentration in the medium affected the solubility of oxygen in

the system. At low starch concentrations, the solubility was more or less the same as the solubility of oxygen in water (at 100% of saturation, the solubility of oxygen in dH_2O is 1.16 mmol/L (Bailey and Ollis, 1986) at 30°C). It has been also observed that changes in sugar concentrations are primarily responsible for changes in oxygen solubility during the course of alcoholic fermentation of glucose and xylose while the inclusion of 1 g/L antifoam does not change oxygen solubility in the medium (Slininger et al., 1989). But at higher starch concentrations, this value was reduced to ca. 70% of saturation.

The dissolved oxygen has displayed a monotonically decreasing trend followed by an increase during the aerobic batch fermentation. The lowest values of DO were observed at the mid-exponential phase of growth where the cells need the highest amounts of oxygen for their growth (**Figure 4.53 (a)-4.57 (a)**). It was reported in the literature that in batch cultures, the maximum oxygen uptake rate was reached when the cells were properly adapted (Sonnleitner and Kappeli, 1986).

When the minimum levels of oxygen in the medium are compared, it was observed that this value was higher at lower starch concentrations, than at higher starch concentrations. This is due to the initial solubility differences of oxygen in starch containing media. The solubility showed a decreasing trend with increasing starch concentration. The oxygen uptake rates ($q_{O_2}x$) were calculated from the slopes of Co_2 versus time plots assuming no nutrient limitation (**Table 4.16**).

TABLE 4.16. Oxygen Uptake Rates

Initial Starch Concentration (g/L)	Time Interval (h)	Oxygen Uptake Rate $-dCo_2/dt$ (mmole O_2 / L / h)
5	15-20	0.093
10	11-20	0.032
17	2-18	0.027
20	2-14	0.016
26	8-22	0.0083

The oxygen uptake rates ($q_{o2}x$) decreased with increasing initial starch concentrations (**Table 4.16**). Due to the increase in the viscosity of the medium with increasing initial starch concentration, mass transfer coefficient (k_l) might decrease which could cause oxygen linitation. In the literature, aerobic metabolism of all yeast cells is determined by the relative sizes of the transport rate of sugar into the cell and the transport rate of respiratory intermediates into the mitochondria. If the rate of sugar uptake exceeds the rate of transport of respiratory intermediates into the mitochondria, the metabolism exhibits the features of ethanol excretion accompanied by a decrease in the specific oxygen uptake rate. If the transport rates of sugar and respiratory intermediates are the same rate, the metabolism is characterized by little or no ethanol excretion and a much higher oxygen uptake rate (Sonnleitner and Kappeli, 1986). In the present case, it was difficult to calculate the glucose uptake rate due to the presence of starch and other reducing sugars.

4.8.2.3 Secretion of α-amylase and glucoamylase. The time profiles of α-amylase and glucoamylase activities in the medium are presented in **Figures 4.53 (c) - 4.57 (c)** for aerobic fermentation and **Figures 4.58 (c) - 4.62 (c)** for anaerobic fermentation. Both α-amylase and glucoamylase activities have displayed a similar trend with each other, both of them either increased or decreased with time in the same manner. The activities and the specific activities of the enzymes obtained at different initial starch concentrations are listed in **Table 4.17** and **4.18**, based on the final operation time, under aerobic and anaerobic conditions respectively.

The protein concentration profiles are presented in **Figures 4.53 (d) - 4.62 (d)**. It was observed that the extracellular protein concentrations increased with an increase in the initial starch concentration from 34.64 mg/L to 47.32 mg/L at 5 g/L and 26 g/L initial starch in case of aerobic fermentation (**Table 4.19**). Also, the activities and specific activities of both enzymes decreased with increasing initial starch concentrations (**Table 4.17** and **4.18**).

TABLE 4.17. Summary of Enzyme Secretion in Aerobic Fermentation

Initial Starch Concentration (g/L)	α-amylase Activity (U/mL)	Specific α-amylase Activity (U/g protein)	Glucoamylase Activity (U/mL)	Specific Glucoamylase Activity (U/g protein)
5	3227	93.15	311	8.96
10	545	12.61	47	1.09
17	548	12.74	47	1.09
20	850	18.88	95	2.11
26	353	7.46	35	0.74

TABLE 4.18. Summary of Enzyme Secretion in Anaerobic Fermentation

Initial Starch Concentration (g/L)	α-amylase Activity (U/mL)	Specific α-amylase Activity (U/g protein)	Glucoamylase Activity (U/mL)	Specific Glucoamylase Activity (U/g protein)
5	829	16.88	670	13.67
20	644	16.17	71	4.46
26	672	10.28	82	1.25
36	405	12.39	11	0.34
50	365	7.89	59	1.27

The reason for the activity loss with respect to starch concentration might be due to the change in the medium composition, since other environmental parameters such as temperature and pH were constant throughout the experiments, or due to the repression of the promoter at higher starch concentrations, or due to the blocking of the secretory mechanism. It is reported that, the construction of YPB-G strain is such that it secretes 96% of the total α-amylase and glucoamylase enzymes (in a 1:3 ratio) into the medium (de Moraes et al., 1995) and since the activity of α-amylase was higher than glucoamylase activity, the accumulation of the reducing sugars in the medium was unavoidable. It is also

remarked in the literature that *Saccharomyces cerevisiae* normally secretes only a very small amount (0.5%) of its own proteins to the medium which is advantageous for product recovery (Das and Shultz, 1987). Similar trends were observed for anaerobic fermentation.

In **Table 4.19**, specific protein concentrations (mg protein per g of cell dry weight) of aerobic and anaerobic fermentations are compared. Higher levels of specific protein concentrations were achieved in anaerobic fermentation, whereas, a decreasing trend in the final specific protein concentrations was observed in both modes of operation with an increase in the initial starch concentration which supports the trend in enzymes activities.

4.8.2.4 Intracellular RNA Concentration. Most classical measurements of biochemistry provide population averaged and thus unsegregated data on the cell population. Measurements of this type can be extended to a very large number of cellular constituents, even to the level of particular proteins, RNA and DNA (chromosomal and plasmid) molecules. Determination of total protein, total RNA content, total DNA content and other average macromolecular content of the cells can be accomplished by well-established methods (Bailey and Ollis, 1986). It is well-known that the intracellular RNA content of many microorganisms increases almost linearly with the specific growth rate (Nielsen et al., 1989). Since RNA constitutes a considerable part of the protein synthesizing system (60%) whose size is a potentially limiting factor for growth, one can expect that the relative size of an active part of the cell can be measured through RNA, assuming the relative size of the different species constant, at all operating conditions (Nielsen et al., 1989).

Throughout the experiments, RNA concentration of the cells were measured by the ethanolic orcinol method as described in Section 3.2.5.9 from the samples taken at regular time intervals. **Figures 4.53 (e) - 4.62 (e)** show the time profiles of RNA concentration at different initial starch concentrations for aerobic and anaerobic fermentations. The behavior of the curves were very similar to the growth pattern of the cells. RNA concentrations increased with time while the cells followed their exponential phase of growth and then leveled off when the cells reached their stationary phase. The amount of RNA is also dependent on the cell mass concentration. At higher initial starch

concentrations, higher RNA concentrations were achieved, just like in the case of biomass concentrations. The amounts of RNA were almost 10% of the cell dry weight, which is in agreement with the RNA concentrations reported as 8-18% of the cell mass by Nielsen et al. (1989).

TABLE 4.19. A Comparison of Specific Protein Concentrations

Aerobic Fermentation			Anaerobic Fermentation		
Initial Starch Concentration (g/L)	Protein Concentration (mg/L)	Specific Protein Concentration (mg/g cell)	Initial Starch Concentration (g/L)	Protein Concentration (mg/L)	Specific Protein Concentration (mg/g cell)
5	34.64	7.61	5	49.11	32.74
10	43.21	7.18	20	39.82	19.91
17	43.00	6.78	26	65.35	17.20
20	45.00	6.00	36	32.67	9.33
26	47.32	4.04	50	46.25	7.71

Table 4.20 summarizes the final RNA concentrations in g/L and g RNA/ g cell dry weight for both case.

TABLE 4.20. A Comparison of RNA Concentrations

Aerobic Fermentation			Anaerobic Fermentation		
Initial Starch Concentration (g/L)	RNA Concentration (g/L)	Specific RNA Concentration (g/g cell)	Initial Starch Concentration (g/L)	RNA Concentration (g/L)	Specific RNA Concentration (g/g cell)
5	0.60	0.131	5	0.25	0.166
10	0.70	0.116	20	0.34	0.170
17	0.68	0.107	26	0.42	0.110
20	0.75	0.100	36	0.36	0.103
26	0.98	0.084	50	0.70	0.116

4.8.2.5 Plasmid Stability. The determination of plasmid DNA concentration within the cell is an important indication of the plasmid stability of recombinant strains. During the experiments, samples withdrawn from the culture medium at regular time intervals were tested for plasmid DNA concentration as described in Section 3.2.5.10. Although the technique required complex analytical procedures compared to the replica plating technique, the sensitivity was higher. In the replica plating technique, only a portion (above 100 single colonies) out of a total of 250 colonies were transferred onto the other agar-plate, which brings an additional probabilistic approach, although it was easier to manipulate. However, the use of an analytical technique for the measurement of plasmid DNA concentration constitutes a more deterministic approach.

The plasmid DNA concentrations increased with time (**Figures 4.53 (f) - 4.62 (f)**) as the biomass concentration increased but their g plasmid DNA per g dry cell weight equivalents (named as specific plasmid DNA concentrations) decreased. These values also decreased with increasing initial starch concentration in both fermentation systems. **Table 4.21** summarizes the final plasmid DNA concentrations in g/L and g plasmid DNA / g cell dry weight for both cases.

TABLE 4.21. A Comparison of Plasmid DNA Concentrations

Aerobic Fermentation			Anaerobic Fermentation		
Initial Starch Concentration (g/L)	Plasmid DNA Concentration (g/L)	Specific Plasmid DNA Concentration (g/g cell)	Initial Starch Concentration (g/L)	Plasmid DNA Concentration (g/L)	Specific Plasmid DNA Concentration (g/g cell)
5	0.97	0.213	5	0.38	0.252
10	0.67	0.145	20	0.23	0.113
17	0.55	0.088	26	0.44	0.116
20	0.15	0.021	36	0.28	0.080
26	0.30	0.020	50	0.28	0.046

4.8.2.6 Ethanol Production. The ethanol production was also measured; however, only barely detectable amounts of ethanol were recorded (ca. 1g/L). This is most probably due

to the amount of initial starch concentration that could be achieved in the medium, since, control experiments have confirmed that the cells were able to produce ethanol when the level of glucose in the starch medium reaches a certain value (greater that 2.5 g/L). Therefore, a suitable pretreatment of the system would be necessary. Furthermore, the experiments in shake flasks (refer to Section 4.6) have shown that when the level of initial starch concentration reaches to 100 g/L, the YPB-G strain manages to convert this into 45 g/L of ethanol. Ethanol production was achieved by modifying the fermenter system Details of this experiment are given in the following section.

Modifications The fermenter vessel was charged with 5% (w/v) of starch medium. Commercial α-amylase and glucoamylase mixture (500 μL each) was added into the system. It was operated at 40°C and at a pH of 5.60 about 3 hours before inoculating with the cell culture. At certain time intervals, samples were withdrawn from the fermenter and analyzed for glucose and reducing sugar concentrations. When the level of glucose and reducing sugar reached 3.76 g/L and 13.93 g/L respectively, the system was cooled down to 30°C and when steady state was reached, inoculation of the cells was done. At the end of 30 hours of operation, ethanol began to accumulate in the fermenter and reached a level of 36 g/L (**Figure 4.63**). In order to confirm that ethanol was produced via YPB-G, an additional experiment was also performed with the host GRF18 *S. cerevisiae*. The same treatment was made with the commercial enzymes, and then, the medium was inoculated with the host strain grown until late exponential phase in YNB-G containing leucine. No ethanol was detected in the system which provided a confirmation on the modification.

4.8.3. Comparison of Aerobic and Anaerobic Fermentation

The Pasteur effect is the control exerted by respiration over glycolytic rate. Under conditions of high dissolved oxygen concentrations, fermentation of the sugar to ethanol is

inhibited. This effect is known as the Pasteur effect. A switch to aerobic conditions can be observed under those conditions (Oliver and Tuite, 1991).

When comparing the two modes of fermentation namely aerobic and anaerobic, the major difference observed was in the production of biomass: 4.22, 3.75 and 3.07 times higher biomass formations were achieved when the system was operated aerobically at 5, 20 and 26 g/L initial starch concentrations respectively. The maximum specific growth rates calculated from the slopes of lnx versus time plots of aerobic fermentation were three times larger than the maximum specific growth rates of anaerobic fermentation up to 20 g/L initial starch. At 26 g/L starch, however, the μ_{max} of both aerobic and anaerobic fermentations converged to the same value (0.033 ± 0.01 hr^{-1}).

The protein secretions were similar in both modes. Oxygen supply to the system seems to decrease the level of protein secretion efficiency. The specific protein concentrations were 32.74, 19.91 and 17.20 mg protein /g cell dry weight under anaerobic fermentation, while these values were reduced to 5.47, 6.00 and 4.04 mg protein /g cell dry weight under aerobic conditions at 5, 20 and 26 g/L initial starch concentrations respectively. It can be concluded that protein secretion increases in the absence of oxygen. The increases in specific protein concentrations did not reflect an increase in the α-amylase and glucoamylase activities. The activities were comparable in each case. This might be explained by the secretion efficiency. The proteins could have stayed within the cell or cell membrane (which we did not test) in aerobic fermentation, so the measured proteins in the culture supernatant might not be 96% as reported by de Moraes et al. (1995) of the total α-amylase and glucoamylase present within the cell, while the conditions for better secretion might have been provided by anaerobic fermentation. The same reasoning can also be used for the explanation of the accumulated reducing sugar and glucose concentrations. The results have displayed that at 20 g/L and 26 g/L of initial starch concentrations, 2.13 g/L and 8.70 g/L of reducing sugar accumulation and 0.22 g/L and 1.72 g/L of glucose accumulation respectively were observed in aerobic fermentation. In anaerobic fermentation, 1.94 g/L and 5.16 g/L of reducing sugars, and 0.14 g/L and 0.66 g/L of glucose were accumulated at 20 g/L and 26 g/L initial starch concentrations respectively. One might expect that at higher biomass concentrations, lower accumulations

of the sugars would be more likely. However, the situation was vice versa. Higher accumulation of the sugars were observed at higher biomass formations (aerobic), than at lower biomass formations (anaerobic), when all other parameters such as medium composition, enzyme activities, pH and temperature were kept constant. This might again be due to the accumulation of the enzymes within the cell or at the cell membrane which prevented to transfer of glucose and small sugar molecules into the cell.

The RNA concentrations were also similar in each case. At higher biomass concentrations, higher RNA concentrations were recorded keeping the level of RNA concentration at ca. 10% of the total cell dry weight regardless of the fermentation mode.

At higher initial starch concentrations, plasmid stability decreased more rapidly in aerobic fermentation. The plasmid stability was even lower at 26 g/L of initial starch in aerobic fermentation (0.020 g plasmid DNA / g cell dry weight) than at 50 g/L of initial starch in anaerobic fermentation (0.046 g plasmid DNA / g cell dry weight). When one compares g plasmid DNA per g cell mass, one should keep in mind that the percentage of plasmid-containing cells in the preculture is an important parameter. The differences observed in the specific protein concentrations could also be explained by plasmid instability. In fact, it has been reported by Porro et al. (1988) that the yield of heterologous protein production depends upon the level of O_2 concentration in the culture. Their findings suggest that the expression efficiency can be altered by increasing the specific growth rate, which is improved with the decrease of EtOH concentration produced. The extra energy can be supplied to the biosynthesis of the protein. Therefore, the recombinant yeast cells can be maintained at a stable level under conditions corresponding to higher dissolved oxygen. This might suggest a parametric study to improve the dissolved oxygen concentrations to keep them at higher levels; the dissolved oxygen concentration dropped to 40% of saturation levels which is known to be near the limiting concentration of dissolved oxygen (Oliver and Tuite, 1991) for the yeast cells.

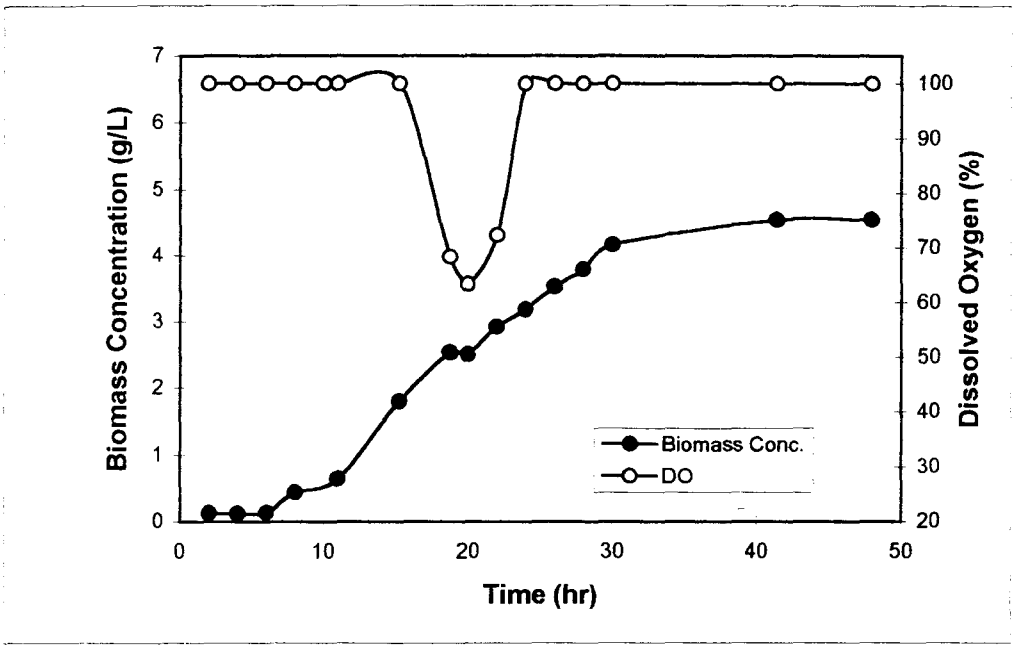


FIGURE 4.53. (a) Time Profiles of Biomass Formation and DO Concentration of YPB-G at 5 g/L Initial Starch under Aerobic Conditions

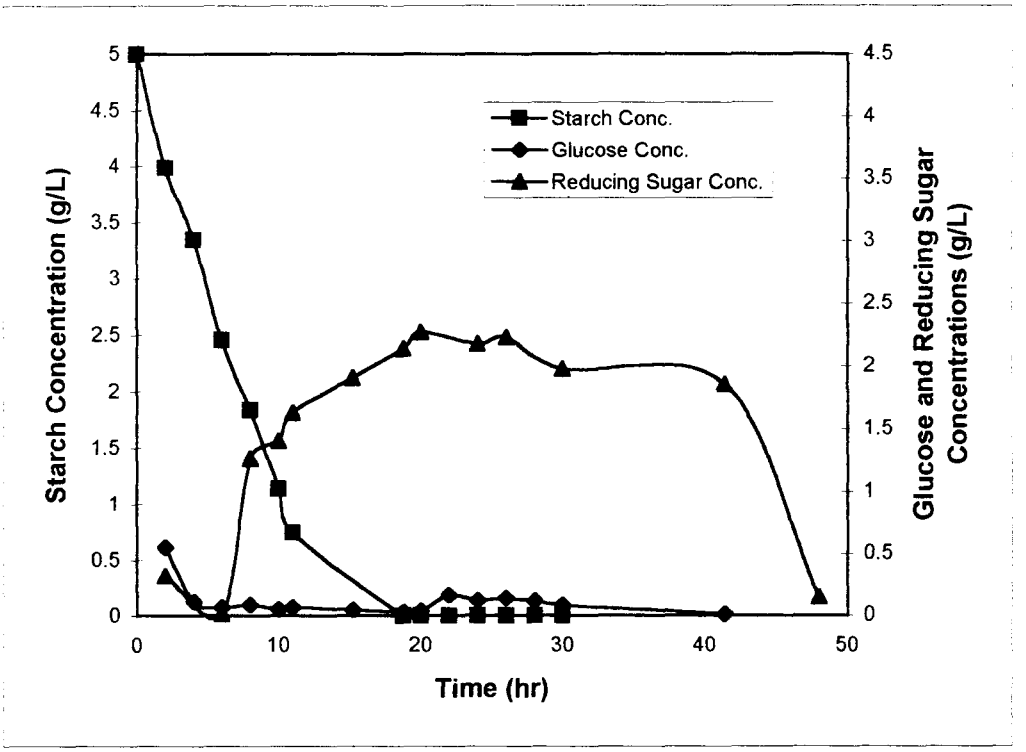


FIGURE 4.53. (b) Time Profiles of Substrate Utilization of YPB-G at 5 g/L Initial Starch under Aerobic Conditions

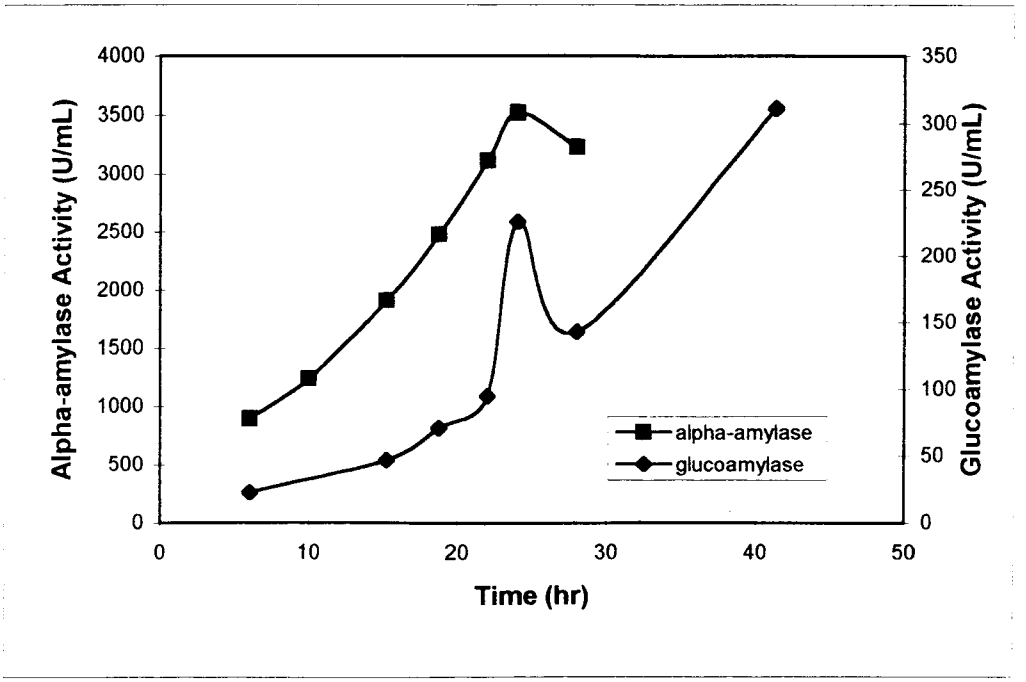


FIGURE 4.53. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 5 g/L Initial Starch Concentration under Aerobic Conditions

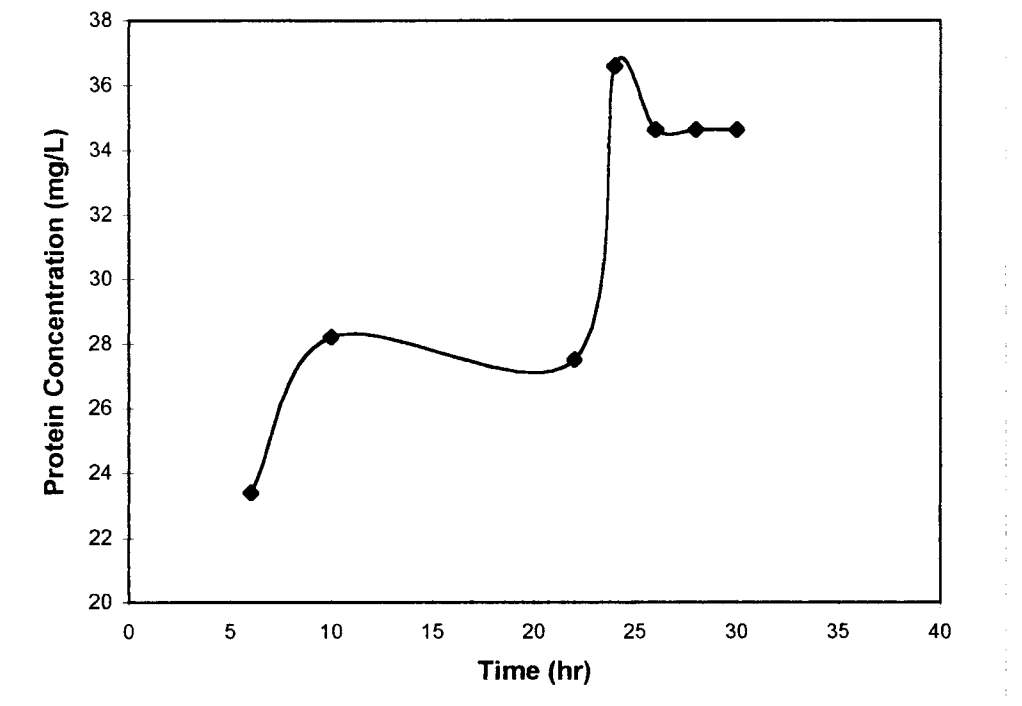


FIGURE 4.53. (d) Time Profiles of Protein Production of YPB-G Strain at 5 g/L Initial Starch Concentration under Aerobic Conditions

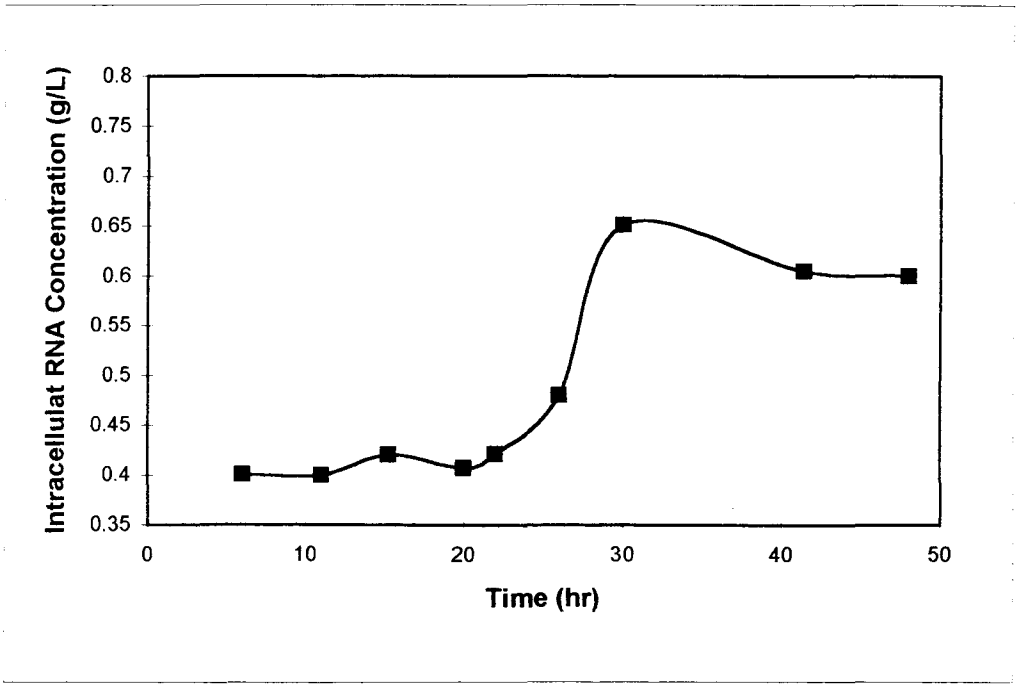


FIGURE 4.53. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 5 g/L Initial Starch Concentration under Aerobic Conditions

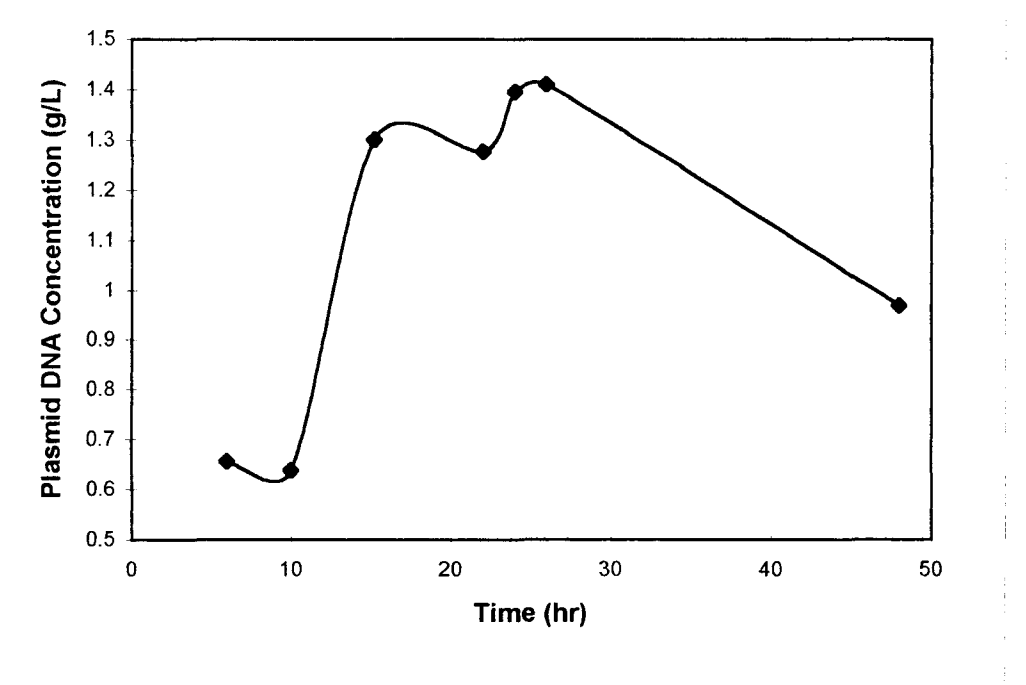


FIGURE 4.53. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 5g/L Initial Starch Concentration under Aerobic Conditions

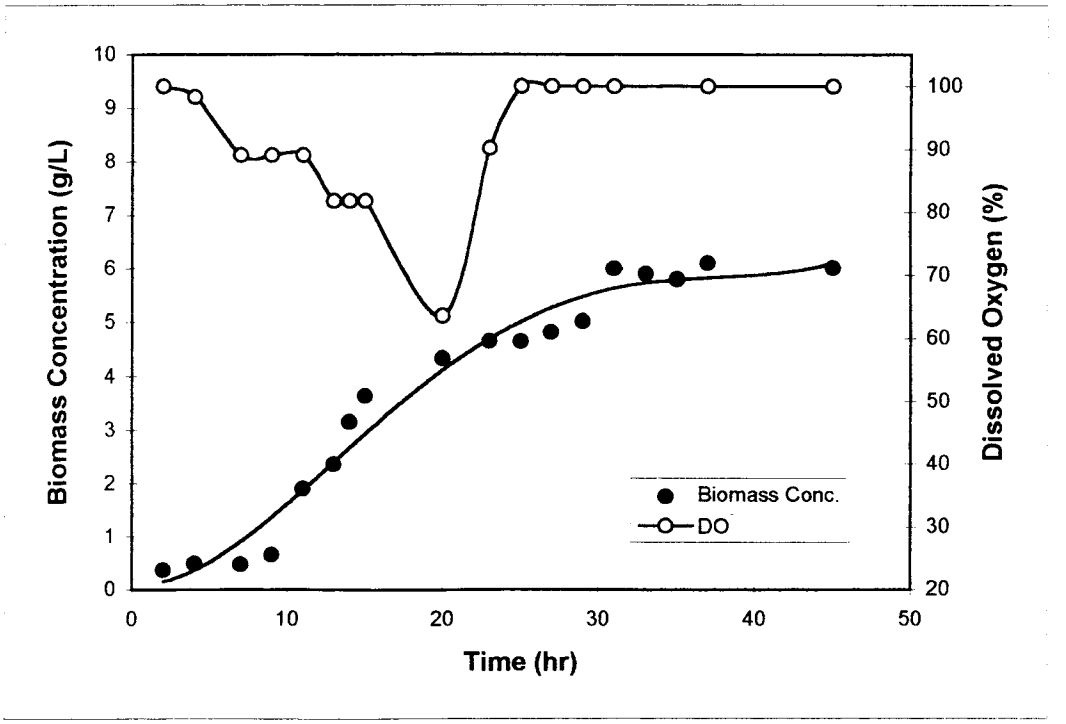


FIGURE 4.54. (a) Time Profiles of Biomass Formation and DO Concentration of YPB-G at 10 g/L Initial Starch under Aerobic Conditions

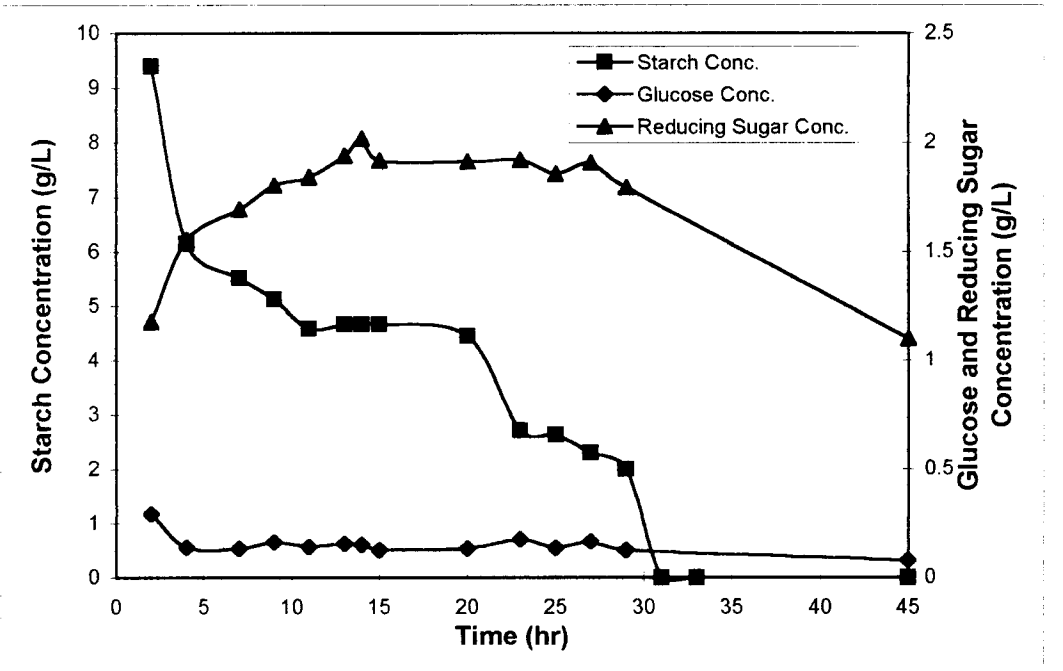


FIGURE 4.54. (b) Time Profiles of Substrate Utilization of YPB-G at 10 g/L Initial Starch under Aerobic Conditions

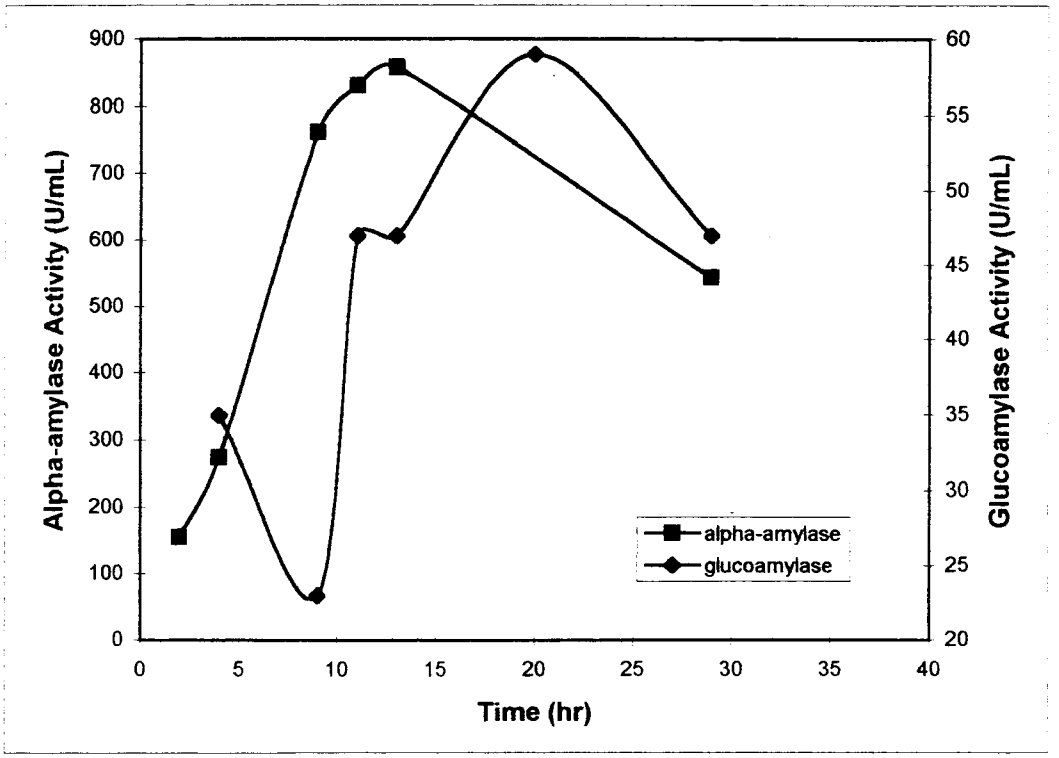


FIGURE 4.54. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 10 g/L Initial Starch Concentration under Aerobic Conditions

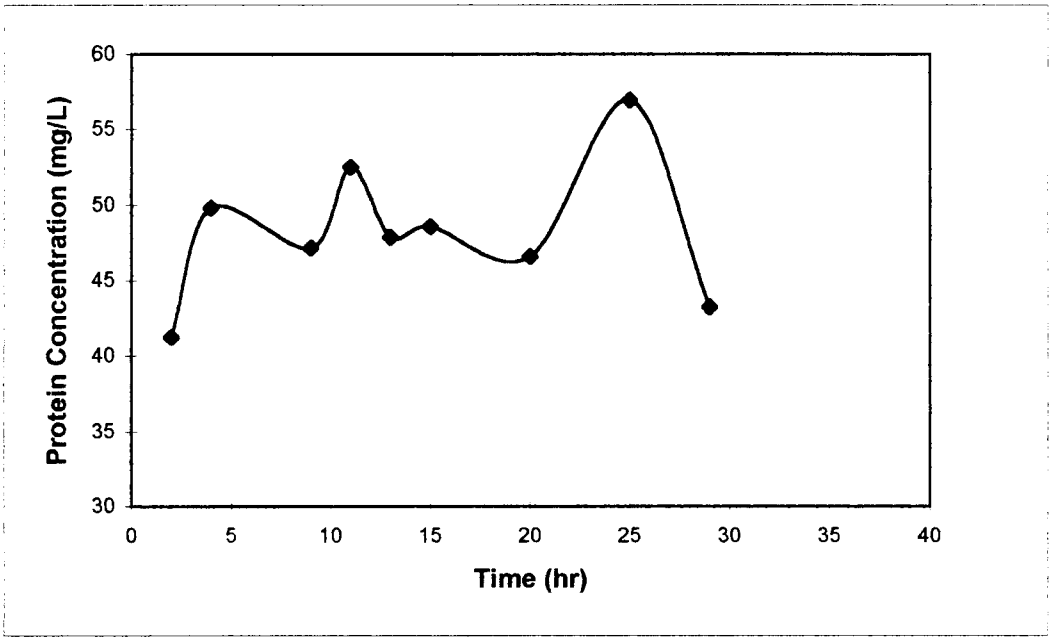


FIGURE 4.54. (d) Time Profiles of Protein Production of YPB-G Strain at 10 g/L Initial Starch Concentration under Aerobic Conditions

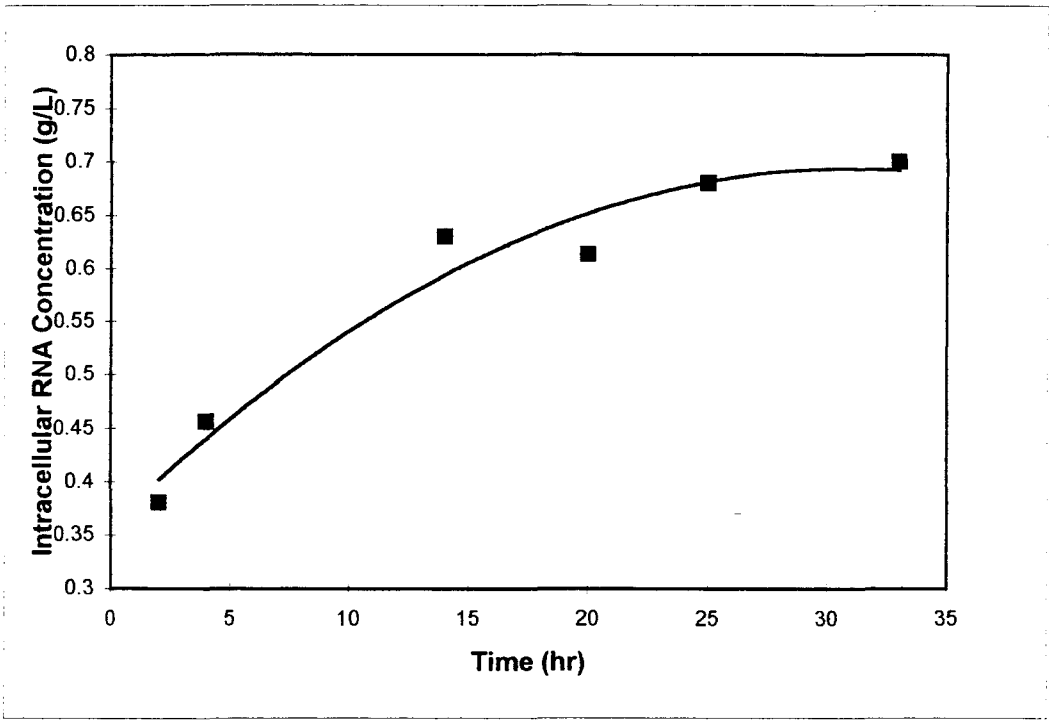


FIGURE 4.54. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 10 g/L Initial Starch Concentration under Aerobic Conditions

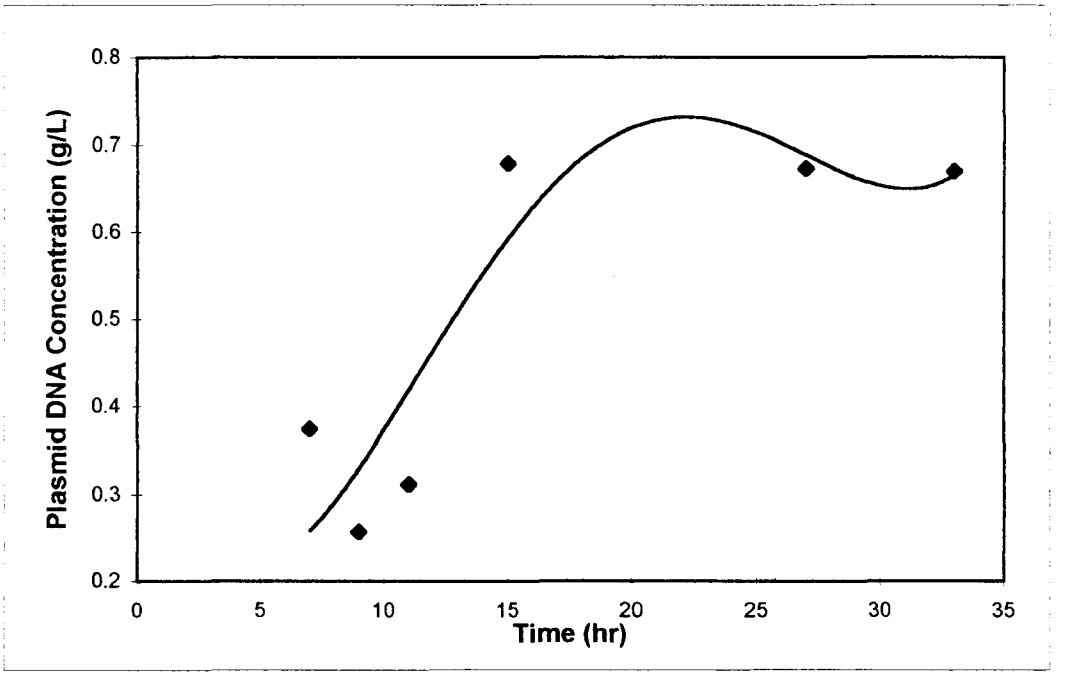


FIGURE 4.54. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 10g/L Initial Starch Concentration under Aerobic Conditions

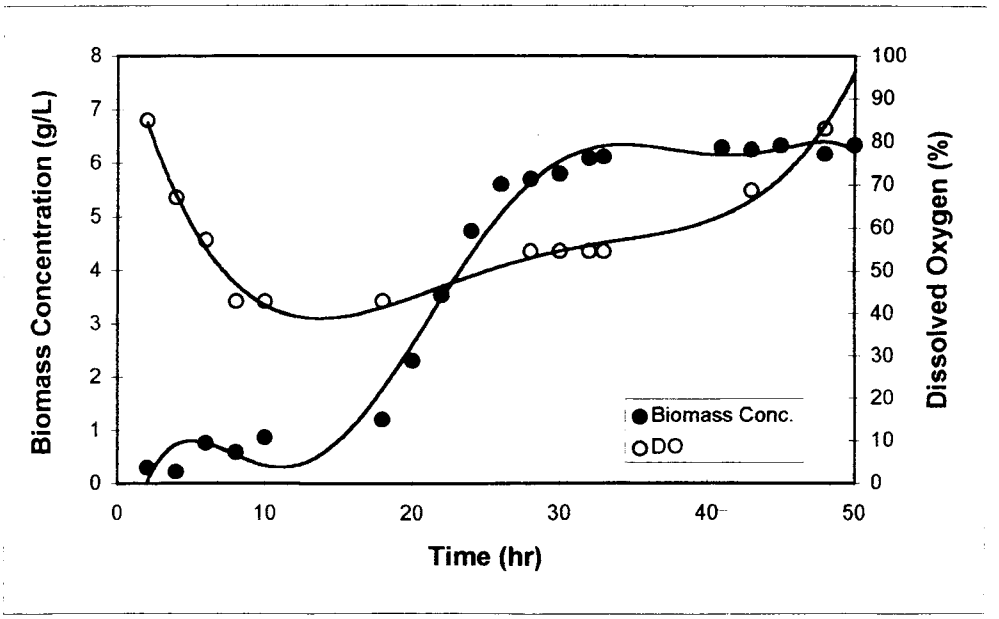


FIGURE 4.55. (a) Time Profiles of Biomass Formation and DO Concentration of YPB-G at 17 g/L Initial Starch under Aerobic Conditions

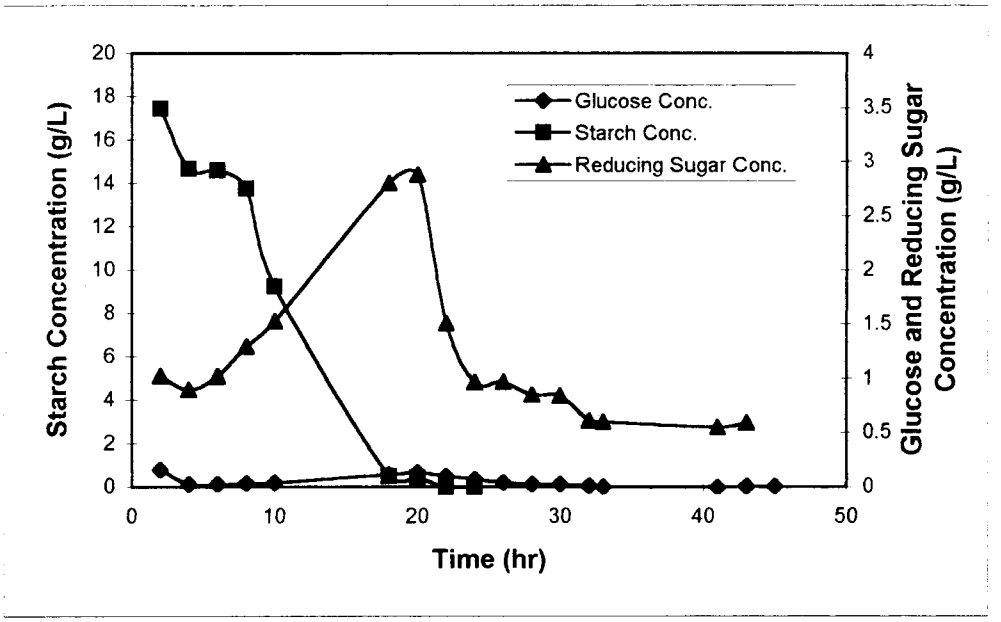


FIGURE 4.55. (b) Time Profiles of Substrate Utilization of YPB-G at 17 g/L Initial Starch under Aerobic Conditions

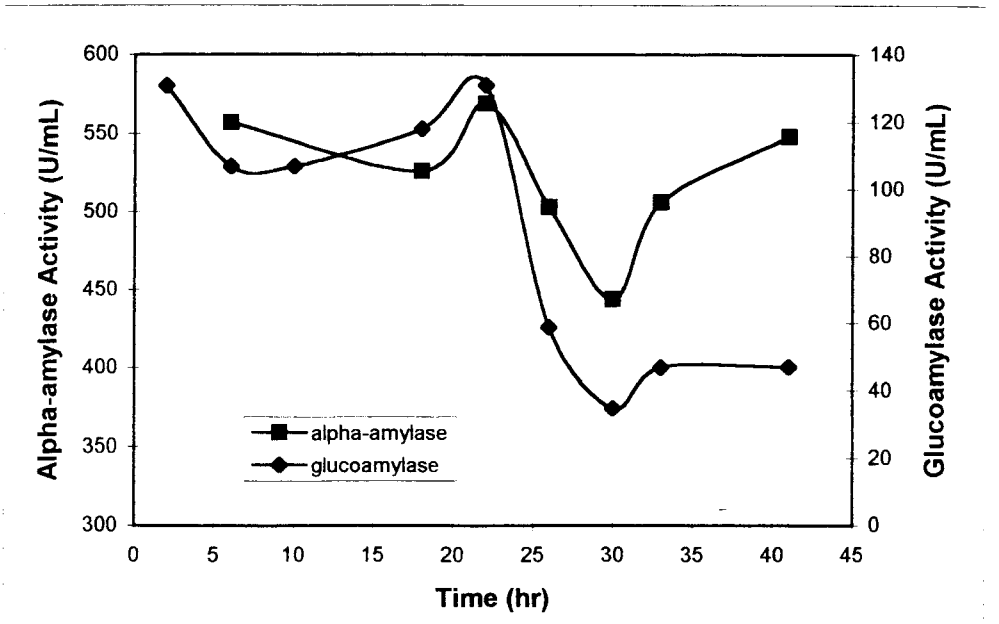


FIGURE 4.55. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 17 g/L Initial Starch Concentration under Aerobic Conditions

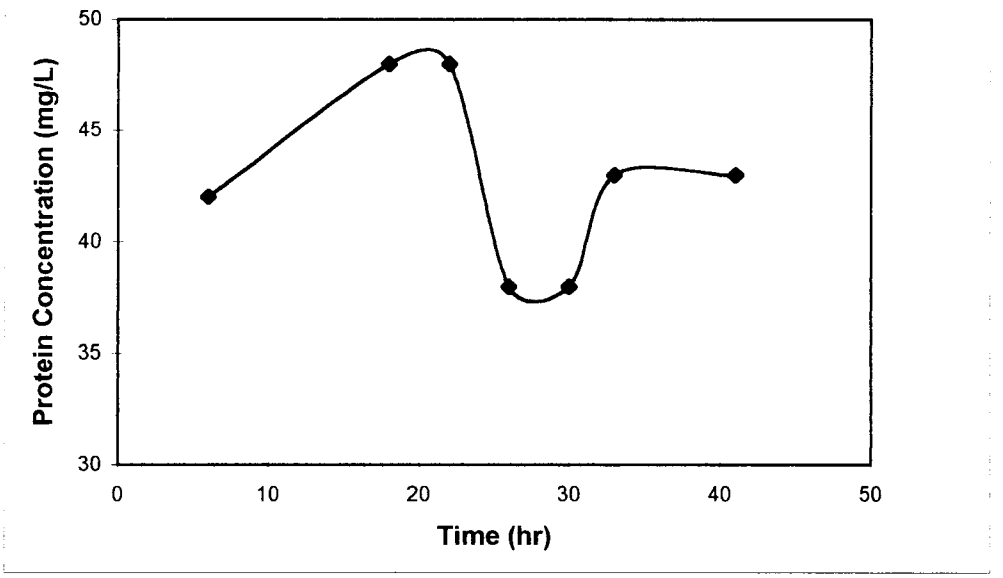


FIGURE 4.55. (d) Time Profiles of Protein Production of YPB-G Strain at 17 g/L Initial Starch Concentration under Aerobic Conditions

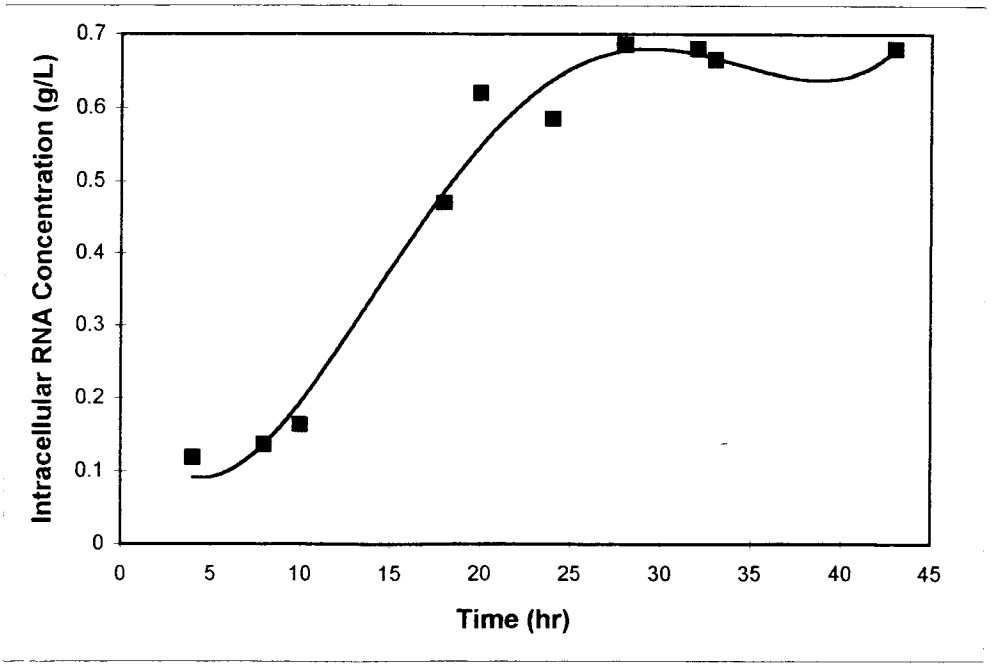


FIGURE 4.55. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 17 g/L Initial Starch Concentration under Aerobic Conditions

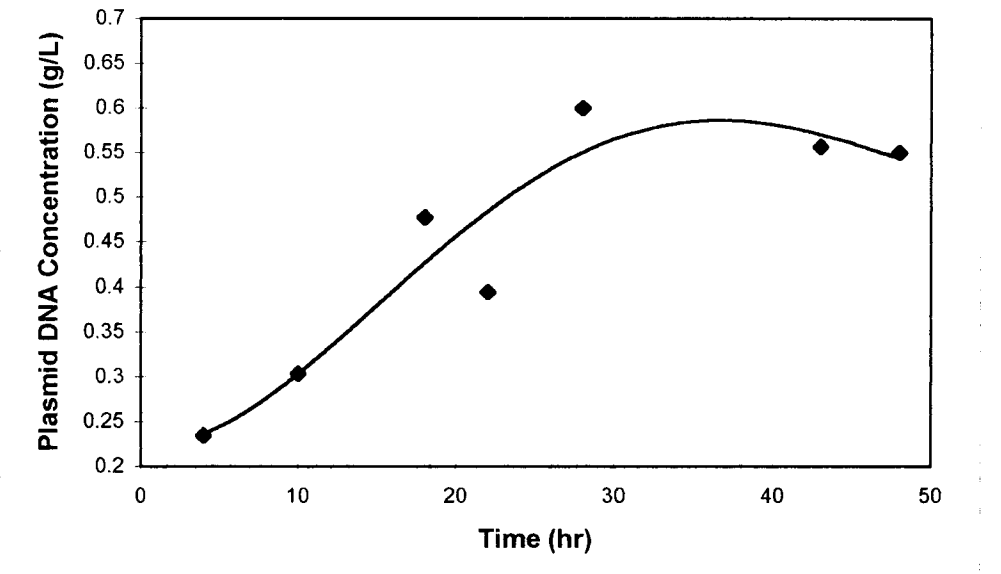


FIGURE 4.55. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 17g/L Initial Starch Concentration under Aerobic Conditions

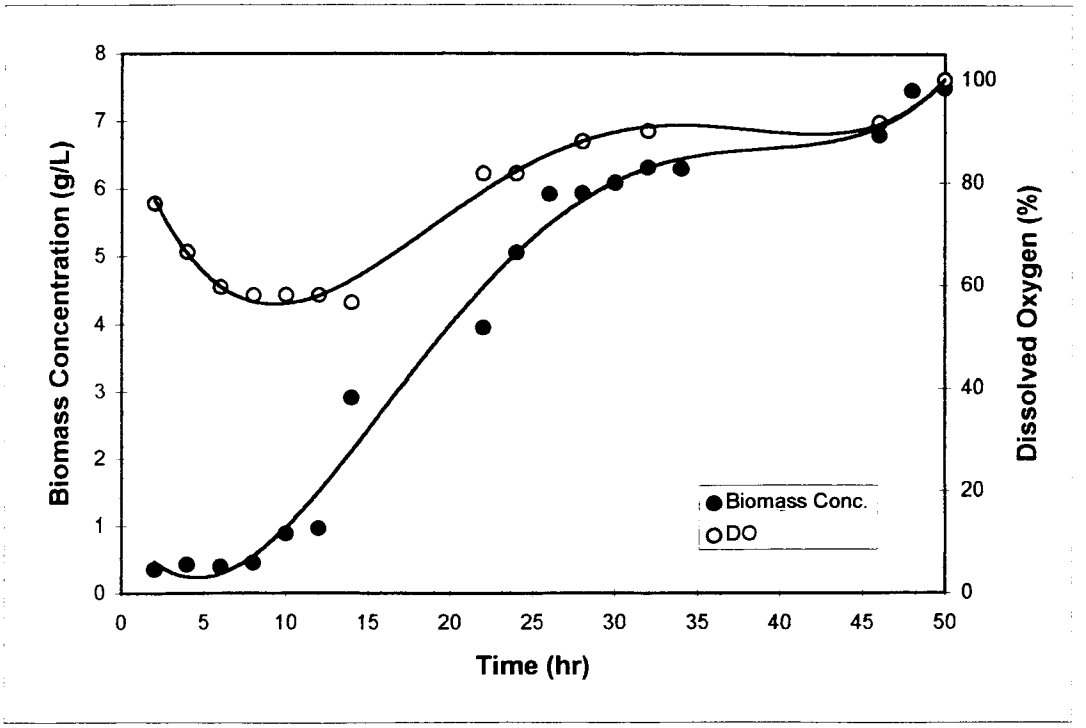


FIGURE 4.56. (a) Time Profiles of Biomass Formation and DO Concentration of YPB-G at 20 g/L Initial Starch under Aerobic Conditions

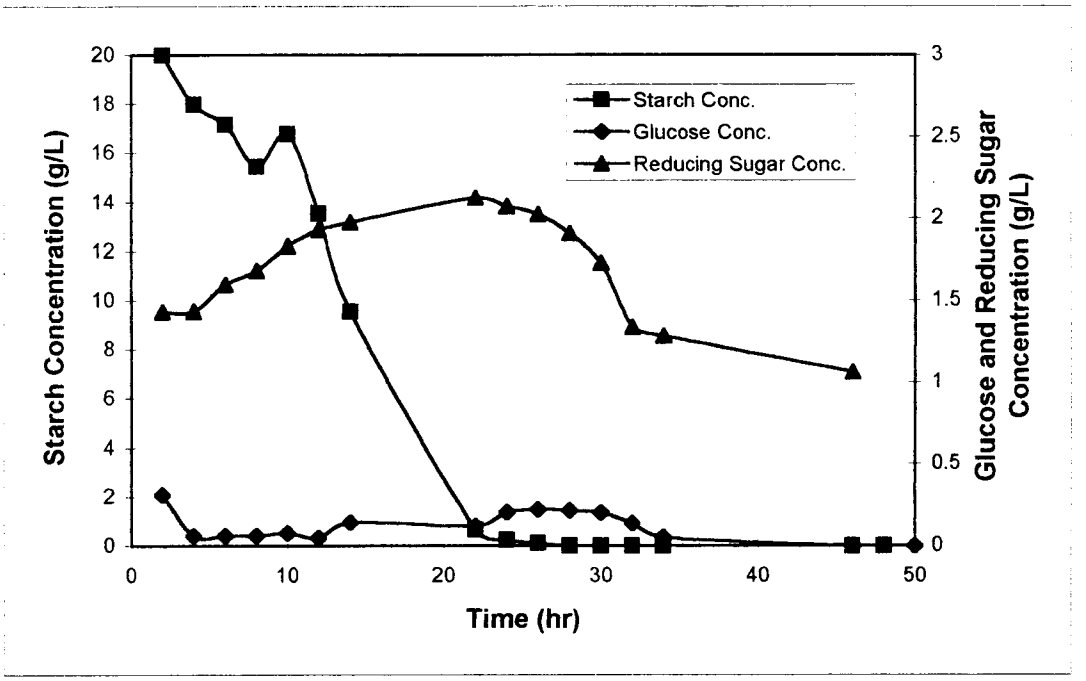


FIGURE 4.56. (b) Time Profiles of Substrate Utilization of YPB-G at 20 g/L Initial Starch under Aerobic Conditions

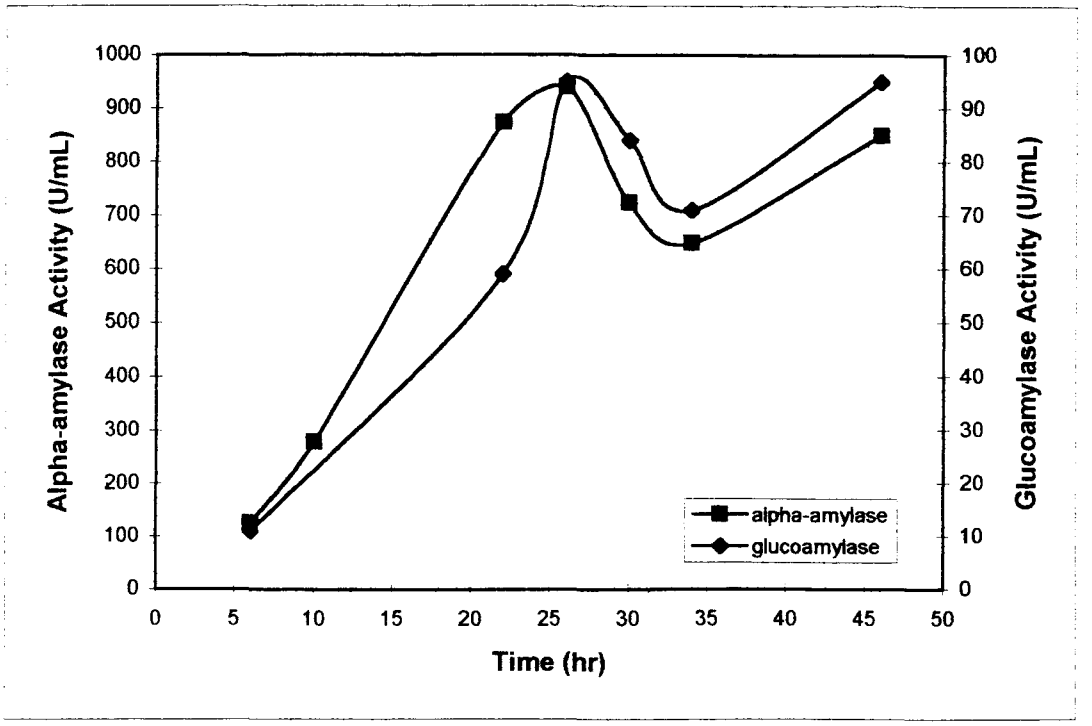


FIGURE 4.56. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 20 g/L Initial Starch Concentration under Aerobic Conditions

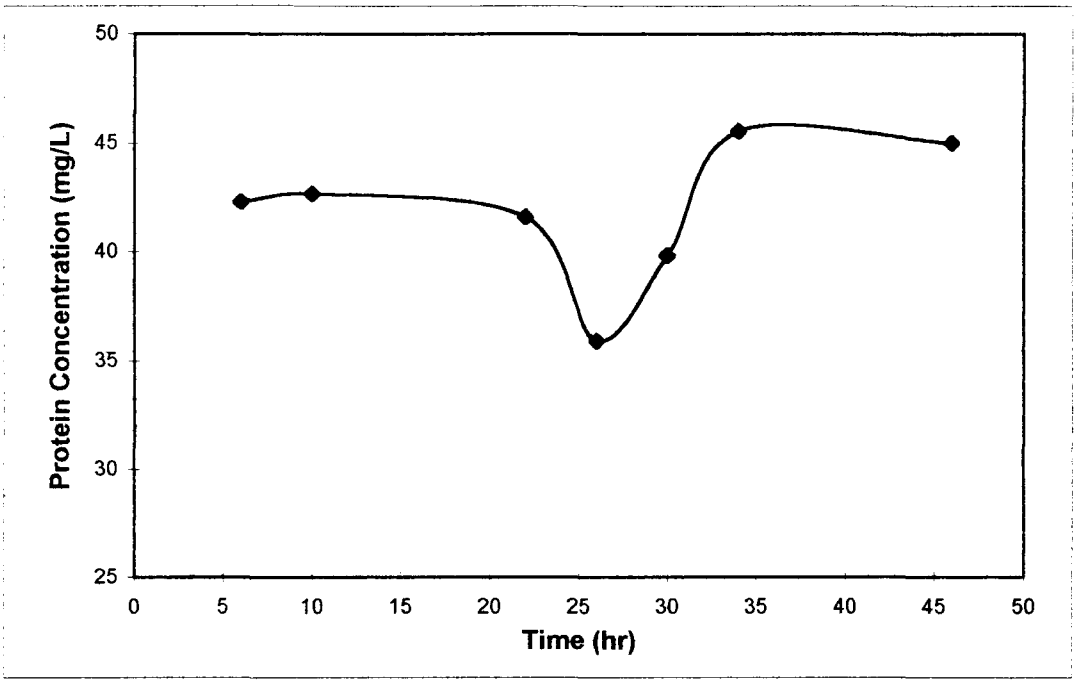


FIGURE 4.56. (d) Time Profiles of Protein Production of YPB-G Strain at 20 g/L Initial Starch Concentration under Aerobic Conditions

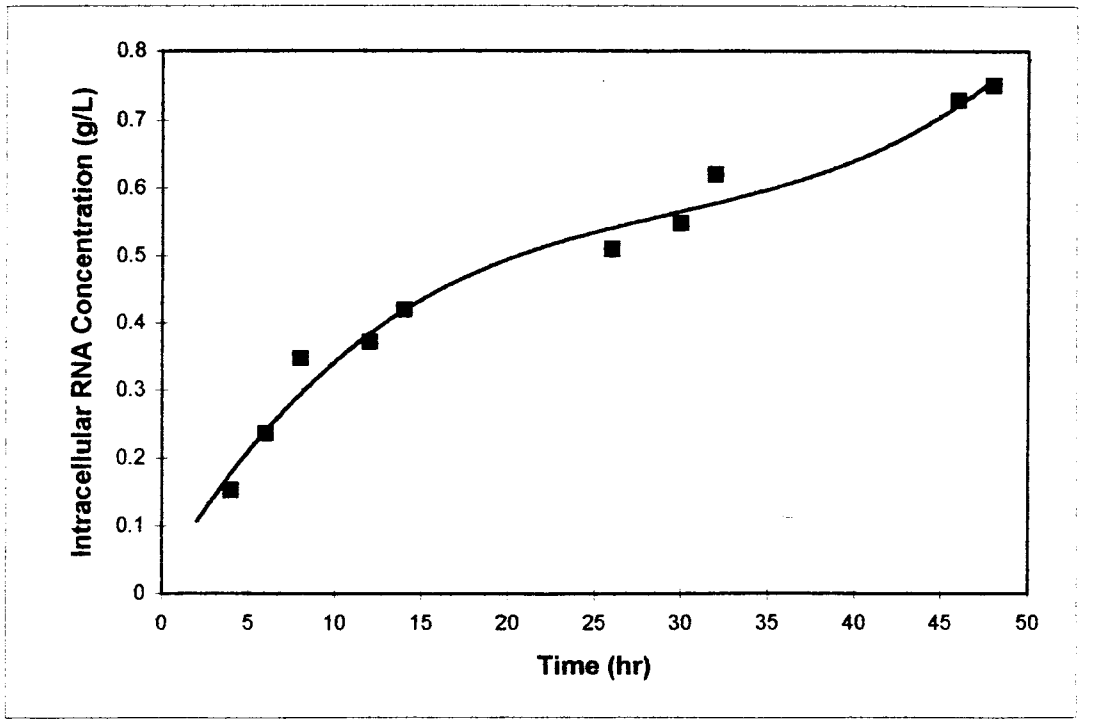


FIGURE 4.56. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 20 g/L Initial Starch Concentration under Aerobic Conditions

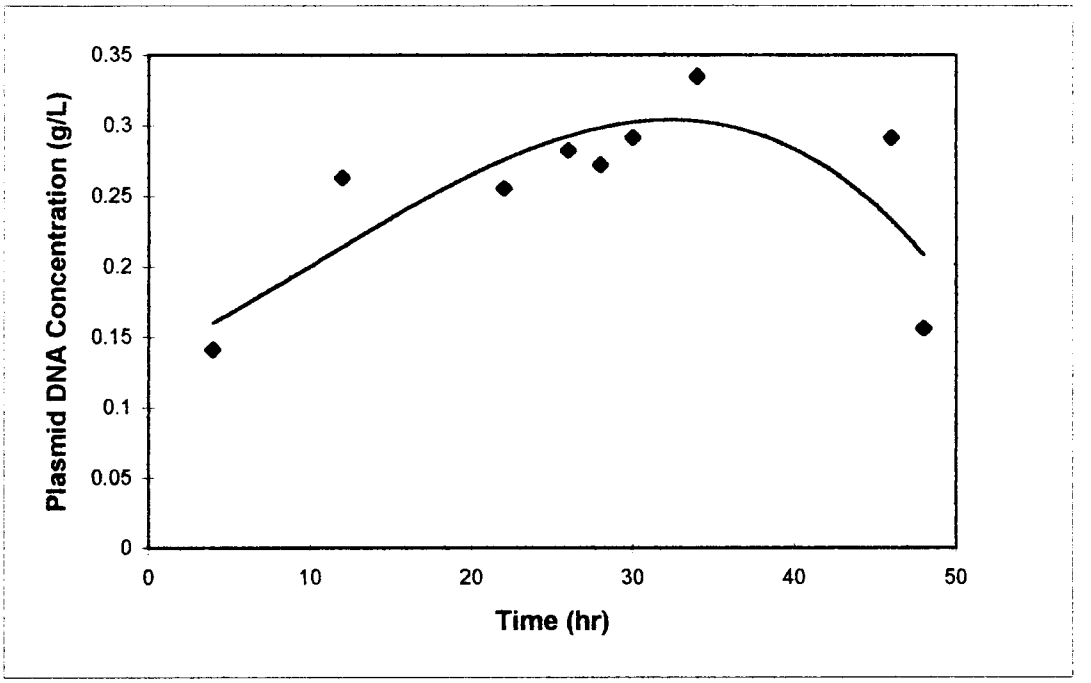


FIGURE 4.56. (f) Time Profiles of Time Course of Plasmid DNA of YPB-G Strain at 20 g/L Initial Starch Concentration under Aerobic Conditions

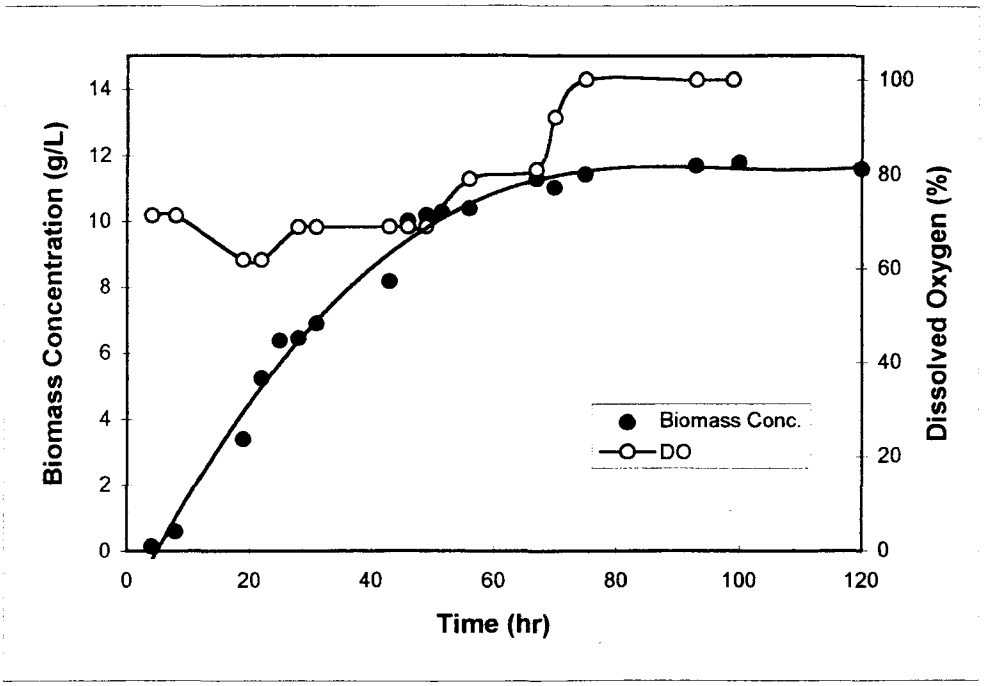


FIGURE 4.57. (a) Time Profiles of Biomass Formation and DO Concentration of YPB-G at 26 g/L Initial Starch under Aerobic Conditions

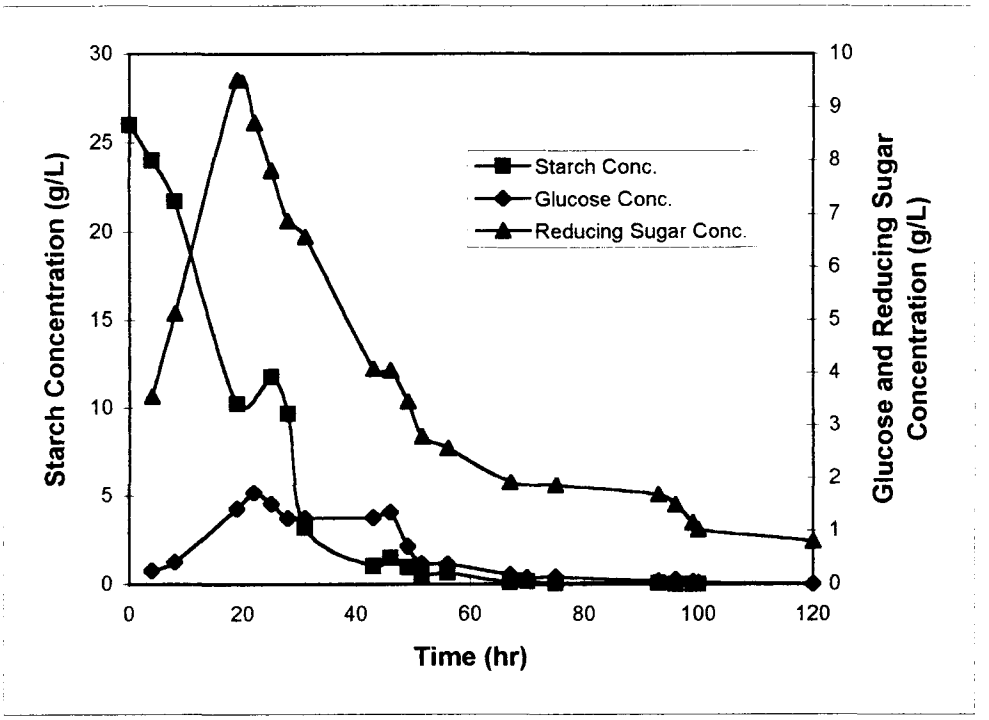


FIGURE 4.57. (b) Time Profiles of Substrate Utilization of YPB-G at 26 g/L Initial Starch under Aerobic Conditions

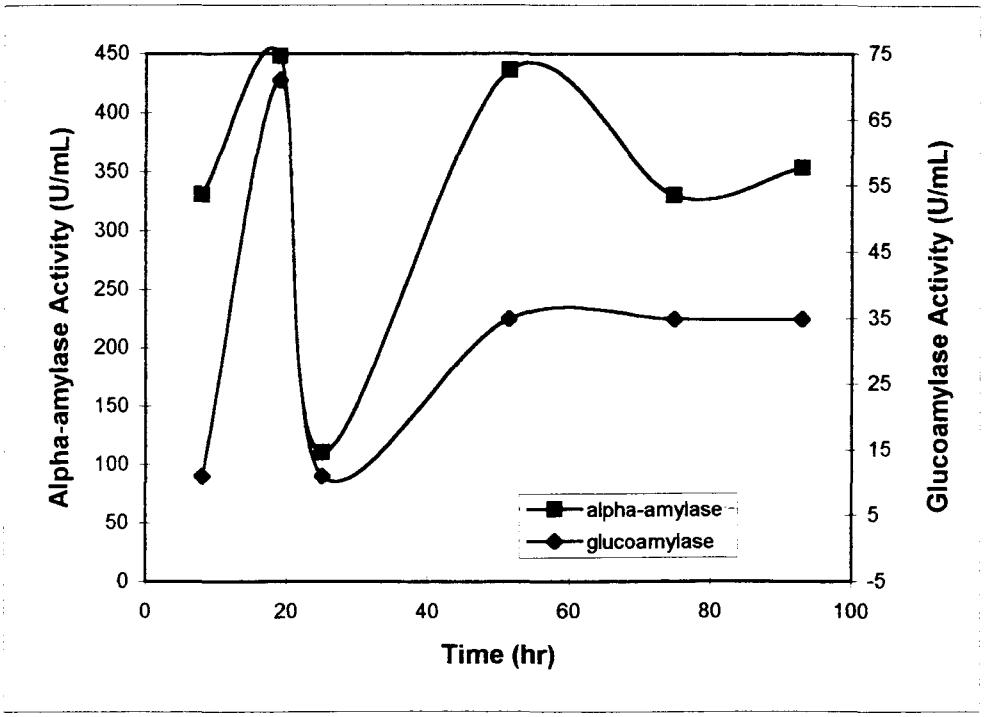


FIGURE 4.57. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 26 g/L Initial Starch Concentration under Aerobic Conditions

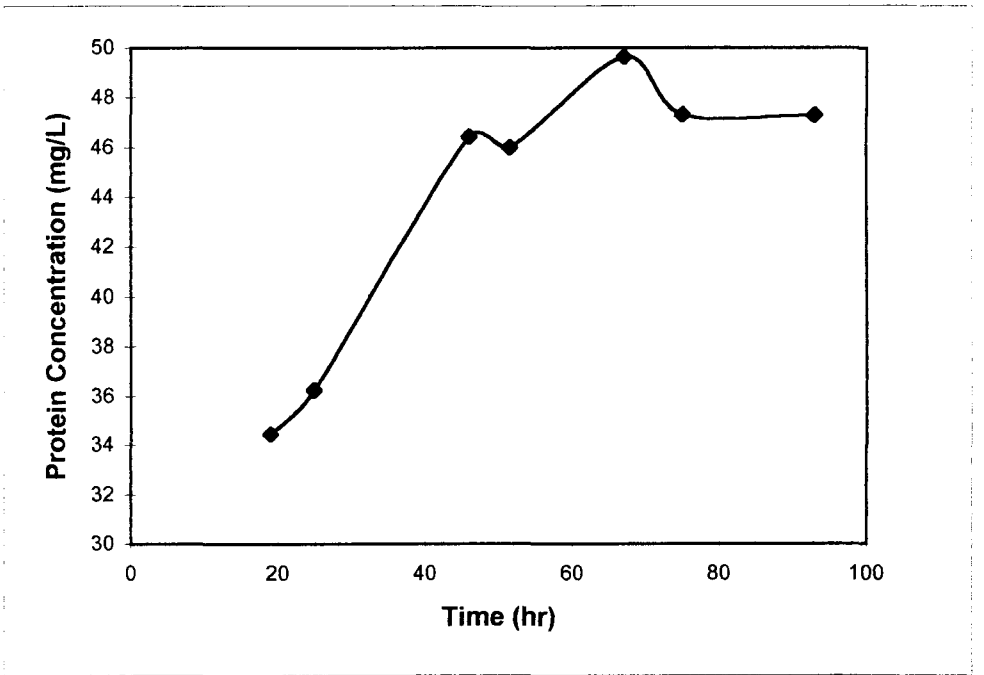


FIGURE 4.57. (d) Time Profiles of Protein Production of YPB-G Strain at 26 g/L Initial Starch Concentration under Aerobic Conditions

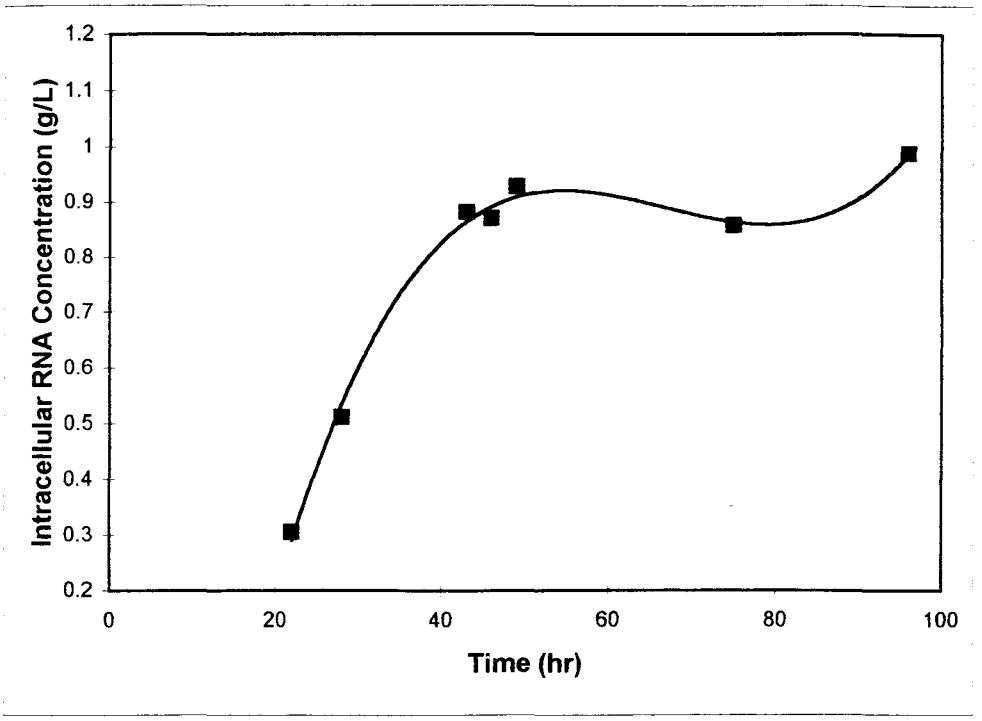


FIGURE 4.57. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 26 g/L Initial Starch Concentration under Aerobic Conditions

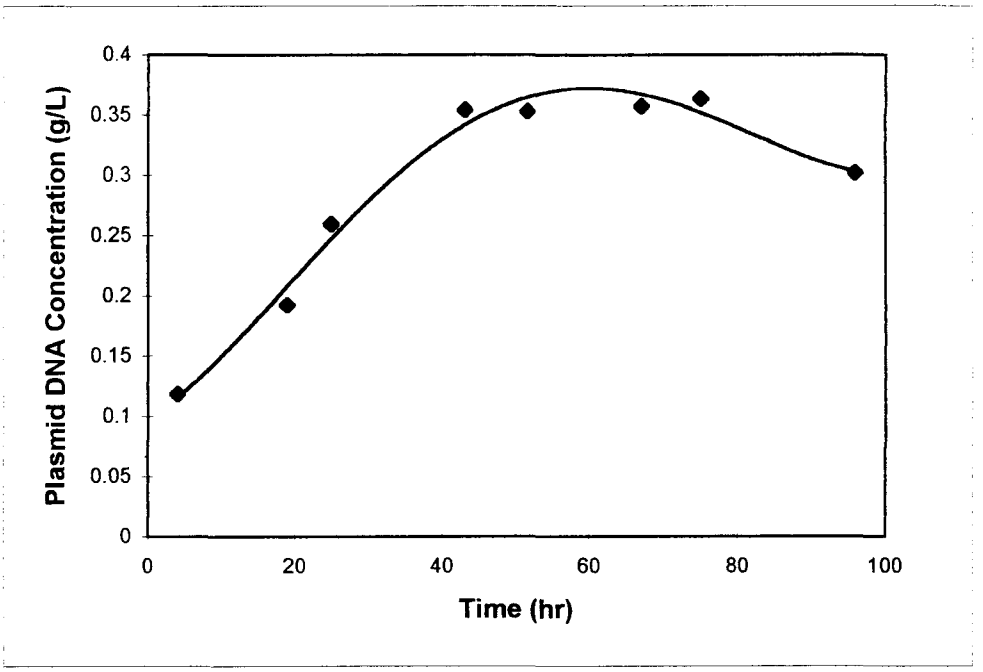


FIGURE 4.57. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 26 g/L Initial Starch Concentration under Aerobic Conditions

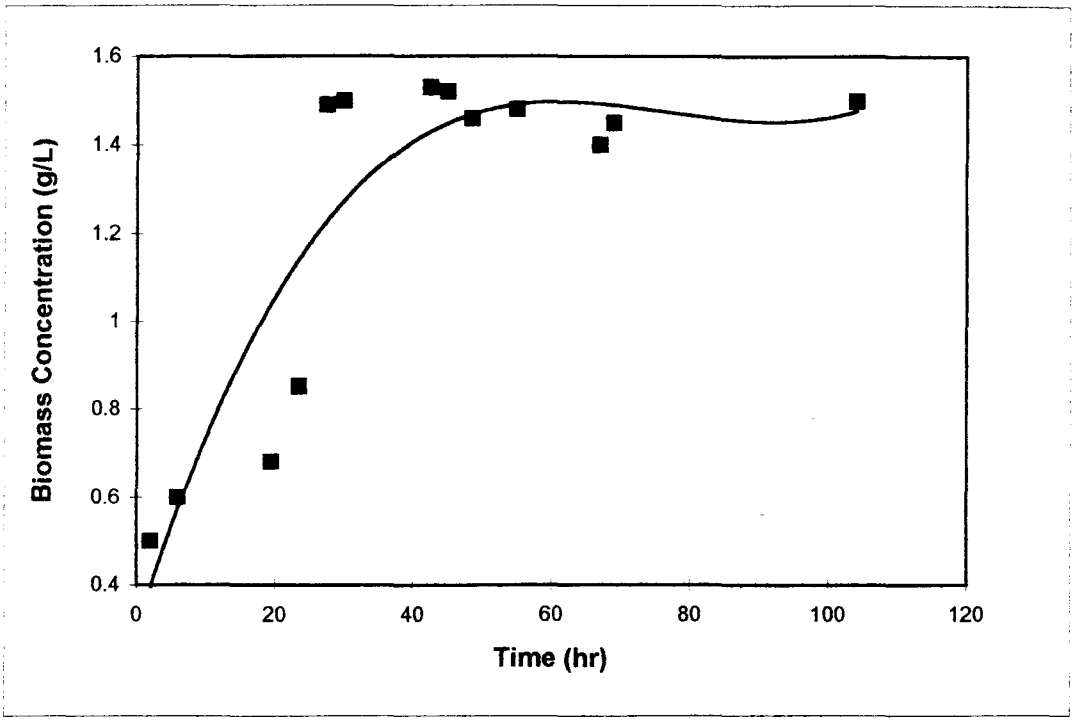


FIGURE 4.58. (a) Time Profiles of Biomass Formation of YPB-G at 5 g/L Initial Starch under Anaerobic Conditions

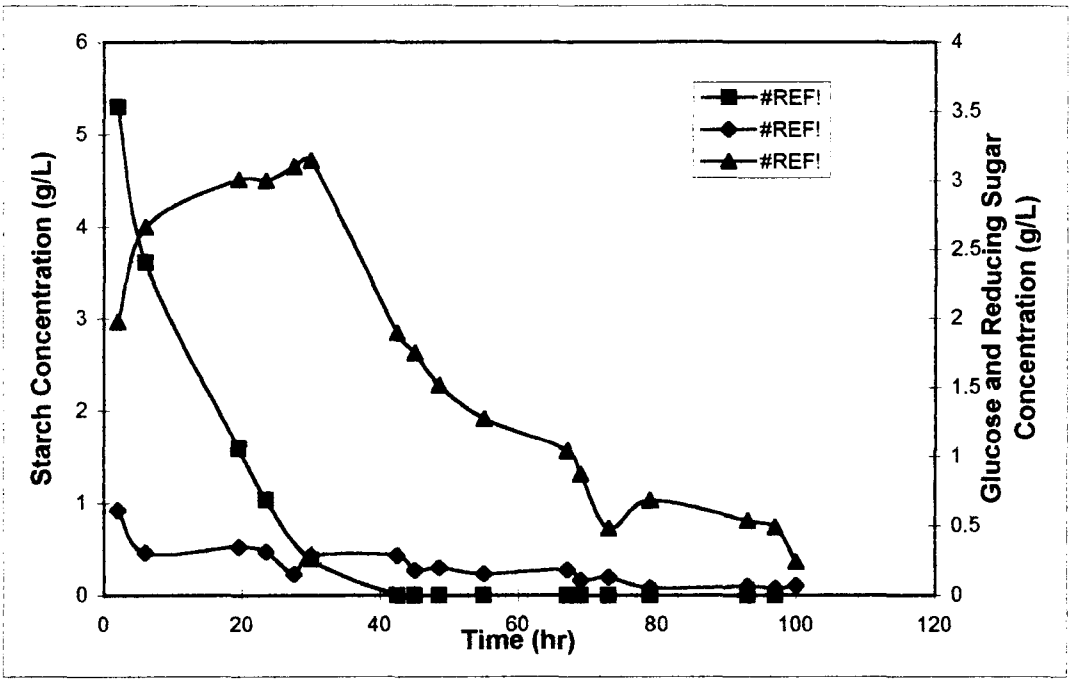


FIGURE 4.58. (b) Time Profiles of Substrate Utilization of YPB-G at 5 g/L Initial Starch under Anaerobic Conditions

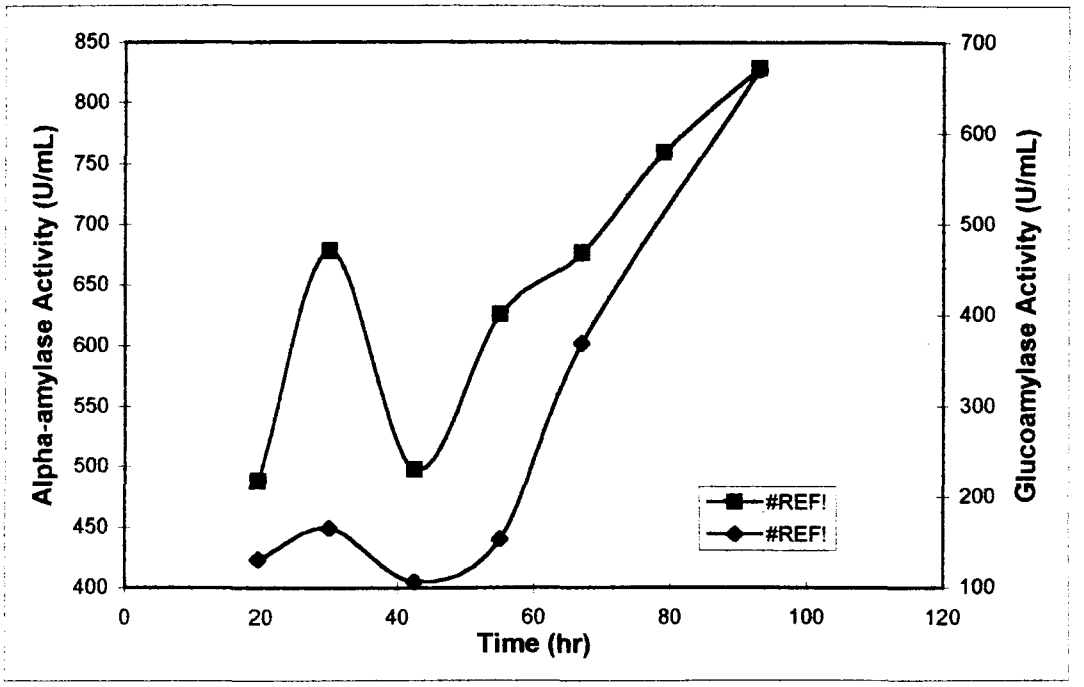


FIGURE 4.58. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 5 g/L Initial Starch Concentration under Anaerobic Conditions

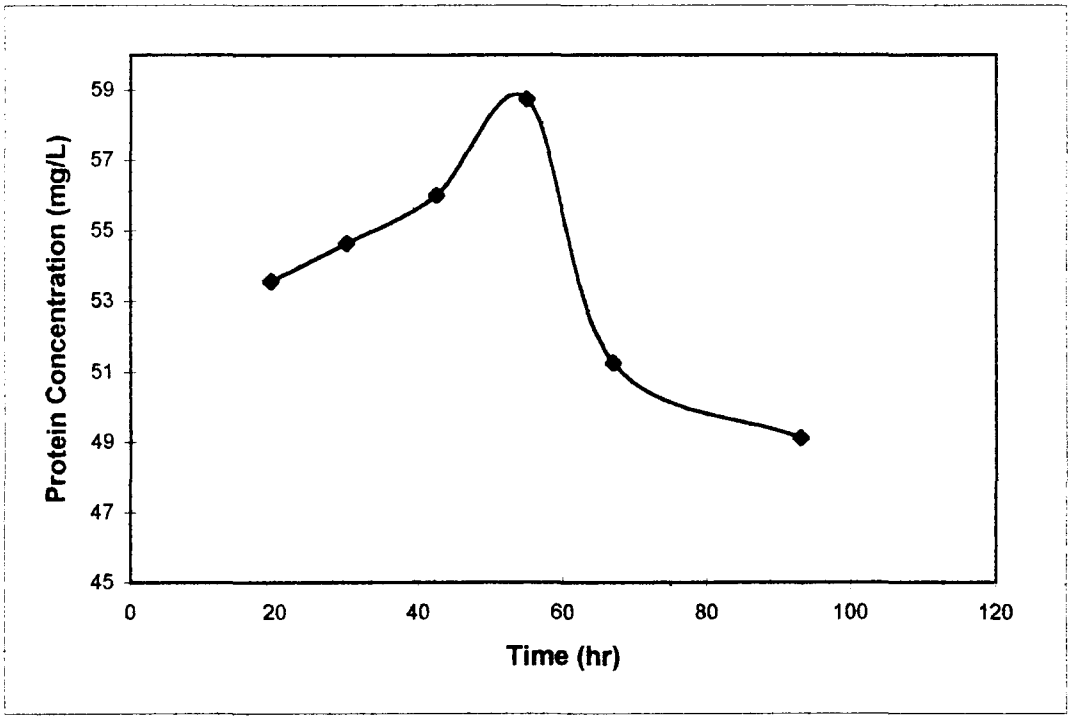


FIGURE 4.58. (d) Time Profiles of Protein Production of YPB-G Strain at 5 g/L Initial Starch Concentration under Anaerobic Conditions

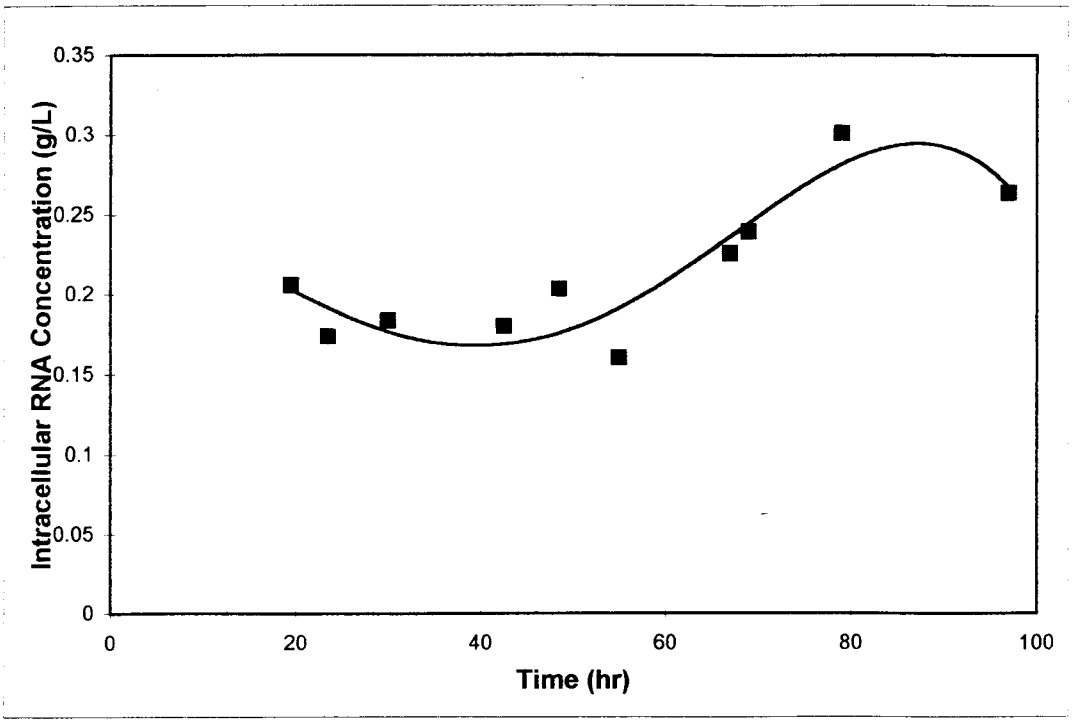


FIGURE 4.58. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 5 g/L Initial Starch Concentration under Anaerobic Conditions

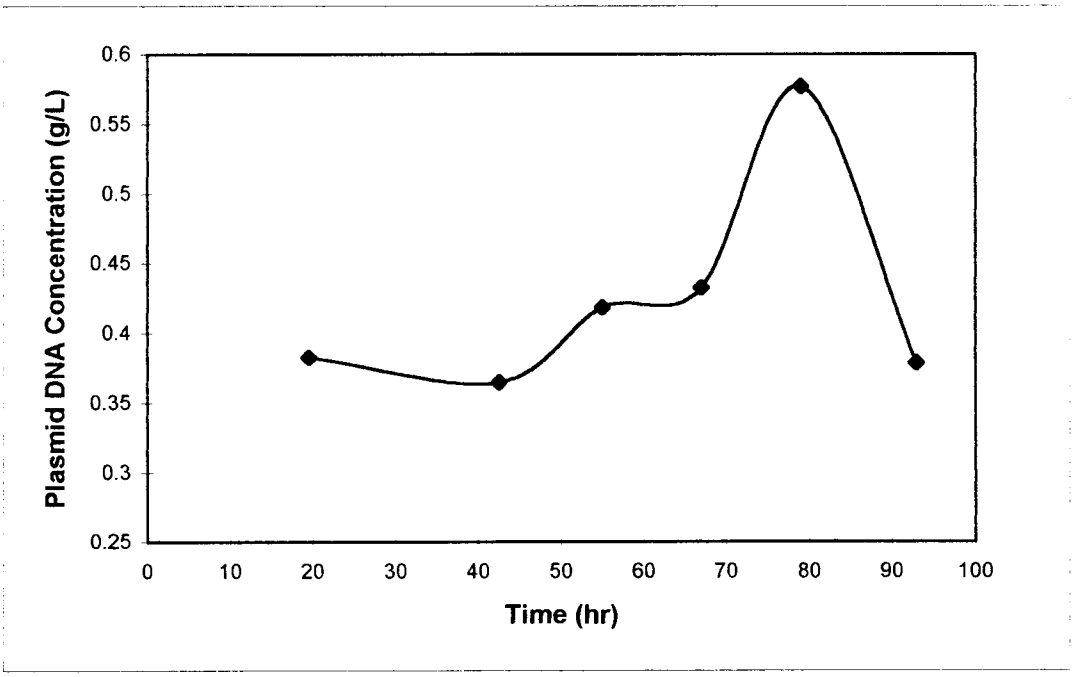


FIGURE 4.58. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 5g/L Initial Starch Concentration under Anaerobic Conditions

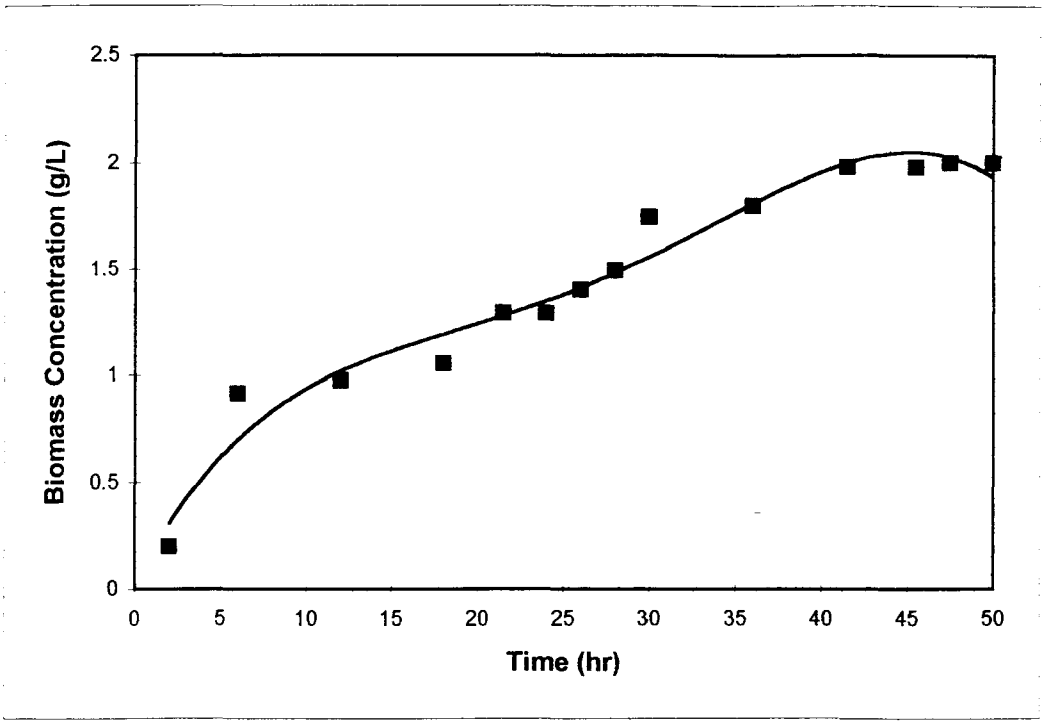


FIGURE 4.59. (a) Time Profiles of Biomass Formation of YPB-G at 20 g/L Initial Starch under Anaerobic Conditions

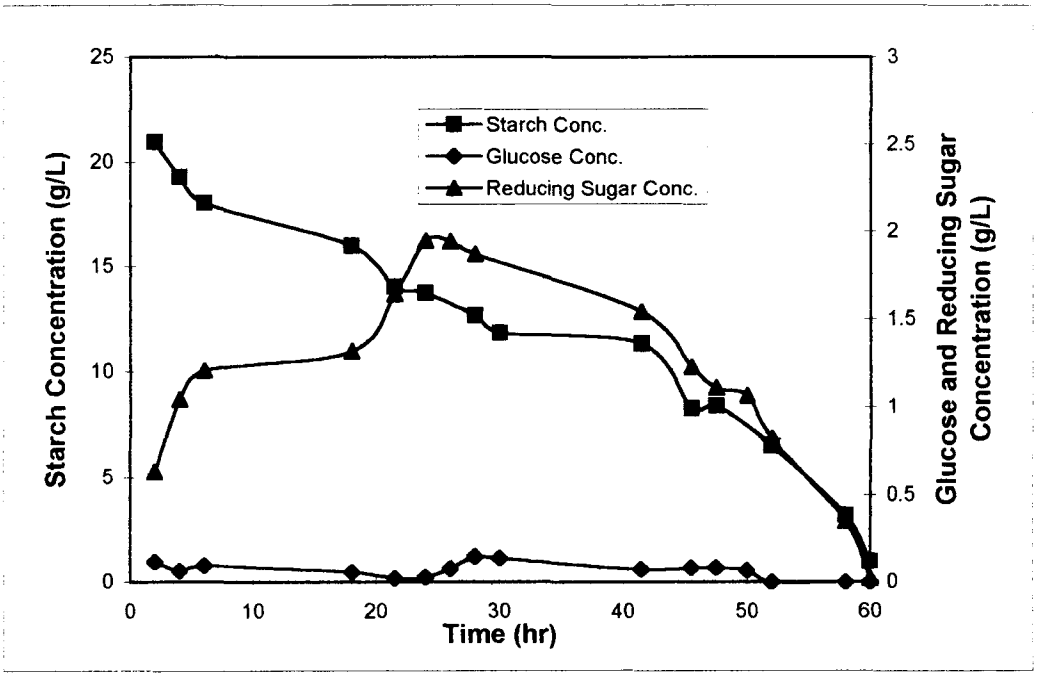


FIGURE 4.59. (b) Time Profiles of Substrate Utilization of YPB-G at 20 g/L Initial Starch under Anaerobic Conditions

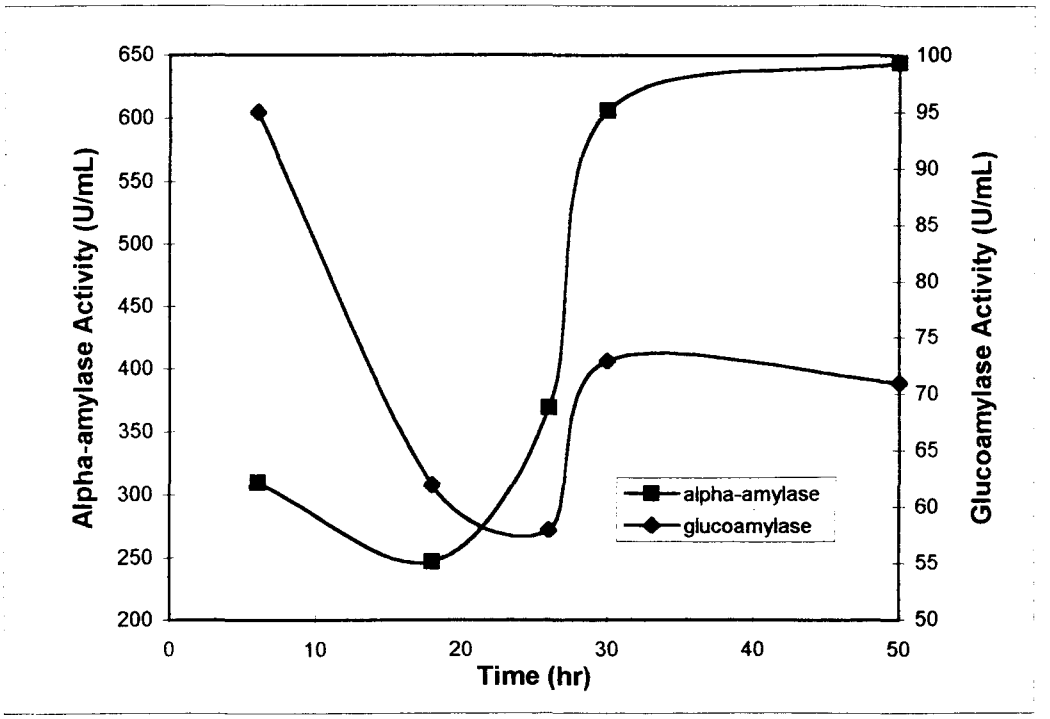


FIGURE 4.59. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 20 g/L Initial Starch Concentration under Anaerobic Conditions

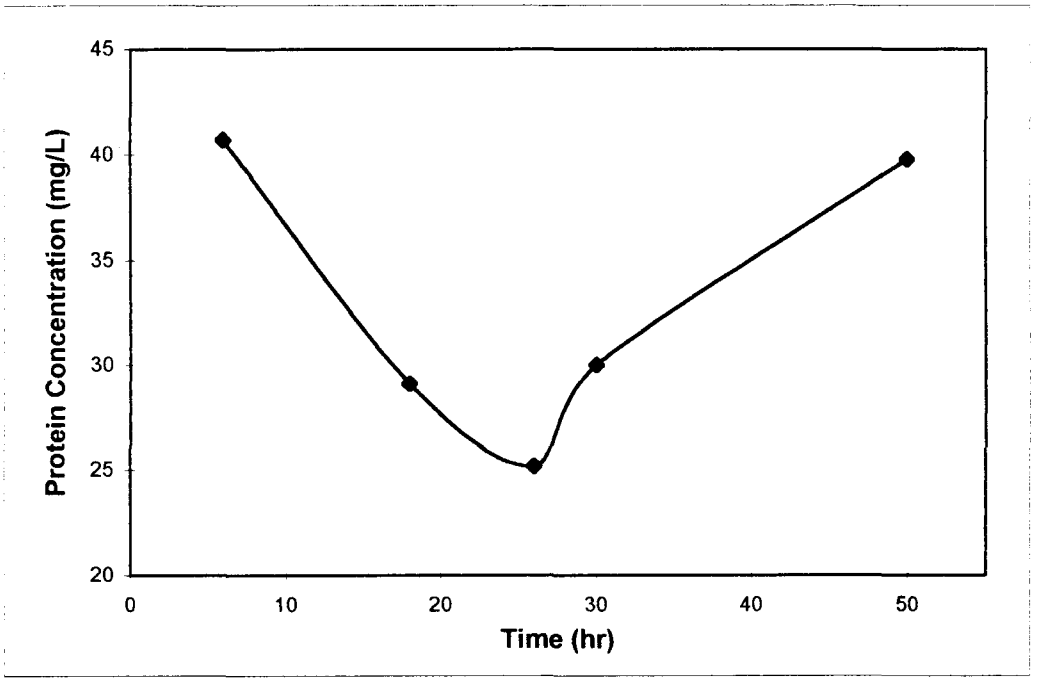


FIGURE 4.59. (d) Time Profiles of Protein Production of YPB-G Strain at 20 g/L Initial Starch Concentration under Anaerobic Conditions

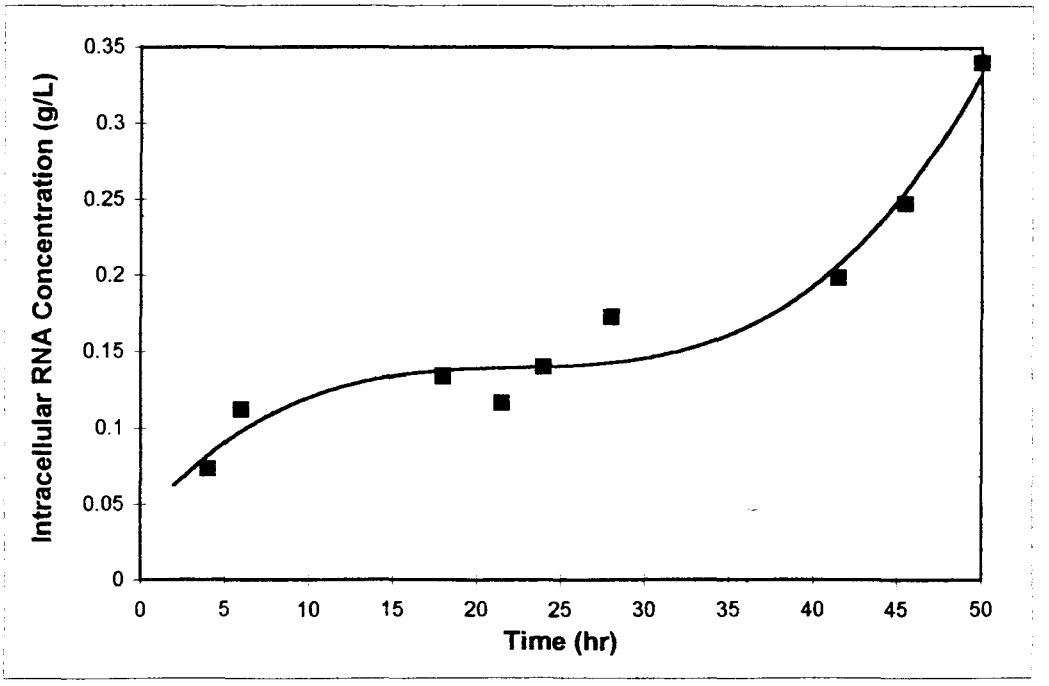


FIGURE 4.59. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 20 g/L Initial Starch Concentration under Anaerobic Conditions

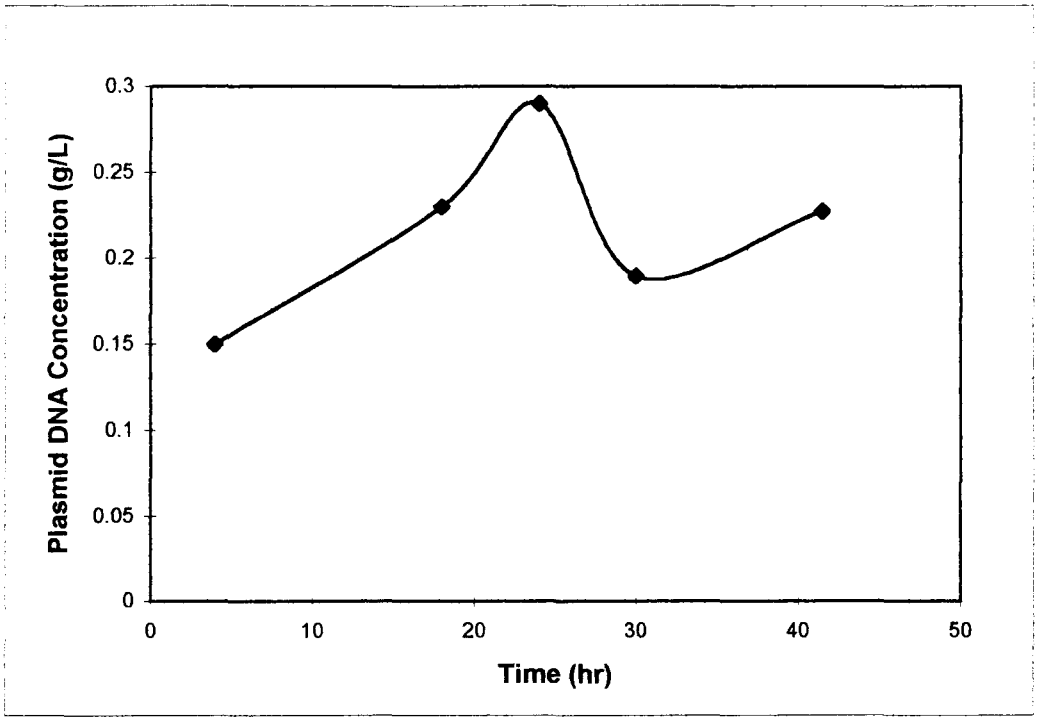


FIGURE 4.59. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 20 g/L Initial Starch Concentration under Anaerobic Conditions

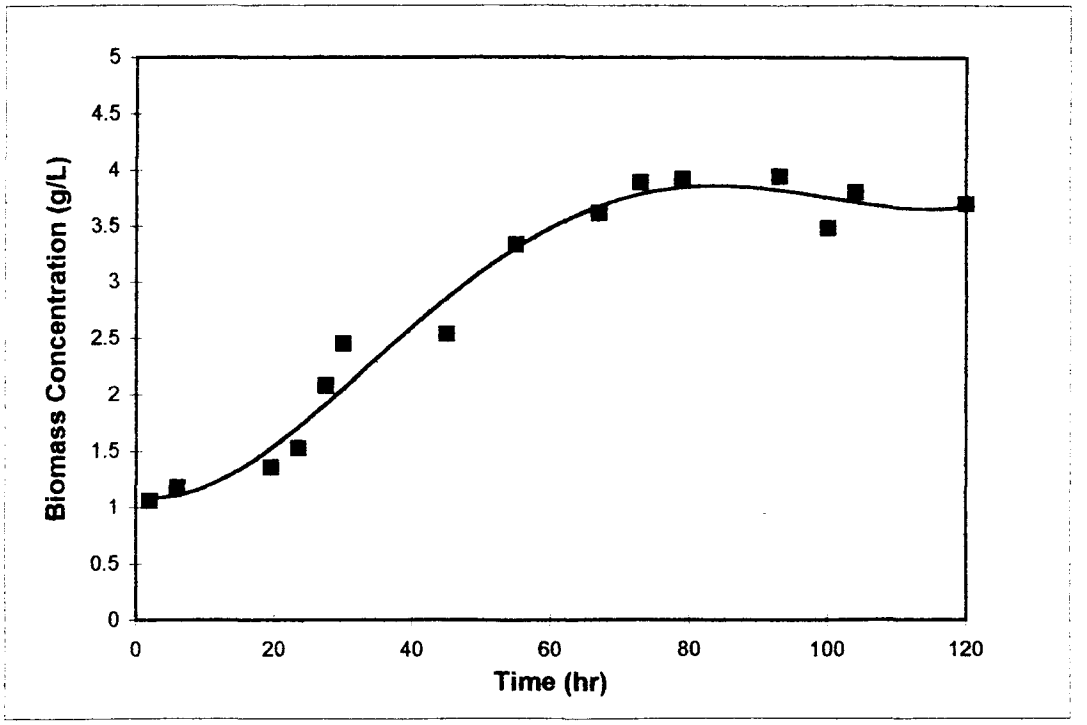


FIGURE 4.60. (a) Time Profiles of Biomass Formation of YPB-G at 26 g/L Initial Starch under Anaerobic Conditions

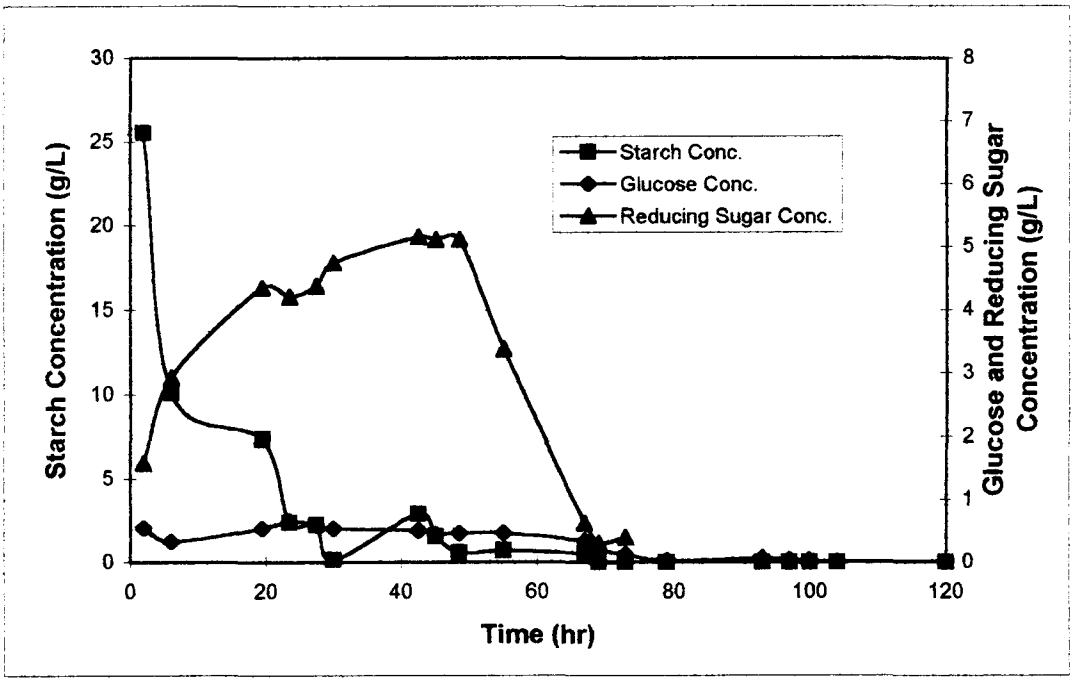


FIGURE 4.60. (b) Time Profiles of Substrate Utilization of YPB-G at 26 g/L Initial Starch under Anaerobic Conditions

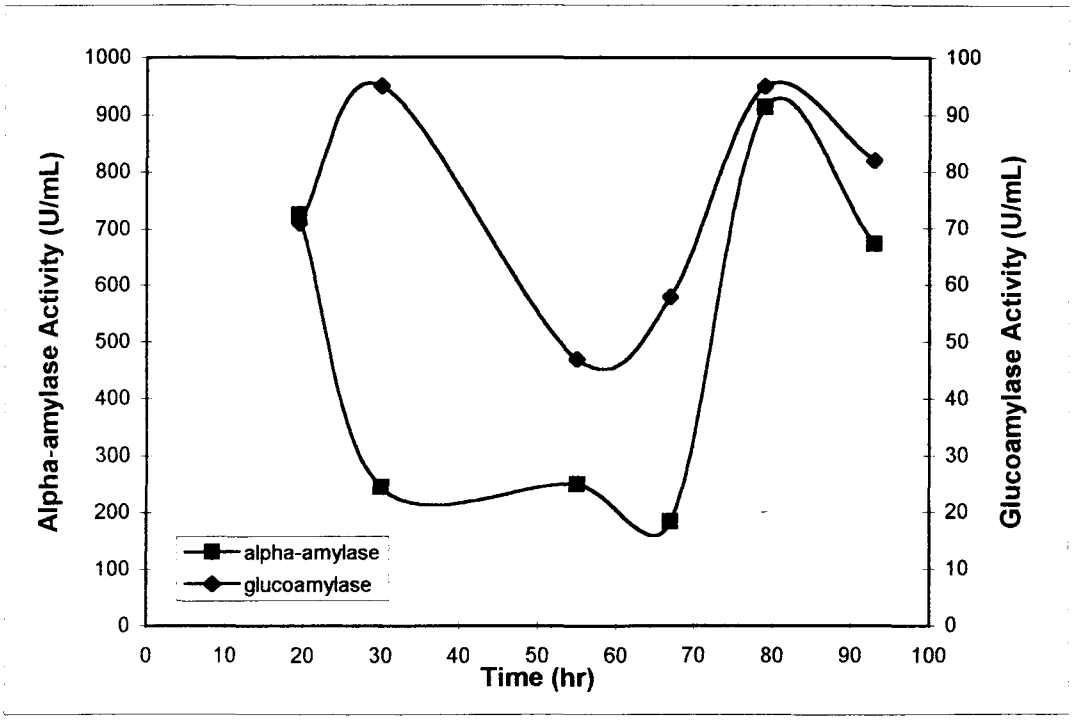


FIGURE 4.60. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 26 g/L Initial Starch Concentration under Anaerobic Conditions

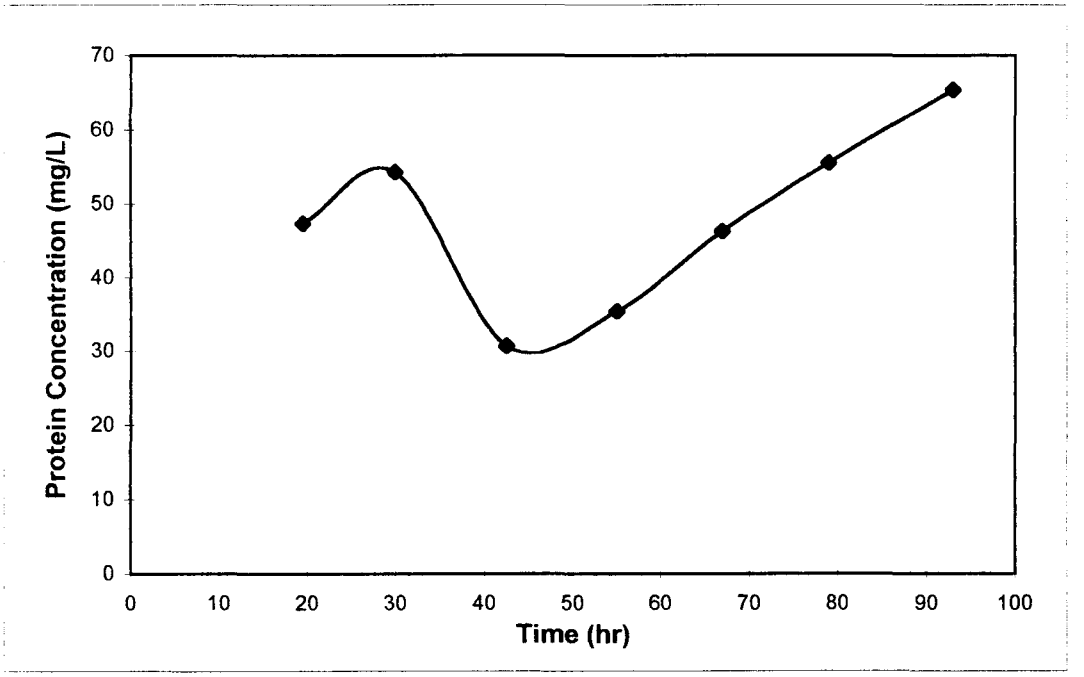


FIGURE 4.60. (d) Time Profiles of Protein Production of YPB-G Strain at 26 g/L Initial Starch Concentration under Anaerobic Conditions

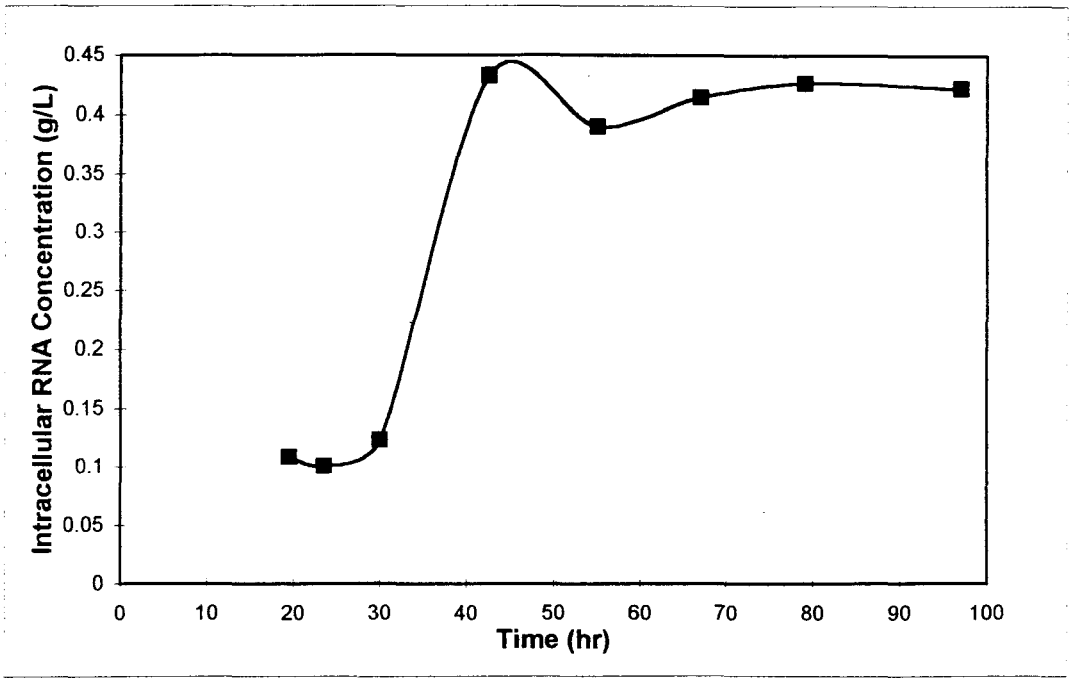


FIGURE 4.60. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 26 g/L Initial Starch Concentration under Anaerobic Conditions

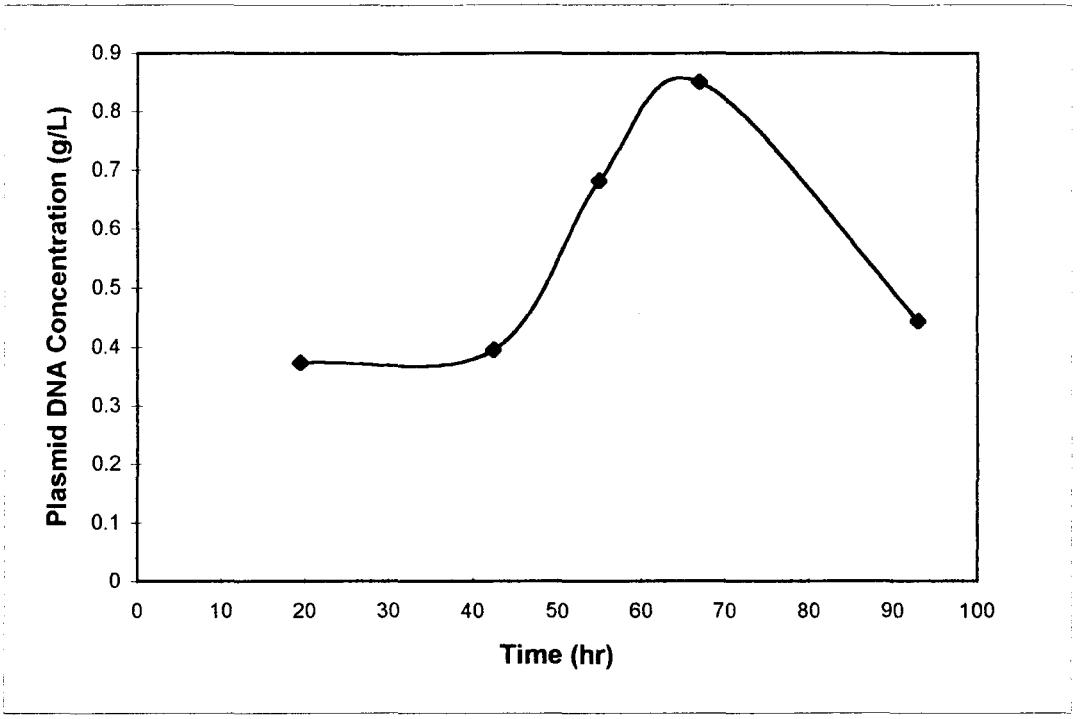


FIGURE 4.60. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 26 g/L Initial Starch Concentration under Anaerobic Conditions

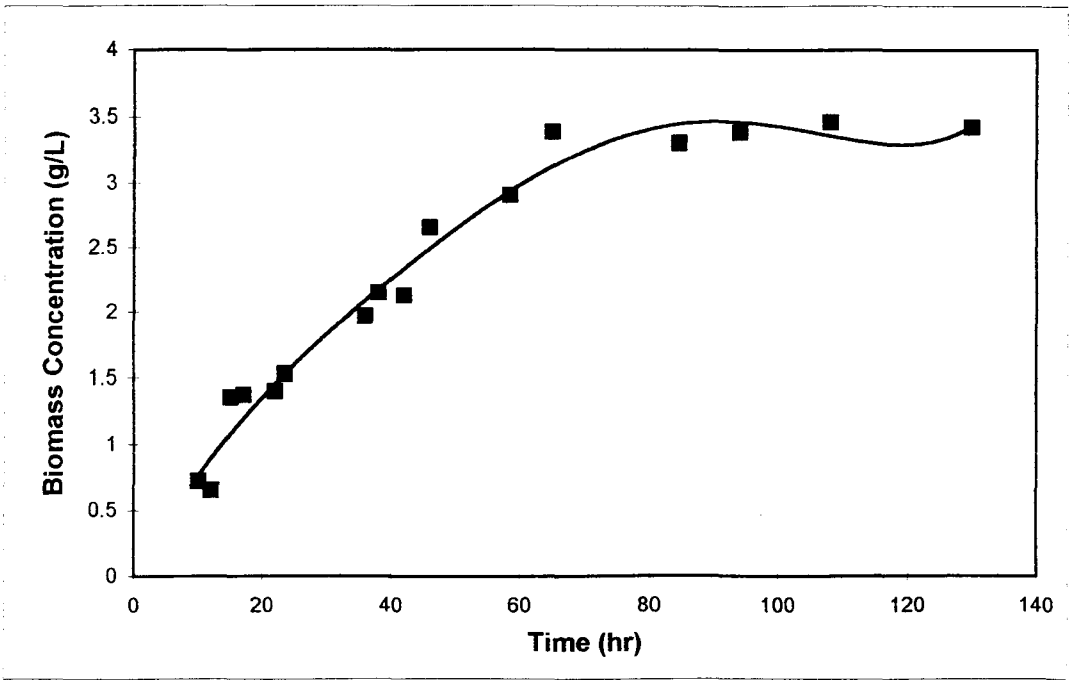


FIGURE 4.61. (a) Time Profiles of Biomass Formation of YPB-G at 36 g/L Initial Starch under Anaerobic Conditions

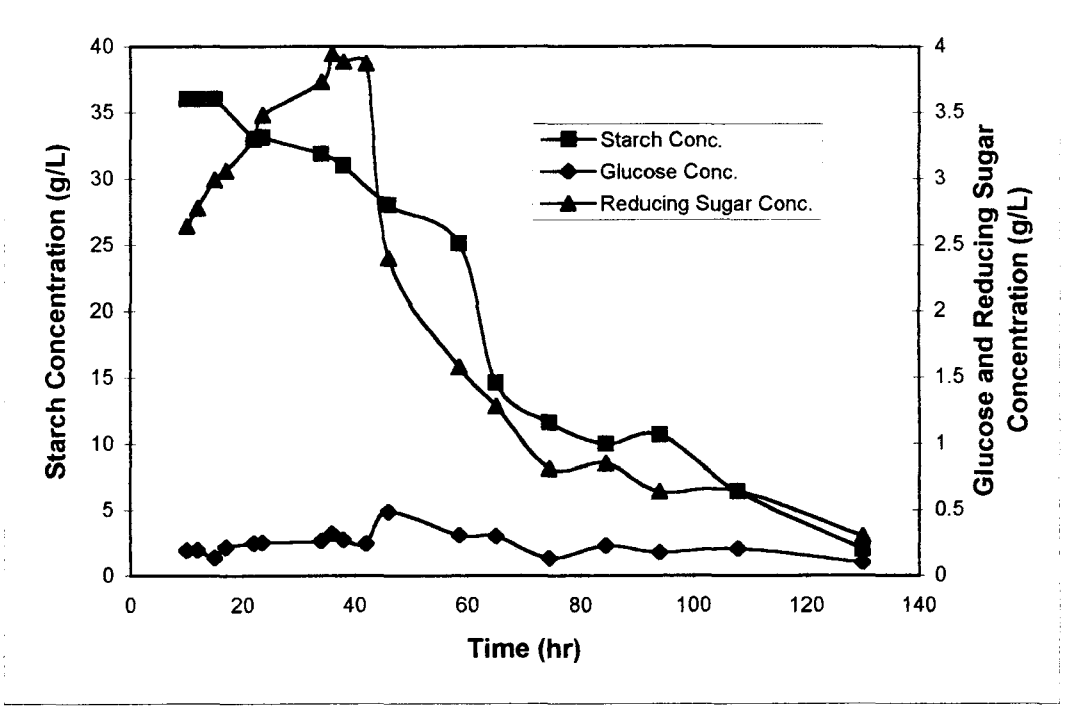


FIGURE 4.61. (b) Time Profiles of Substrate Utilization of YPB-G at 36 g/L Initial Starch under Anaerobic Conditions

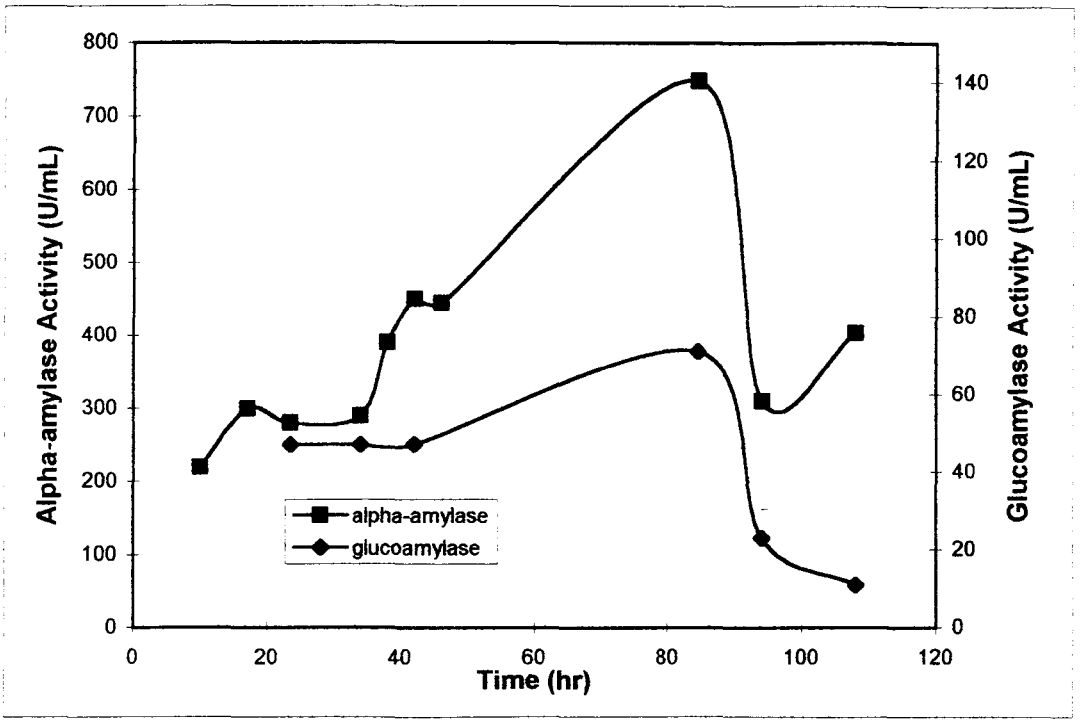


FIGURE 4.61. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 36 g/L Initial Starch Concentration under Anaerobic Conditions

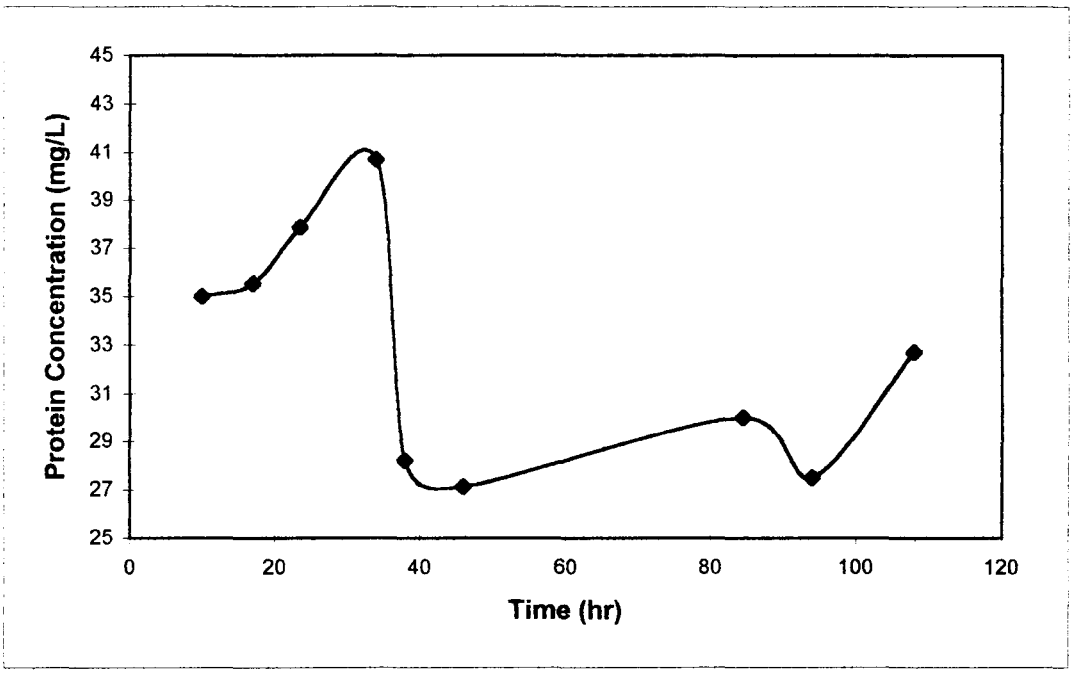


FIGURE 4.61. (d) Time Profiles of Protein Production of YPB-G Strain at 36 g/L Initial Starch Concentration under Anaerobic Conditions

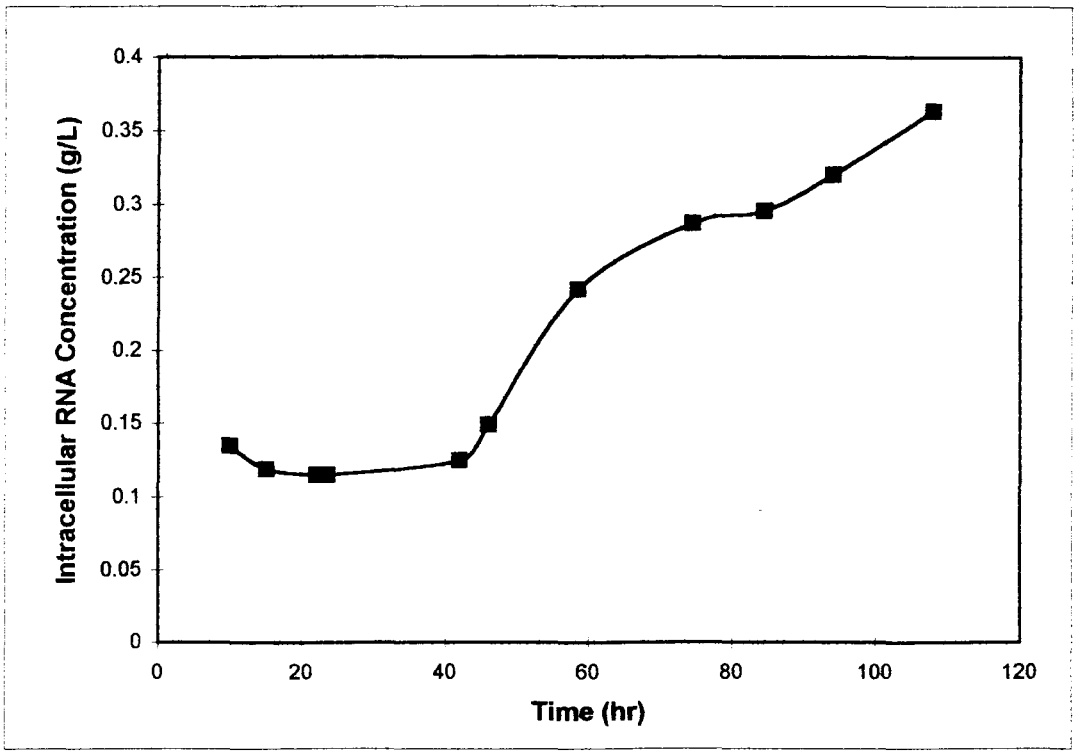


FIGURE 4.61. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 36 g/L Initial Starch Concentration under Anaerobic Conditions

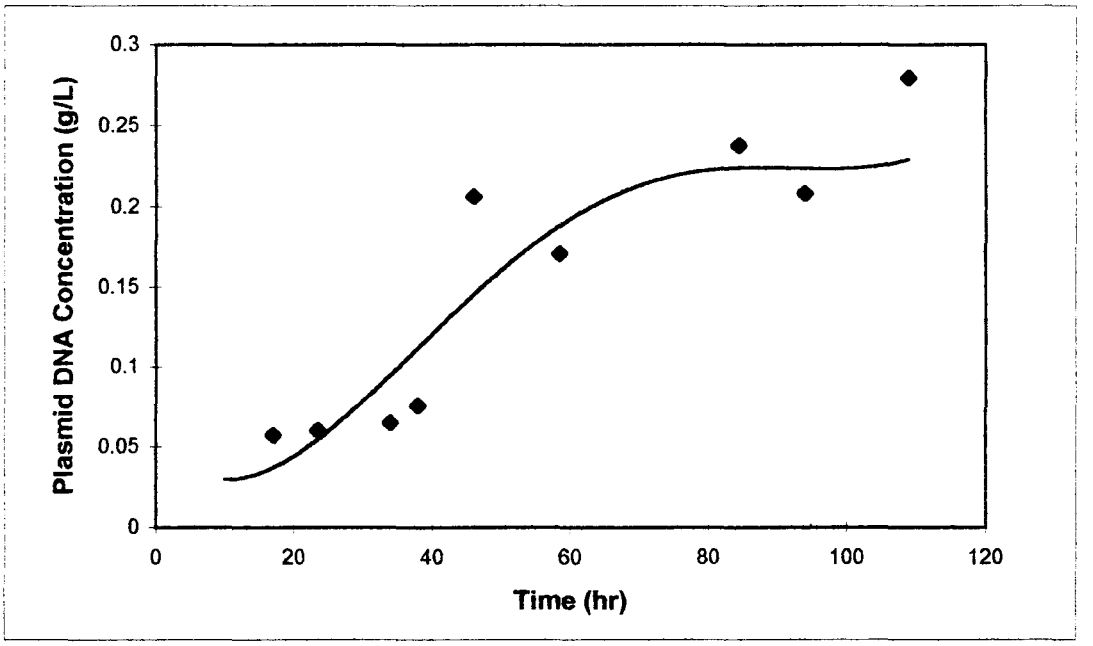


FIGURE 4.61. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 36 g/L Initial Starch Concentration under Anaerobic Conditions

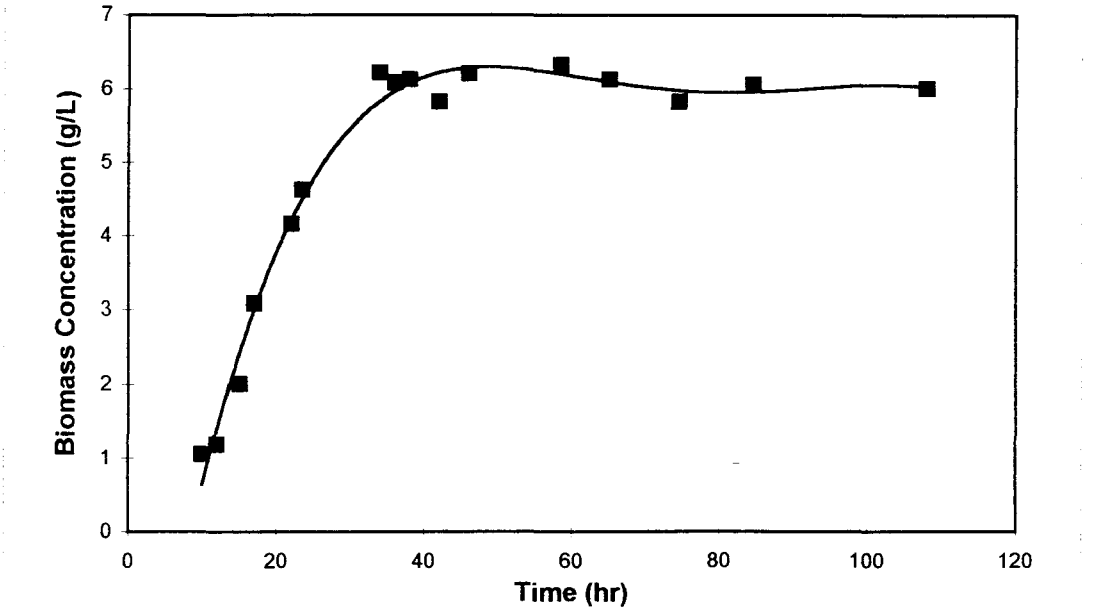


FIGURE 4.62. (a) Time Profiles of Biomass Formation of YPB-G at 50 g/L Initial Starch under Anaerobic Conditions

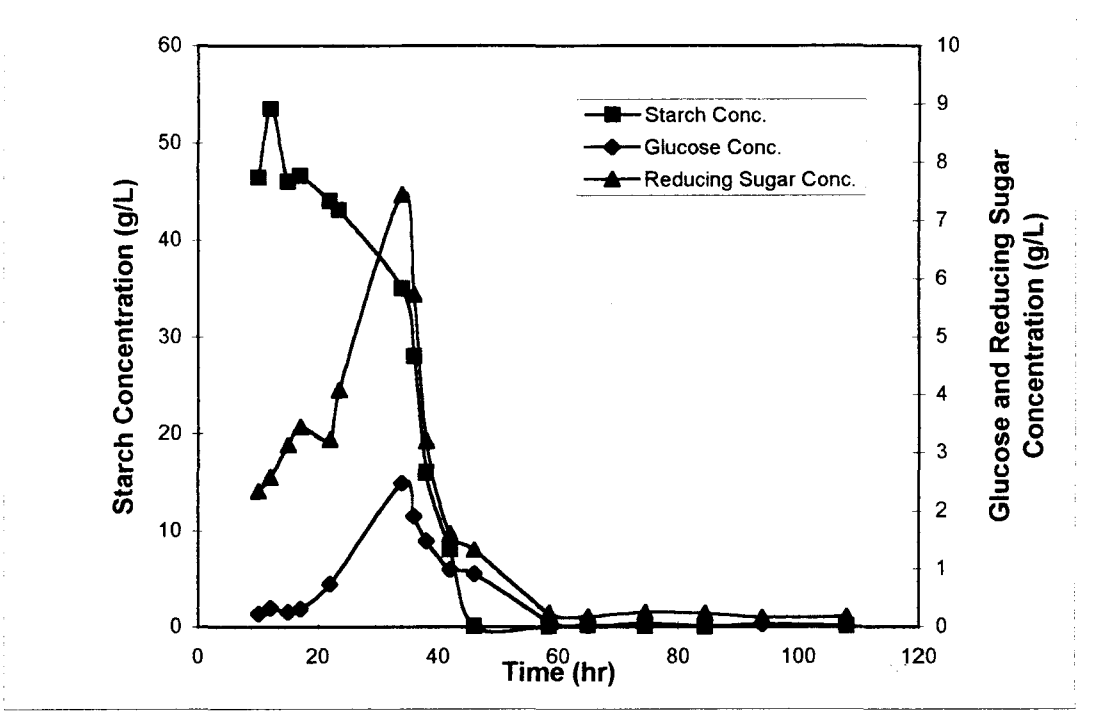


FIGURE 4.62. (b) Time Profiles of Substrate Utilization of YPB-G at 50 g/L Initial Starch under Anaerobic Conditions

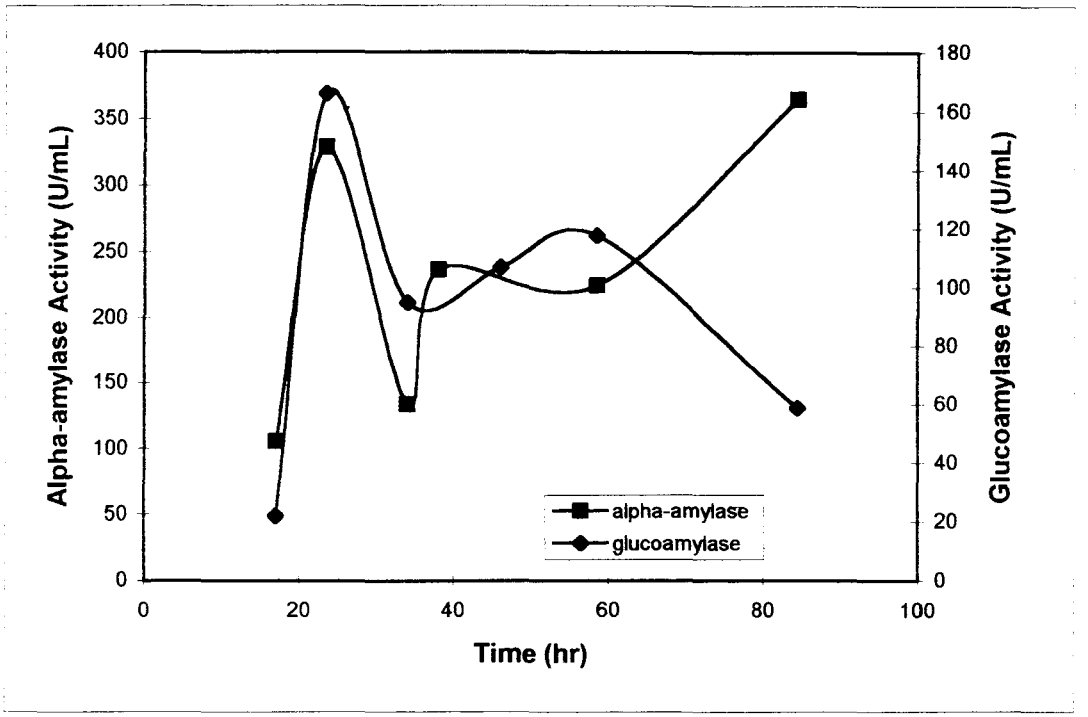


FIGURE 4.62. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 50 g/L Initial Starch Concentration under Anaerobic Conditions

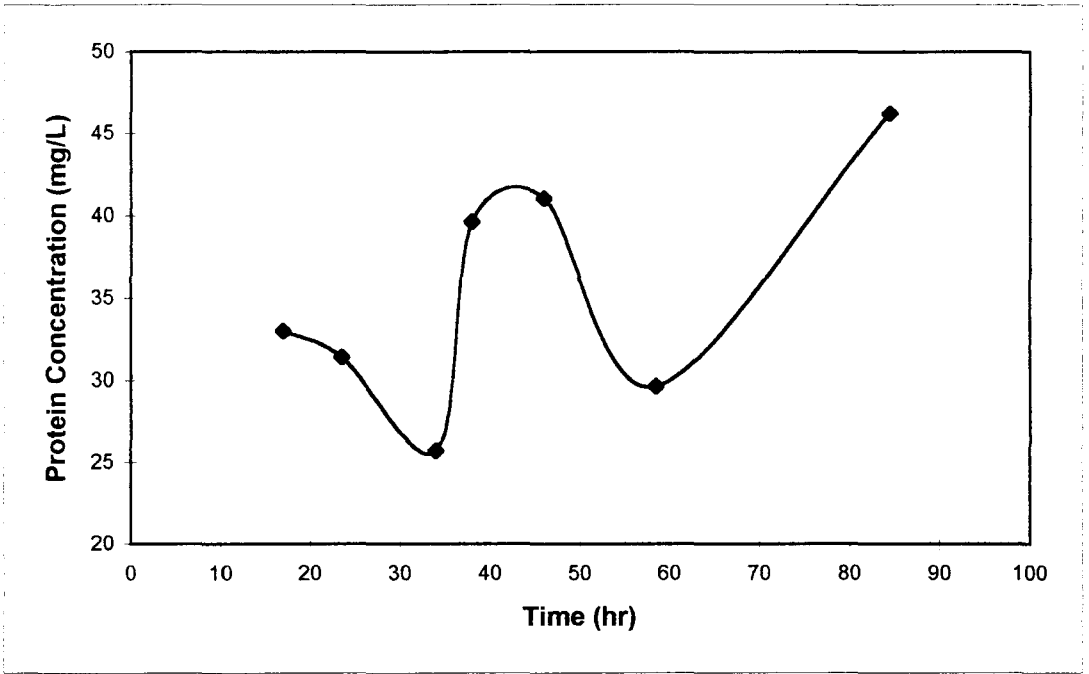


FIGURE 4.62. (d) Time Profiles of Protein Production of YPB-G Strain at 50 g/L Initial Starch Concentration under Anaerobic Conditions

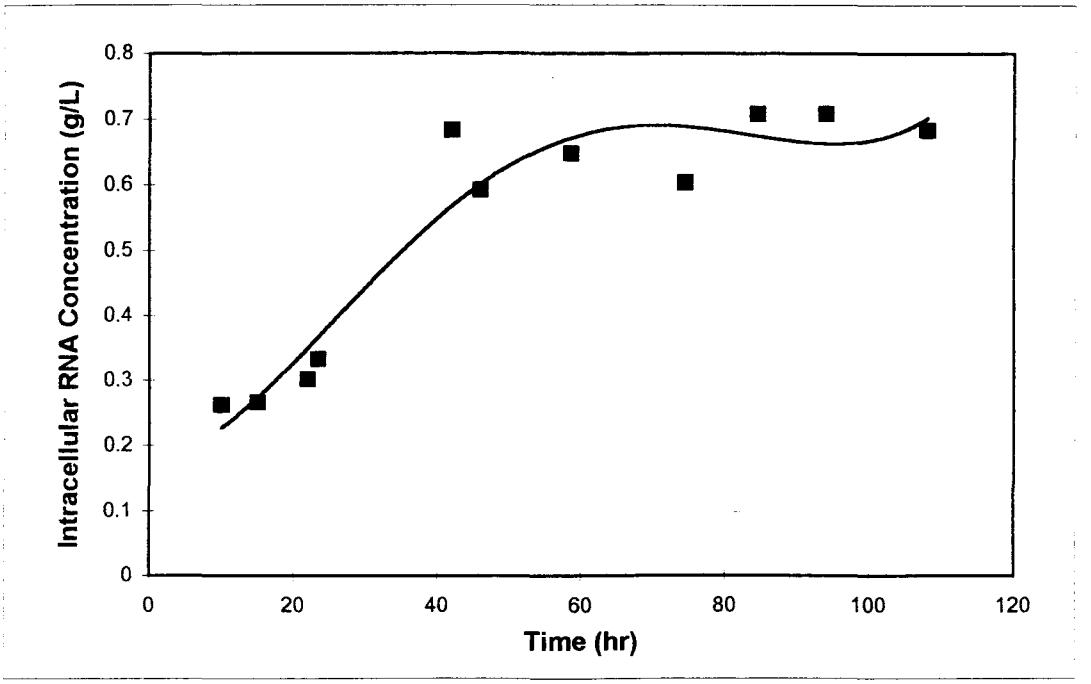


FIGURE 4.62. (e) Time Profiles of Intracellular RNA Concentration of YPB-G Strain at 50 g/L Initial Starch Concentration under Anaerobic Conditions

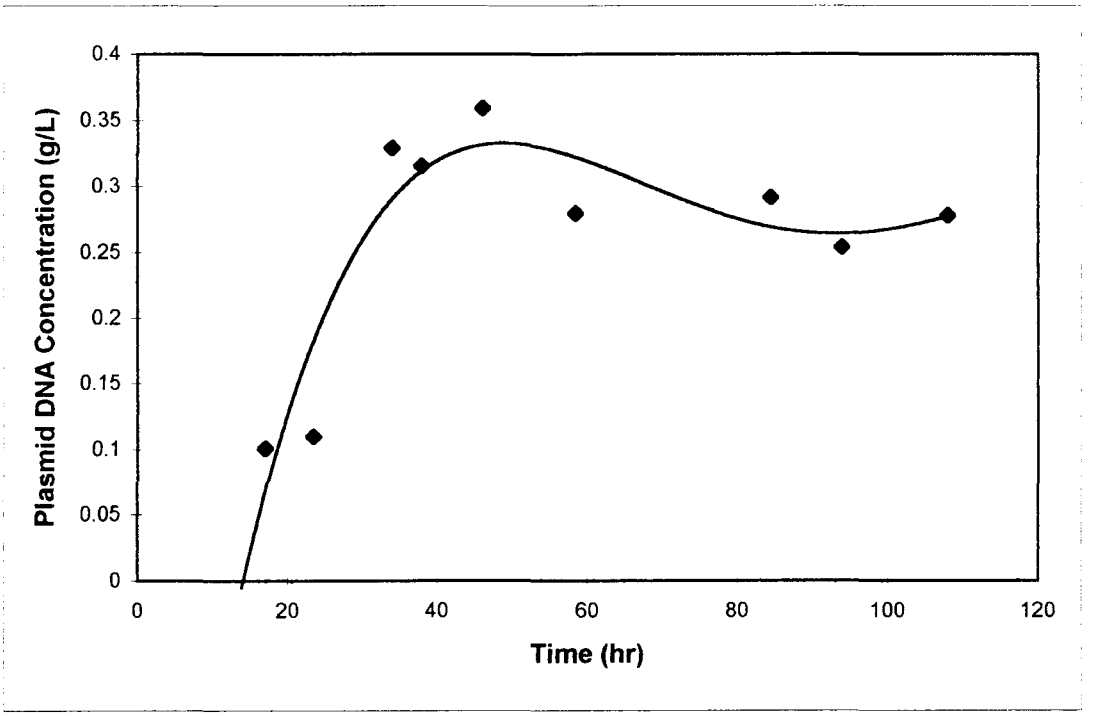


FIGURE 4.62. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 50 g/L Initial Starch Concentration under Anaerobic Conditions

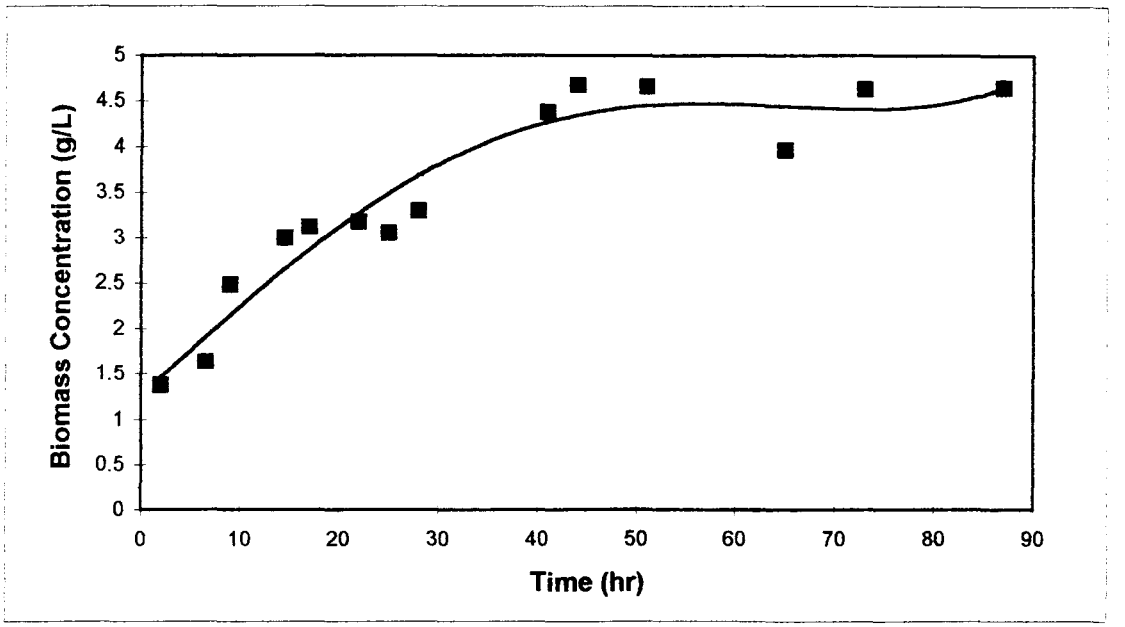


FIGURE 4.63. (a) Time Profiles of Biomass Formation of YPB-G at 50 g/L Initial Starch with Modification

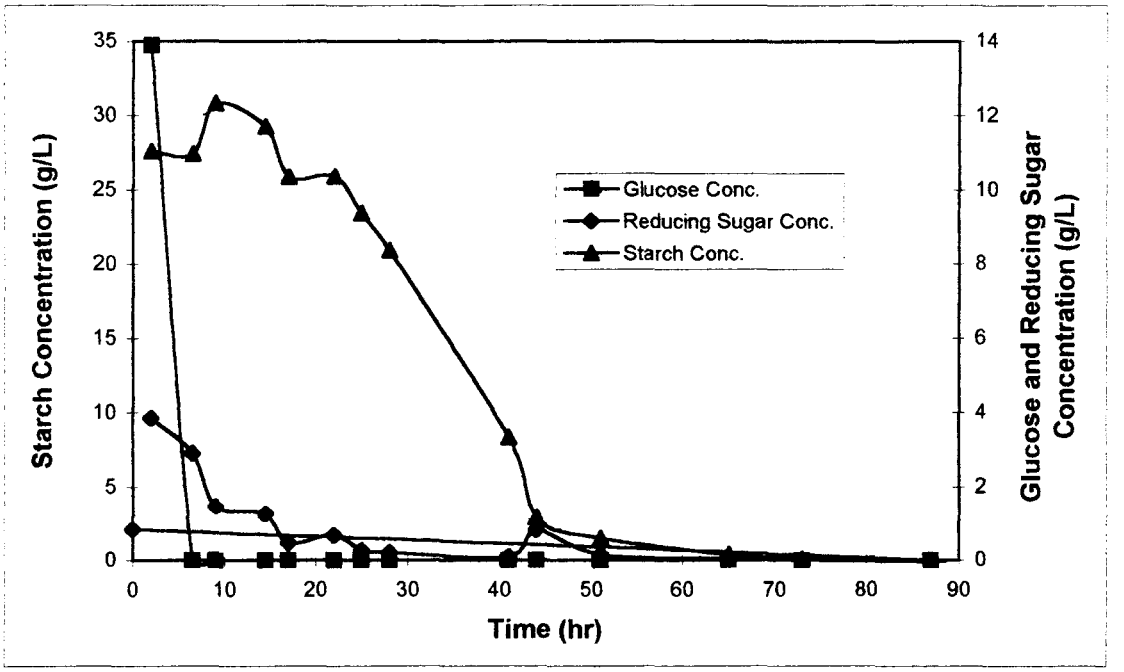


FIGURE 4.63. (b) Time Profiles of Carbon Source Utilization of YPB-G at 50 g/L Initial Starch with Modification

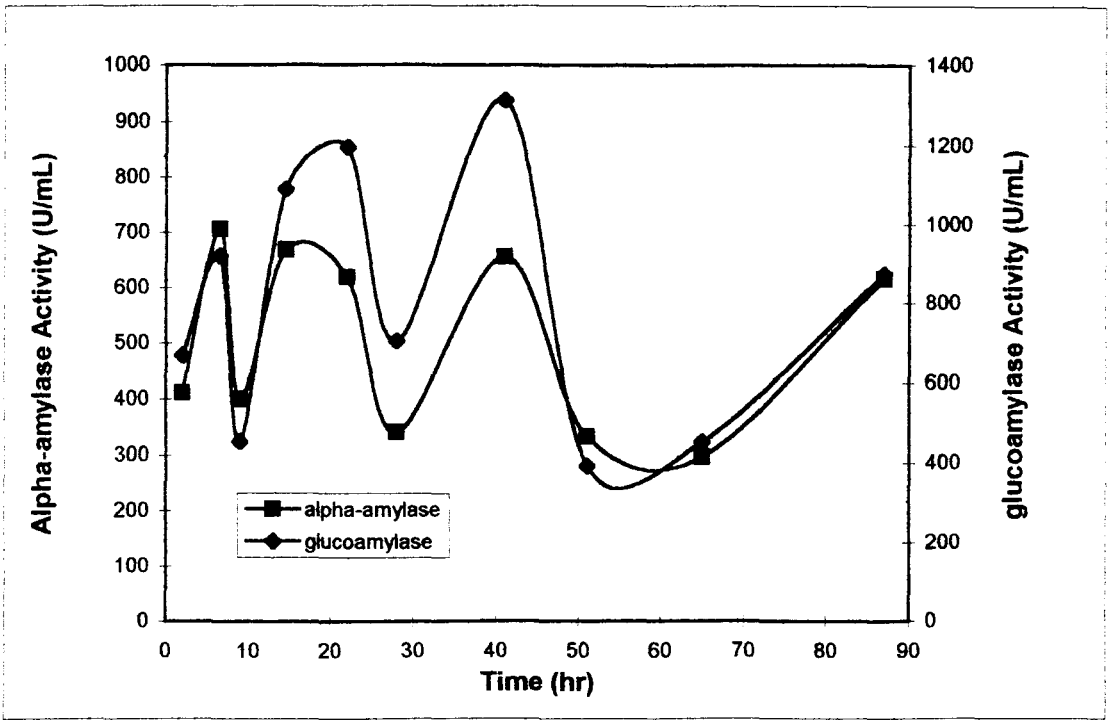


FIGURE 4.63. (c) Time Profiles of Enzyme Secretion of YPB-G Strain at 50 g/L Initial Starch with Modification

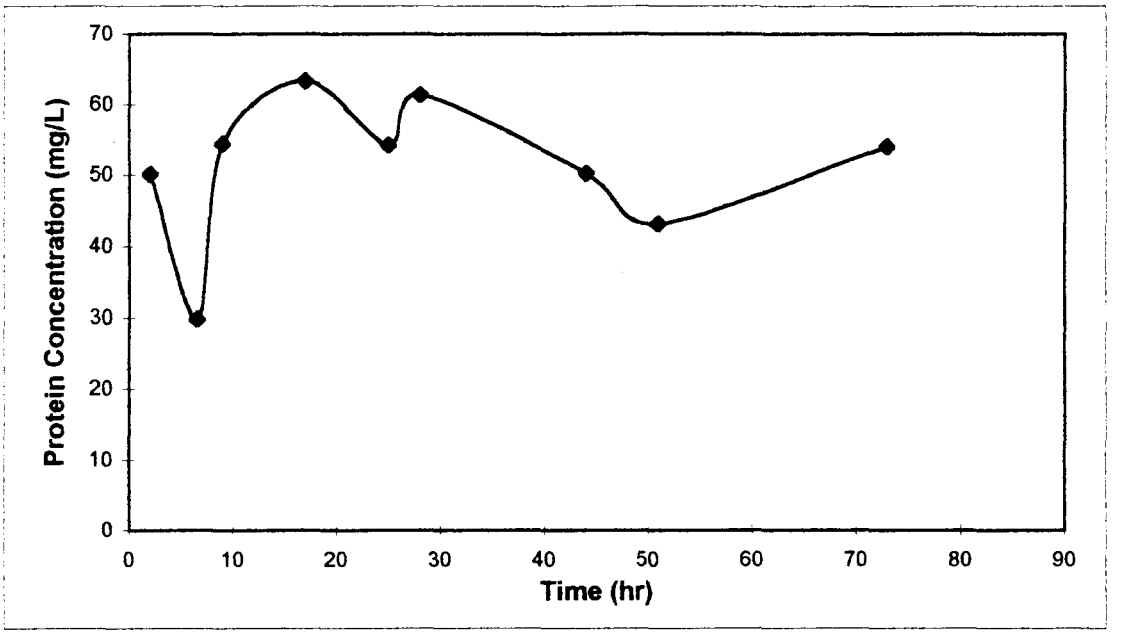


FIGURE 4.63. (d) Time Profiles of Protein Production of YPB-G Strain at 50 g/L Initial Starch with Modification

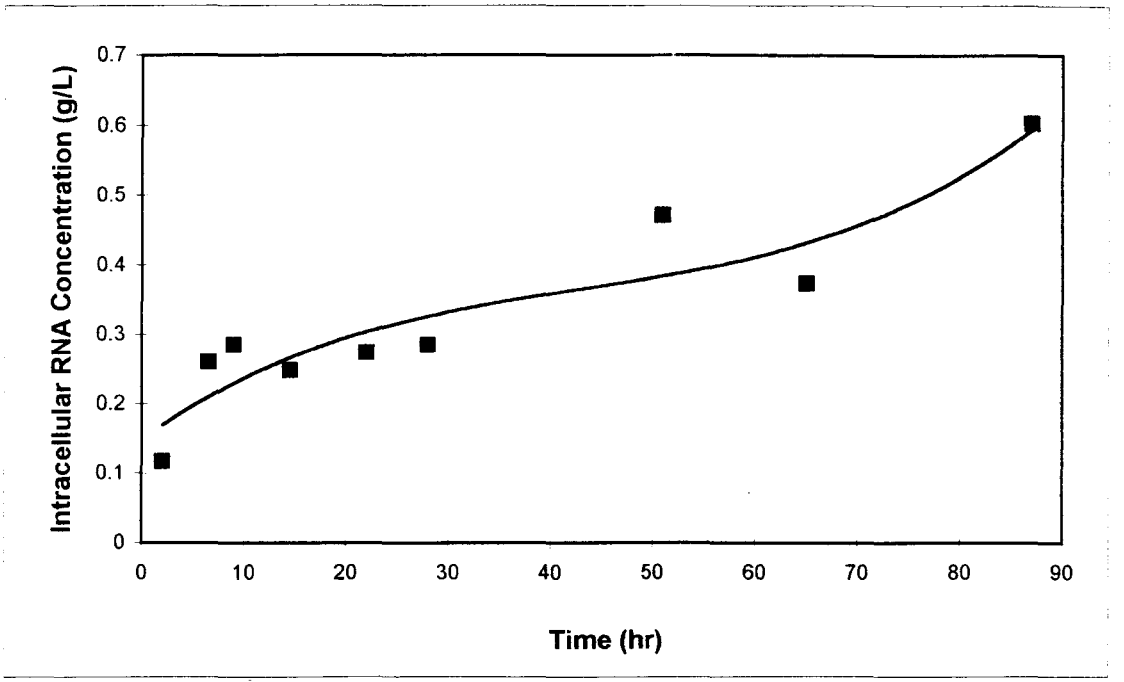


FIGURE 4.63. (e) Time Profiles of Intracellular RNAConcentration of YPB-G Strain at 50 g/L Initial Starch with Modification

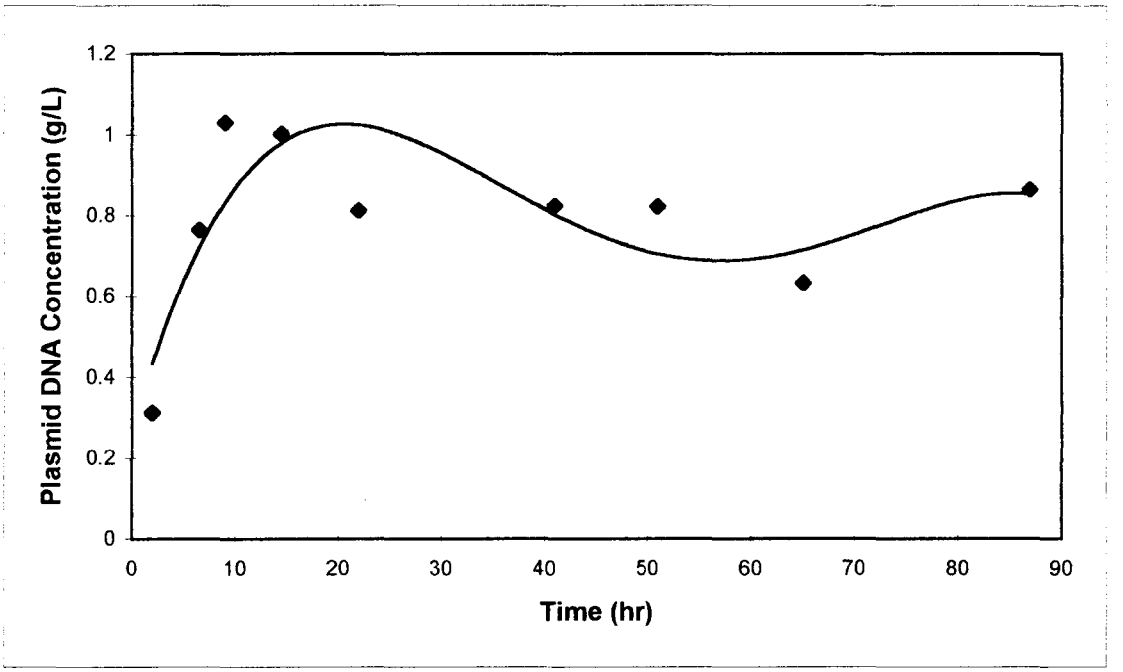


FIGURE 4.63. (f) Time Profiles of Plasmid DNA of YPB-G Strain at 50 g/L Initial Starch with Modification

4.9. Structured Modeling of Enzyme and Biomass Production by Recombinant Yeast Cells

The simplest representation of microbial kinetics is termed the *unstructured model* and describes the growth of biomass, consumption of substrate, and the formation of metabolic products. The unstructured model, includes the most fundamental observations concerning microbial growth processes: the rate of cell mass production is proportional to biomass concentration, there is a saturation limit for growth rate on each substrate, and the cells need substrate and may synthesize products even when they do not grow (Montesinos et al., 1995).

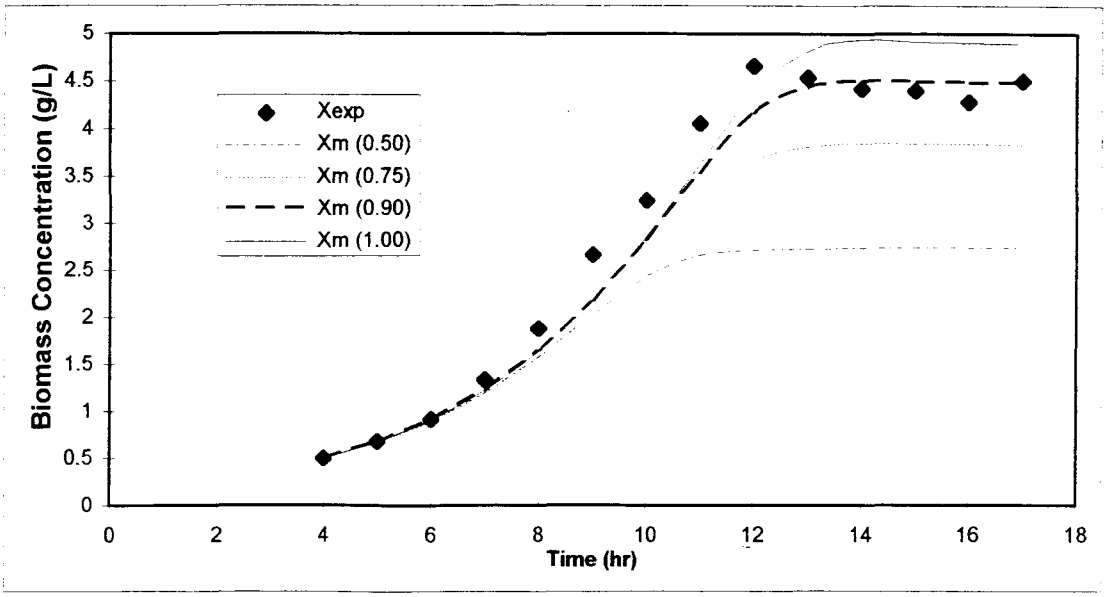
This kind of model does not recognize any internal structure of the cell, nor a diversity between cell forms, which is so evident in plant and animal cell cultures and which may also be an important feature of certain microbial cell cultures. These models are quite satisfactory in many situations, such as when the balanced-growth condition is accomplished or in many control and optimization problems in fermentation processes with minimum mathematical complexity. However, they are not suitable for describing situations in which the cell composition and/or the morphology of the cell culture are important variables, and are strongly time-dependent. The solution is to develop the models termed *structured models* that include aspects of the microbial physiology and structure for the mathematical description of the metabolism of microorganisms (Nielsen and Viladsen, 1992; Montesinos et al., 1995). Some authors give emphasis to the simple structured models against the highly structured models (Roels and Kossen, 1978) while some do not recommend the use of a sophisticated structured model with a high degree of complexity unless strictly necessary (Montesinos et al., 1995). Hence, a complete metabolic description of the microbial kinetics may not be required, and a simple description of transport, diffusion and excretion phenomena, together with some intracellular concentrations, may be sufficient to describe correctly the dynamic behavior of the process (Montesinos et al., 1995).

In this part of the study, enzyme (α-amylase and glucoamylase) and ethanol production of the recombinant *S.cerevisiae* strain (YPB-G) from glucose were modeled through a structured model in order to explain the dynamic behavior of the process. This model described correctly the cell growth and the production of enzymes.

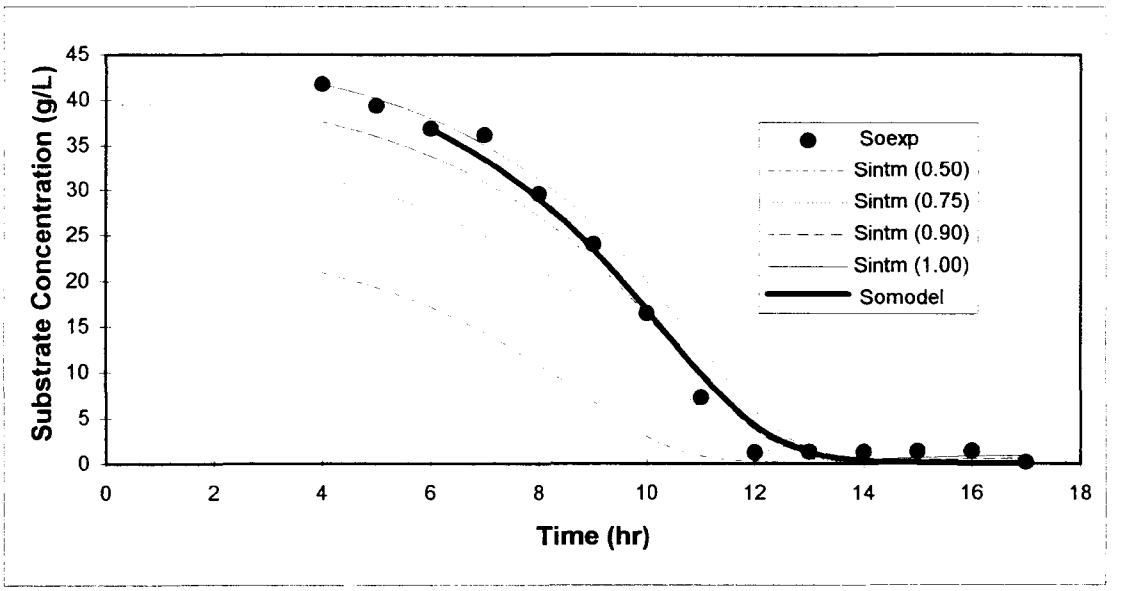
4.9.1. Experimental Observations

Experiments were carried out either in a Bioflo III New Brunswick Fermenter operating in batch mode or in shake flasks under conditions previously described in Section 3.2.3. YPB-G strains were grown in YPG containing 50 g/L glucose in the fermenter. The biomass formation, α-amylase and glucoamylase production, substrate (glucose) consumption and ethanol production were measured as a function of time (**Figure 4.64**). The results are summarized in **Table 4.22**.

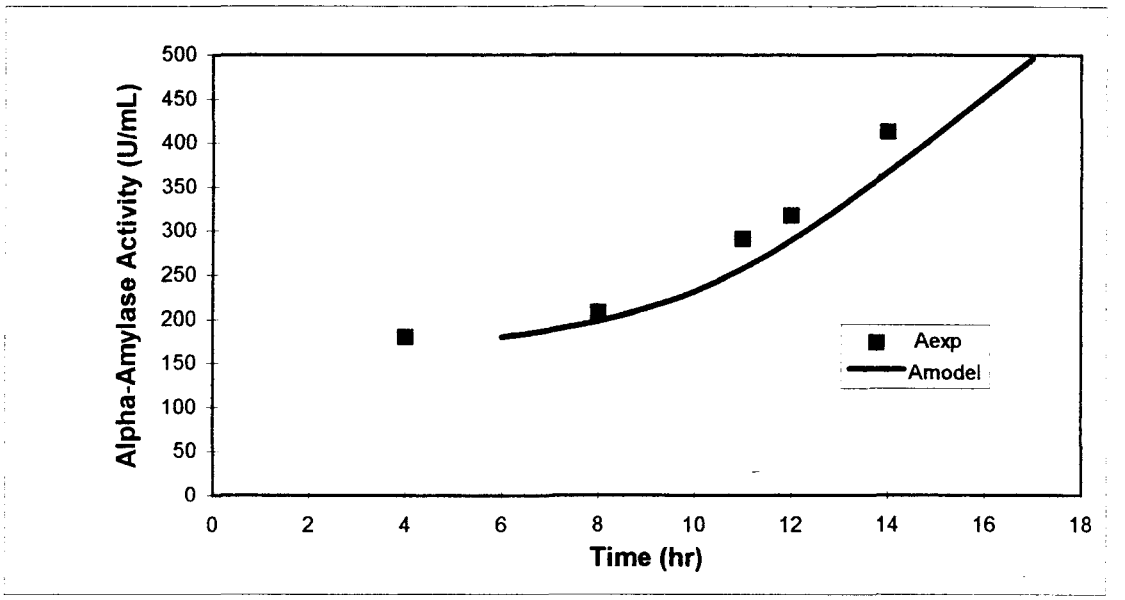
Several shake flask fermentation experiments were carried out to study the effect of glucose concentration on α-amylase and glucoamylase secretion and ethanol production. Observations of the cell growth revealed that the maximum specific growth rate (observed) increased with increasing glucose concentration. 0.047, 0.157 and 0.203 h^{-1} maximum specific growth rates (μ_{max}) were calculated for 5, 10 and 25 g/L glucose containing YPG medium respectively in shake flask experiments (see **Table 4.22**). The biomass/substrate yield was constant at a value close to 0.1 g biomass/g glucose. The S-shape behavior of biomass formation and glucose consumption were observed in all experiments. At the higher glucose concentrations used, the production of ethanol reached higher levels (**Table 4.22**). The activities of α-amylase and glucoamylase also changed with initial glucose concentration, i.e. at lower initial glucose concentrations the activities were relatively higher. In shake flask experiments with 5, 10 and 25 g/L initial glucose concentration, the α-amylase activities were 700, 700, and 500 U/mL respectively (**Table 4.22**), but in most cases, a growth-associated behavior was observed.


Biomass Concentration (g/L)
Time (hr)
Xexp
Xm (0.50)
Xm (0.75)
Xm (0.90)
Xm (1.00)

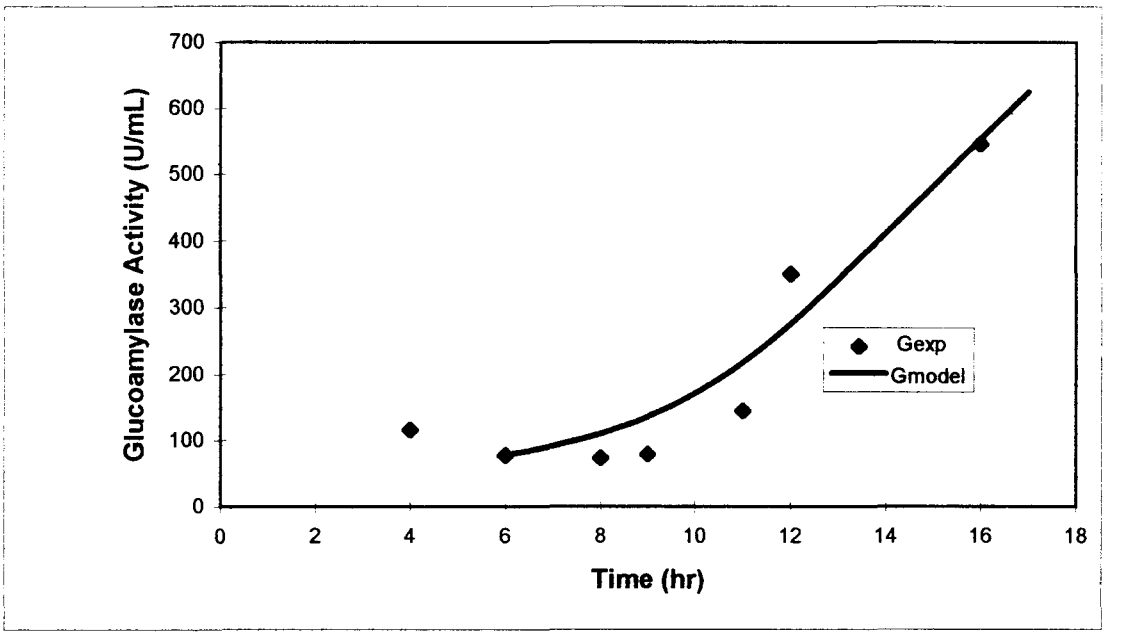
(a)


Substrate Concentration (g/L)
Time (hr)
Soexp
Sintm (0.50)
Sintm (0.75)
Sintm (0.90)
Sintm (1.00)
Somodel

(b)



(c)



(d)

FIGURE 4.64. Model Calibration for (a) Biomass Concentration, (b) Substrate Concentration, (c)Alpha-Amylase and (d) Glucoamylase Activities for YPB-G Grown on YPG Containing 50 g/L Glucose

TABLE 4.22. Summary of Experimental Results

Initial Glucose Concentration (g/L)	Final Biomass Concentration (g/L)	μ_{max} (h^{-1})	α-Amylase Activity (U/mL)	Glucoamylase Activity (U/mL)	Final Ethanol Concentration (g/L)
5*	1.66	0.047	700	350	nd
10*	1.75	0.157	700	460	2.87
25*	2.02	0.203	500	450	4.60
50**	4.78	0.249	200	545	25.40

nd: not detected, * shake flask and ** fermenter experiments.

4.9.2. Reaction Scheme

In this model, the substrate decomposes into an internal substrate (S) and an external substrate (So). The dynamic model consists of a set of differential equations: growth of the microorganism, transport of the substrate into the cell, consumption of the internal substrate, α-amylase and glucoamylase excretion (**Figure 4.65**).

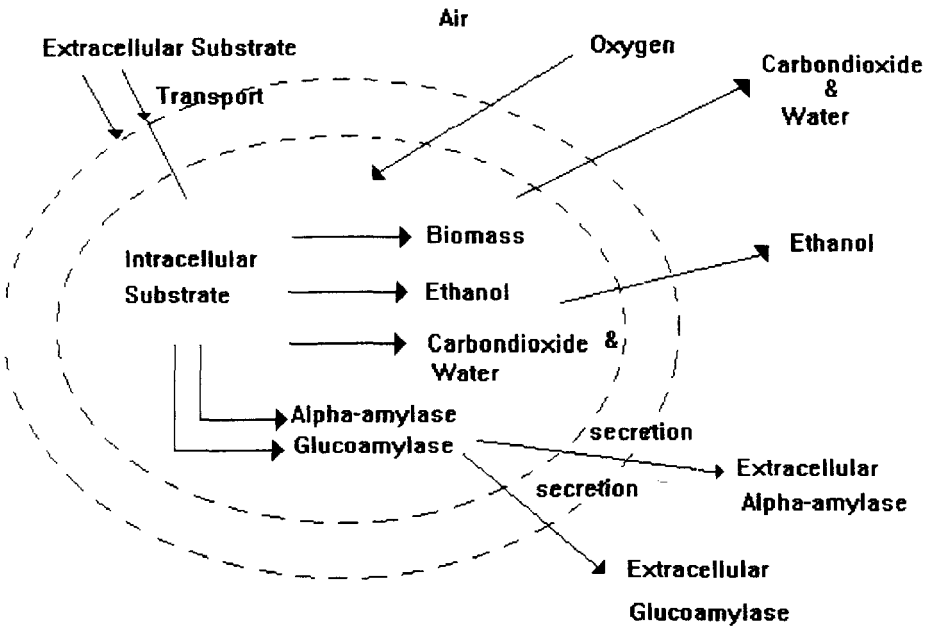


FIGURE 4.65. Representation of the Reaction Scheme

Cell Growth

The cellular growth is assumed to be modulated by the intracellular concentration of substrate, described by Monod's equation with a linear product formation term assuming no inhibition of product (Luong 1985). Furthermore, 100% plasmid stability was assumed.

$$\mu = \frac{\mu_{max} S}{Ks + S} \qquad (h^{-1}) \qquad (4.1)$$

$$\frac{dx}{dt} = \mu x (1 - KP) \qquad (\text{g biomass/ L/h}) \qquad (4.2)$$

where, x is the biomass (g/L), S is the internal substrate concentration (g/L), P is the ethanol concentration (g/L). μ_{max}, K_s and K are the model parameters in h^{-1}, g/L and g/L respectively.

The glucose is transported toward the cell by means of an active transport with saturation, where it is consumed (Montesinos et al., 1995). The active transport is the transport of the nutrients through the cell membrane into the cell against its chemical gradient, from regions of low to high concentration. This process requires metabolic energy.

Intracellular Substrate

$$\frac{dS}{dt} = -\frac{dS_o}{dt}\frac{1}{x} - Y_{sx}\mu - \mu S \quad \text{(g intracellular substrate/g biomass /h)} \qquad (4.3)$$

where S is in g/g/h and S_o and x are in g/L. Furthermore, μ and Y are in h^{-1} and g/g.

Extracellular Substrate

$$\frac{dS_o}{dt} = -\frac{k_{so}S_o}{K_{so} + So}x \quad \text{(g extracellular substrate/L /h)} \qquad (4.4)$$

Here, ks and Ks are in g/g/h and g/L respectively.

In differential equation in (4.14), corresponding to internal concentrations per unit biomass, has a term that takes into account the cellular growth contribution in the mass balance. When the microorganism grows, intracellular substrate is affected by a dilution factor, μS derived from the mass balancing. The total substrate added to the bioreactor (Z) can be considered to be equal to the external substrate (S_o), total internal substrate (xS), and the consumed substrate ($Y_{sx}x$).

$$Z = S_o(t) + x(t)(S(t) + Y_{sx}) \qquad (4.5)$$

Extracellular α-Amylase

Since, it is known that YPB-G strain secretes normally 96% of α-amylase and glucoamylase into the medium (de Moraes et al., 1995). The secretion is again described

by an active transport with saturation (Bailey and Ollis, 1986), assuming that a negligible amount of substrate is accumulated and 100% of the α-amylase is secreted.

$$\frac{dA}{dt} = \frac{k_A A}{K_A + A} x \tag{4.6}$$

Extracellular Glucoamylase

The glucoamylase secretion is also treated in the same way as α-amylase. Here again, 100% secretion is assumed. Since, YPB-G produces fusion proteins, and their action on the substrate is such that they complete each other. For that reason, it is reasonable to assume similar secretion efficiencies.

$$\frac{dG}{dt} = \frac{k_G G}{K_G + G} x \tag{4.7}$$

The target function(s) selected in the optimization methodology to obtain the parameter values was related to the least squares sum between experimental and simulated data. In other words, the objective function, J, was optimized:

$$J = \sum (y_i - y_\theta(x_i))^2 \tag{4.8}$$

where y_i's are the experimental, and $y_\theta(x_i)$'s are the simulated values with data x_i and the parameter set θ. Since the model equations are nonlinear in parameters, the optimization of J was done using the steepest descent method. Due to the nature of the steepest descent algorithm, a set of initial guesses was necessary. To ensure that the optimal parameter values reached at the end of the optimization procedure were the global ones, various initial points were used. The results obtained indicated that the global optima for the described model equations have been found.

The intracellular substrate concentration was treated by using two assumptions: (1) intracellular substrate concentration was assumed to be equal to the extracellular

(measured) substrate concentration or (2) transport of the substrate into the cell was assumed to take some time. Thus starting from the beginning of the exponential phase of growth allowed the assumption that the cells took only a certain fraction of the extracellular substrate inside. When the first assumption was considered, an overshoot was observed in the estimation of the biomass concentration at the stationary phase, and the intracellular substrate concentration exceeded the extracellular substrate concentration in the course of time. Therefore, a parametric study was conducted for the second assumption, trying various initial values for the intracellular to extracellular substrate concentration ratio and the most reasonable ratio for a good fit was found to occur at ca. 0.9.

Model parameters obtained with the optimization routine are tabulated in **Table 4.23**. Results obtained in the model calibration procedure for a batch fermentation with 50 g/L initial glucose concentration are presented in **Figure 4.64**. The model represents correctly the general behavior of standard fermentations, showing only slight differences between simulated and experimental results. The whole model was validated against experiments (shake flask experiments) that were not used in the parameter optimization (fermenter experiments) showed satisfactory results (**Figures 4.66**).

TABLE 4.23. Optimal Parameter Set Obtained on the Basis of Experimental Data with 50 g/L Initial Glucose

Parameter	Value
μ_{max}	0.308 (1/hr)
K_{ss}	1.369 (g/g)
K	0.0439 (L/g)
k_s	4.416 (g/g hr)
K_s	13.295 (g/L)
k_g	16.837 (U/mg hr)
K_g	29.490 (U/mL)
k_a	12.984 (U/mg hr)
K_a	150.82 (U/mL)

In order to determine the sensitivity of model predictions to the values of the parameters used to simulate cell growth and enzyme secretion, the value of each parameter was increased by 1% and the resulting relative absolute change in χ^2 is reported in **Table 4.24**. Changes in χ^2 significantly below the median indicate that the model results are not sensitive to that parameter value in comparison to the others, and thus are not needed by the model. Absolute changes are compared because the direction of changes in χ^2 does not have an impact on model sensitivity. Parameter changes in μ_{max}, K_{ss} and K were expected to affect the χ^2's of all the fits, since they were used in modeling the dynamics of x, and all other observables were modeled through x. While the 1% changes in other parameters would affect only the relevant observable. The results showed that the parameter K did not play an important role in the model, since the product showed up only at the stationary phase of growth. As a general tendency, model equations showed greater sensitivity to the parameters in the numerator, i.e., μ_{max}, k_g, k_a and k_s, rather than the ones in the denominator, i.e., K_{ss}, K_g, K_a and K_s., which is quite reasonable since the parameters in the numerator act as coefficient while the ones in the denominator are additive quantities. The results can be seen in **Table 4.24**.

TABLE 4.24. Sensitivity of model predictions to parameter value

		1% change in								
		μ_{max}	K_{ss}	K	k_s	K_s	k_g	K_g	k_a	K_a
	A	8.9	0.7	-	-	-	-	-	8.5	3.2
$\delta\chi^2$	G	2.2	0.1	-	-	-	2.6	0.3	-	-
(%)	X	10.7	0.9	-	-	-	-	-	-	-
	S	9.6	0.5	-	8.4	3.2	-	-	-	-

- no change

As can be seen from **Table 4.24**, K does not play an "active" role in the modeling of the system. However, it is affected by the system and the determined value predicts the reciprocal of the final product. This fact is obvious from equation (4.13), which can be written as,

$$P = \frac{1}{K}(1 - \frac{1}{\mu x}\frac{dx}{dt}) \qquad (4.20)$$

In the stationary phase of growth $\frac{dx}{dt}$ vanishes and,

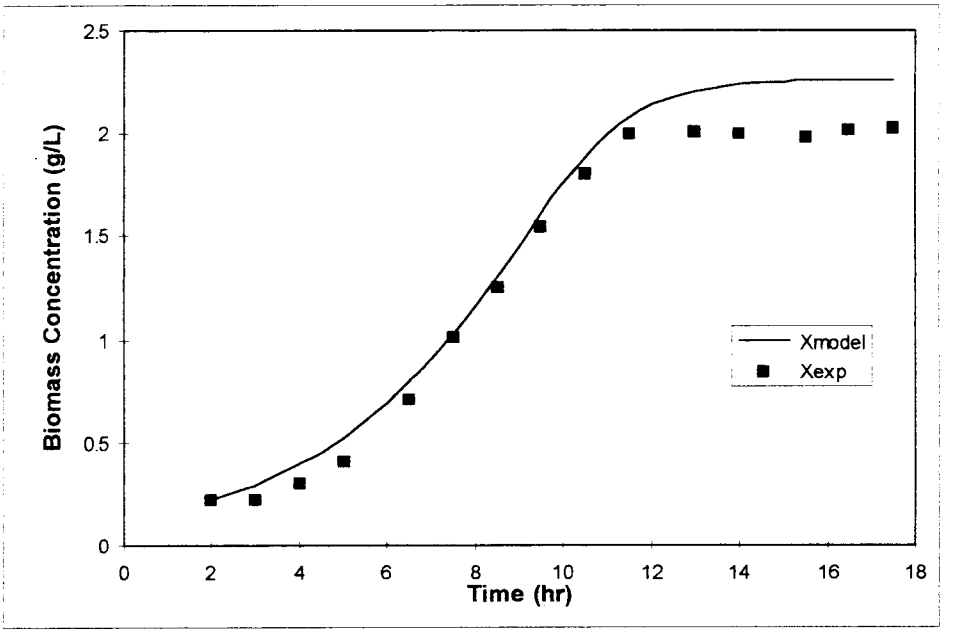
$$P_s = \frac{1}{K} \qquad (4.21)$$

This can be concluded that it is a "passive" parameter which leads to the final product of the system. Indeed, the parameter, K, was found to be 0.031 and the final product concentration for this experiment is 35 g/L, supporting the idea. A parametric study changing the initial glucose concentration should be performed to model K as a function of the initial glucose concentration.

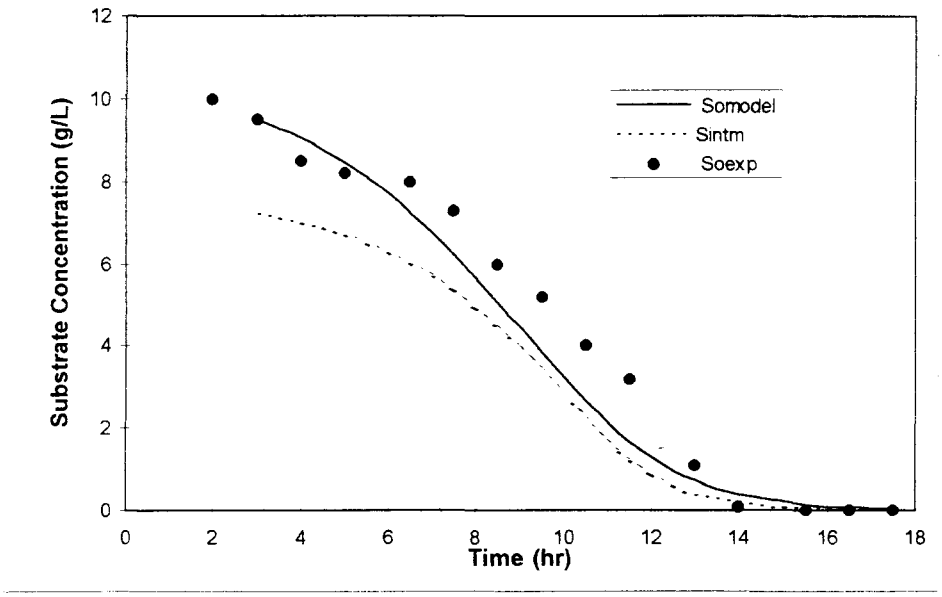
This part of the study provided to describe the enzyme and biomass production via a simple structured model in batch cultures of recombinant *S.cerevisiae* grown in YPG medium. With a simple description of transport and excretion phenomena, and the intracellular substrate concentration (S), the model was shown to be useful in explaining

the production process avoiding a sophisticated structured model with a high degree of complexity.

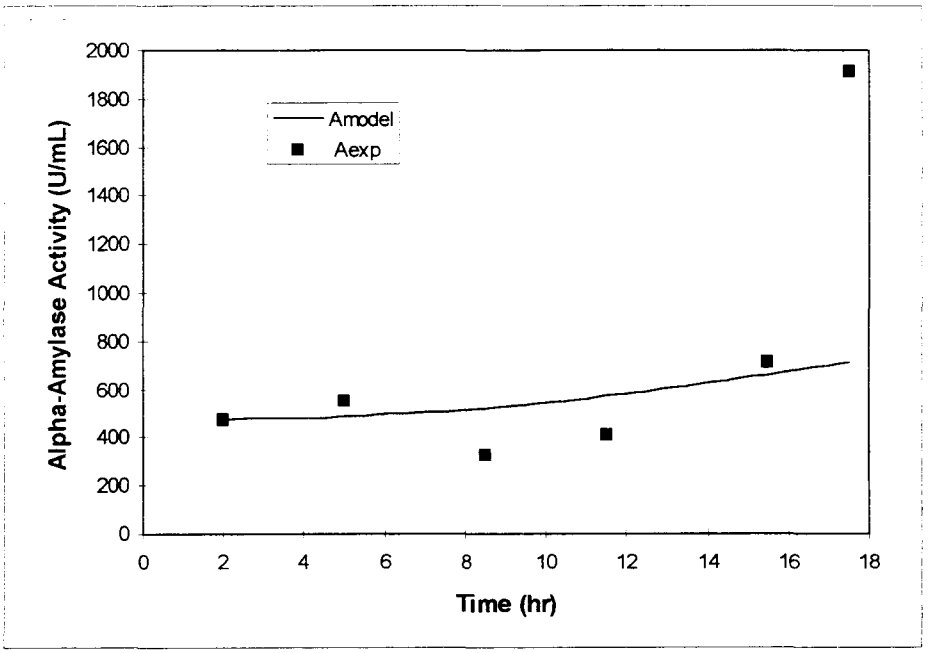
The model developed constitutes the first stage of modeling the growth and fermentation characteristics of the recombinant *S.cerevisiae* strains grown in starch containing media. It is important to know the behavior of the strains on glucose, keeping in mind the fact that starch is degraded via the enzymes into glucose for the utilization by the cells. The next step should be the correlation of the passage from starch to glucose, so that it will be possible to model the whole system beginning from starch. The model developed can be used to operate in the optimal range of substrate concentration and to design strategies of substrate feeding in order to improve enzyme production.



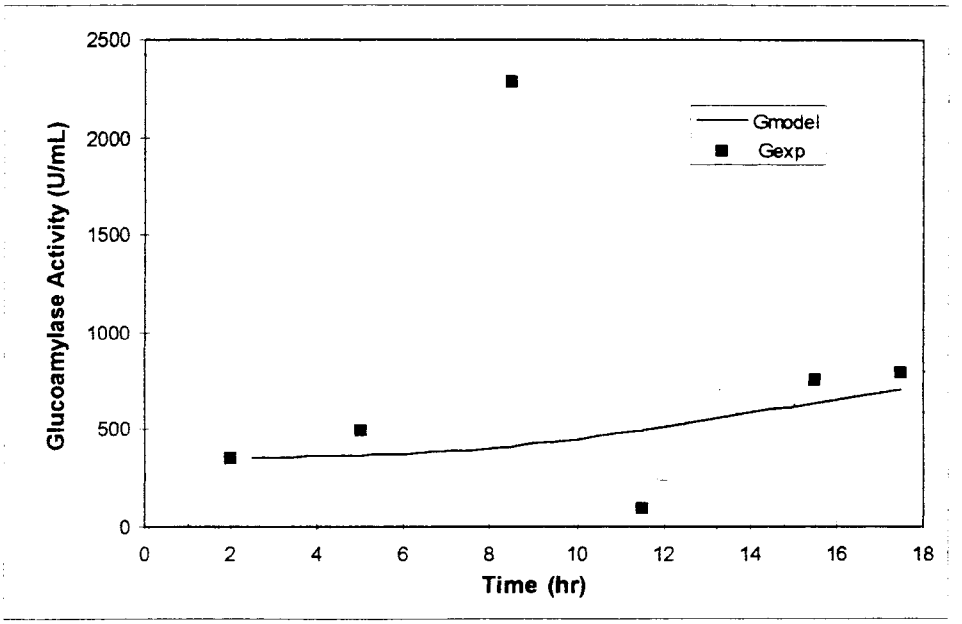
(a)


Substrate Concentration (g/L)
Time (hr)
Somodel
Sintm
Soexp
0
2
4
6
8
10
12
14
16
18

(b)


Alpha-Amylase Activity (U/mL)
Time (hr)
Amodel
Aexp
0
200
400
600
800
1000
1200
1400
1600
1800
2000
2
4
6
8
10
12
14
16
18

(c)



(d)

FIGURE 4.66. Model Verification showing (a) Biomass Formation, (b) Substrate Utilization, (c) Alpha-Amylase and (d) Glucoamylase for YPB-G grown on YPG Containing 10 g/L Glucose

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The major aim of this study was to investigate the growth, fermentation and ethanol production characteristics of four different genetically engineered yeast strains in various growth conditions. A comparative study was conducted on certain characteristics of the yeast strains. The following conclusions may be drawn on the basis of the results presented:

(1) The strains had different growth and fermentation characteristics in various growth conditions. YPG/AB and YA7 strains which secretes separate proteins (BsAase and GAAse) showed similar growth characteristics in all media types. They had high secretion efficiency of α-amylase and glucoamylase that give rise to ethanol production from starch. YPB-G strain which produces fusion proteins (BsAase and GAAse), also effectively used starch for its growth and ethanol production with a good secretion of the enzymes. On the other hand, YPG/MM that secretes separate proteins (MAAase and GAAse), could grow on starch, but due to the very low levels of glucoamylase secretion, it failed in the production of ethanol from starch.

(2) When comparing the growth characteristics of YPG/AB strain on YEP-Sg and YEP-S, the time for 50% utilization of initial starch was longer on YEP-S medium than YEP-Sg medium. Similarly, this strain has entered the exponential phase of its growth after a 10 hour lag period on YEP-Sg, while it took about 20 hours to finish its lag phase on YEP-S. The adaptation time of the strains to the starch containing medium was decreased to one half by the presence of glycerol in the medium. Whereas, the maximum specific growth rates were more than doubled in YEP-S without glycerol.

(3) Keeping parameters like medium formulation, medium sterilization and culture growth conditions constant, the optimum time of inoculum was found to be the late exponential phase in terms of biomass formation for YPB-G and YPG/AB strains. Furthermore, the initial inoculum size was found to influence the apparent lag time and the amount of biomass in the subsequent culture. An inoculum size of 10% was found to be the optimum for maximum biomass production and starch utilization.

(4) Mouse pancreatic and *B. subtilis* α-amylases have shown a wide range of activity. Mouse pancreatic α-amylase displayed a constant activity up to pH 7.00, then its activity decreased rapidly, while *B. subtilis* α-amylase showed remarkable pH stability between pH 2.70 to 9.25. In addition to that, the α-amylase from *B. subtilis* (YPG/AB) has shown considerable activity over the 20 - 40°C , then the activity decreased. Glucoamylase had a stable and high activity at 40°C and a decrease in the activity of the enzyme was observed at higher temperatures.

(5) The cell mass increased by a factor of 1.31 in both YPB-G and in YA7 when the initial glucose concentration was changed from 5 to 20 g/L. The use of a higher glucose concentration (50 g/L) did not result in any remarkable increase in the final cell mass which reached 3.23 g/L and 3.40 g/L in YPB-G and YA7 respectively, when they were grown in YNB-G medium. The behavior of protein production has displayed a parallelism to the biomass formation. As the biomass increased with increase in the initial glucose concentration, the protein concentrations also increased. YA7 strain showed a superiority over YPB-G strain in terms of secretion efficiencies of the proteins to the medium. The Yx/s and Yp/s values decreased with increasing initial glucose concentration for both strains. On the other hand, the Yp/x values showed that protein formation increased up to 20 g/L and 10 g/L for YPB-G and YA7 respectively, and it leveled off for YPB-G while it has decreased for YA7.

(6) Among the various kinetic models tested, the fit of the models to the data of the YA7 strain was either poor or there was no correlation in the data sets. However, the fits obtained for the YPB-G strain were quite good. As a general tendency, the models for YPB-G strain resulted in better fits with increasing initial glucose concentration.

(7) Batch cultures in shake flasks of the three strains (YPB-G, YPG/AB and YPG/MM) demonstrated that in each case superior growth was associated with media containing glucose. The ethanol production of the strains increased with increasing starch concentration in the medium resulting in higher levels of reducing sugar. Furthermore, YPG/AB strain was found to consume the reducing sugars more rapidly, while YPB-G and YPG/MM strains accumulated them even after 120 - 130 hours, thus showing a weak efficiency for the utilization of the hydrolysis products of starch. Ethanol was not produced by YPG/MM in starch containing media because of its very poor secretion of glucoamylase into the medium. YPG/AB strain, which secretes the enzymes separately, showed a higher ethanol productivity than YPB-G strain which secretes fusion proteins in YEP-S.

(8) From the results of shake flask experiments, It is found that the α-amylase activity of the YPG/AB strain is about 2.2 times higher than that of YPB-G, while the glucoamylase activities secreted by YPG/AB and YPB-G are at the same level. This indicates that the more efficient utilization of starch by YPG/AB strain is due to the higher levels of α-amylase activity in the medium. YPG/MM yields an α-amylase activity that is lower than that of YPB-G and almost no glucoamylase activity.

(9) All strains showed higher plasmid stabilities when grown on glucose containing media (YEP-G and YNB-G) in shake flasks. The studies on plasmid stability have indicated the superiority of YPG/AB which has displayed a plasmid stability of 80 - 85 % in YEP-S. A similar plasmid stability was also observed for the other two strains only in the medium containing low starch concentration. However, lower plasmid stabilities were detected in the medium containing higher concentration of starch.

(10) The use of lower starch concentration in the medium had no detectable effect on the maximum growth rates and/or biomass formation of YPG/AB, YPB-G and YPG/MM.

(11) When the cultures were grown in shake flasks, the production of ethanol was 43.8 g/L and 18.8 g/L for YPG/AB strain in YEP-S containing 100 g/L and 50 g/L starch

respectively. YPB-G strain did not produce ethanol when it was grown in YEP-S medium containing 50 g/L starch. However, in YEP-S medium containing 100 g/L starch, 35.2 g/L of ethanol production was observed. While, YPG/MM strain did not produce any ethanol in YEP-S media.

(12) From the experiments in fermenter, comparing the two modes of fermentation namely aerobic and anaerobic, the major difference observed was in the production of biomass for YPB-G: 4.22, 3.75 and 3.07 times higher biomass formations were achieved when the system was operated aerobically at 5, 20 and 26 g/L initial starch concentrations respectively. The maximum specific growth rates were three times larger than the maximum specific growth rates of anaerobic fermentation up to 20 g/L initial starch. At 26 g/L starch, however, the μ_{max} of both aerobic and anaerobic fermentations converged to the same value (0.033 ± 0.01 hr^{-1}).

(13) The protein secretions were similar in both fermenter modes for YPB-G strain. Oxygen supply to the system seems to decrease the level of protein secretion efficiency. The specific protein concentrations were 32.74, 19.91 and 17.20 mg protein /g cell dry weight under anaerobic fermentation, while these values were reduced to 5.47, 6.00 and 4.04 mg protein /g cell dry weight under aerobic conditions at 5, 20 and 26 g/L initial starch concentrations respectively. It can be concluded that protein secretion increases in the absence of oxygen.

(14) At higher biomass concentrations, higher RNA concentrations were recorded keeping the level of RNA concentration at ca. 10% of the total cell dry weight regardless of the growth conditions.

(15) At higher initial starch concentrations, plasmid stability decreased more rapidly in aerobic fermentation for YPB-G strain, than in anaerobic fermentation

(16) With a modification in the fermenter system, when the level of glucose and reducing sugar reached 3.76 g/L and 13.93 g/L respectively, ethanol began to accumulate in the fermenter and reached a level of 36 g/L.

(17) The model developed constitutes the first stage of modeling the growth and fermentation characteristics of the recombinant *S.cerevisiae* strains grown in starch containing media. It is important to know the behavior of the strains on glucose, keeping in mind the fact that starch is degraded via the enzymes into glucose for the utilization by the cells. The model represents correctly the general behavior of standard fermentations, showing only slight differences between simulated and experimental results. The whole model was validated against experiments (shake flask experiments) that were not used in the parameter optimization (fermenter experiments) showed satisfactory results

5.2. Recommendations

The recommendations that can be drawn in the light of this study for the future work is as follows:

The determination of glucose, reducing sugars and ethanol can be analyzed through HPLC which is advantageous in terms of time and in detection of all the polysaccharides.

The fermenter can be operated in fed-batch mode in order to maintain a certain level of starch in the system to enhance ethanol production.

The fermenter can be operated by the addition of glucose into the starch containing medium rather than the addition of the commercial α-amylase and glucoamylase to reduce the cost for the production of ethanol under fermenter conditions.

The recombinant strains could be tested by growing them in raw starch containing media.

A parametric study in terms of temperature and rpm may be conducted in order to achieve higher levels of enzymes' activities which might lead to higher levels of ethanol production.

The mathematical model developed could be improved by taking other intracellular components into account such as intracellular RNA concentration and plasmid DNA concentrations. Also, as the next step, the correlation of the passage from starch to glucose via the enzymes can be modeled, so that it will be possible to model the whole system beginning from starch to ethanol production.

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APPENDIX A. DATA ANALYSIS APPROACH

A.1. Data Treatment

When models of the type described in the following section are concerned, one has to consider the time derivatives of the measured cell concentrations. This differentiation is to be done numerically. Numerical differentiation is to be avoided whenever possible, especially when the data are subject to experimental error; since differentiation is a roughening process, errors in measurements can affect numerical derivatives. To diminish this effect, it is advised to smooth the data before differentiation (Perry and Green, 1985). Furthermore, since some of the kinetic models require data points taken at equally spaced time intervals, smoothing of raw data is necessary.

A.1.1. Smoothing

Most common smoothing techniques involve the approximation of the tabular data by a least-squares fit of the data by using some known functional form such as a polynomial. Instead of approximating a set of measurements x_i by a single least squares polynomial of degree n over the entire range of tabulation, it is often more suitable to replace the tabulated value, by the value taken on by a least-squares polynomial of degree n relevant to a sub-range of $2M-1$ points centered, when possible, at the point for which the entry is to be modified. The method necessitates the measurements to be made in equal time intervals. For instance, when M was 1, one should consider 3 data points, hence 2 time intervals and the smoothed values were computed from (Perry and Green, 1986)

$$y_0 = \tfrac{1}{6}(5x_0 + 2x_1 - x_2),$$

$$y_1 = \tfrac{1}{3}(x_0 + x_1 + x_2), \qquad \text{(A.1)}$$

$$y_2 = \tfrac{1}{6}(-x_0 + 2x_1 + 5x_2).$$

For those measurements that do not have equal time intervals with the successors or predecessors, no smoothing was done. On the other hand, data were checked to see whether they belong to a set taken with equal time intervals for *M=2* and *M=3*, for which the smoothed values were computed from,

$$y_0 = \tfrac{1}{5}(3x_0 + 2x_1 + x_2 - x_4),$$

$$y_1 = \tfrac{1}{10}(4x_0 + 3x_1 + 2x_2 + x_3),$$

$$y_2 = \tfrac{1}{5}(x_0 + x_1 + x_2 + x_3 + x_4), \qquad \text{(A.2)}$$

$$y_3 = \tfrac{1}{10}(x_0 + 2x_2 + 3x_3 + 4x_4),$$

$$y_4 = \tfrac{1}{5}(-x_0 + x_2 + 2x_3 + 3x_4)$$

and,

$$y_0 = \tfrac{1}{42}(39x_0 + 8x_1 - 4x_2 - 4x_3 + x_4 + 4x_5 - 2x_6),$$

$$y_1 = \tfrac{1}{42}(8x_0 + 19x_1 + 16x_2 + 6x_3 - 4x_4 - 7x_5 + 4x_6),$$

$$y_2 = \tfrac{1}{42}(-4x_0 + 16x_1 + 19x_2 + 12x_3 + 2x_4 - 4x_5 + x_6),$$

$$y_3 = \tfrac{1}{21}(-2x_0 + 3x_1 + 6x_2 + 7x_3 + 6x_4 + 3x_5 - 2x_6), \qquad \text{(A.3)}$$

$$y_4 = \tfrac{1}{42}(x_0 - 4x_1 + 2x_2 + 12x_3 + 19x_4 + 16x_5 - 4x_6),$$

$$y_5 = \tfrac{1}{42}(4x_0 - 7x_1 - 4x_2 + 6x_3 + 16x_4 + 19x_5 + 8x_6),$$

$$y_6 = \tfrac{1}{42}(-2x_0 + 4x_1 + x_2 - 4x_3 - 4x_4 + 8x_5 + 39x_6),$$

respectively. Furthermore, whenever a data point happened to fall in a range where more than one smoothing value were applicable; (*i*) the smoothed value coming from the set with higher M was considered and (*ii*) the smoothed value arising from equal M's were averaged.

A.1.2. Differentiation

After smoothing the measured data, derivatives were taken making use of the Lagrange interpolation formula (Perry and Green, 1986). If three succeeding measurements were made with equal time intervals, three-point formulas,

$$\dot{x}_0 \approx \tfrac{1}{2h}(-3x_0 + 4x_1 - x_2),$$

$$\dot{x}_1 \approx \tfrac{1}{2h}(-x_0 + x_2), \qquad \text{(A.4)}$$

$$\dot{x}_2 \approx \tfrac{1}{2h}(x_0 - 4x_1 + 3x_2),$$

were used, where h was the time increment between the measurements. Similarly, for five succeeding measurements that were recorded with equal time intervals, five-point formulas,

$$\dot{x}_1 \approx \frac{1}{12h}(-25x_1 + 48x_2 - 36x_3 + 16x_4 - 3x_5),$$

$$\dot{x}_2 \approx \frac{1}{12h}(-3x_1 - 10x_2 + 18x_3 - 6x_4 + x_5),$$

$$\dot{x}_3 \approx \frac{1}{12h}(x_1 - 8x_2 + 8x_4 - x_5), \qquad \text{(A.5)}$$

$$\dot{x}_4 \approx \frac{1}{12h}(-x_1 + 6x_2 - 18x_3 + 10x_4 + 3x_5),$$

$$\dot{x}_5 \approx \frac{1}{12h}(3x_1 - 16x_2 + 36x_3 - 48x_4 + 25x_5),$$

were used. For the data points recorded with inhomogeneous time steps, the crude Euler approximation,

$$\dot{x}_1 \approx \frac{1}{h}(-x_1 + x_2) \qquad \text{(A.6)}$$

or,

$$\dot{x}_2 \approx \frac{1}{h}(-x_1 + x_2) \qquad \text{(A.7)}$$

was used, if it was a first or a last point in such a sequence respectively. If more than one estimate were applicable to a single data point, the average of the highest order estimations was taken as the estimate for the derivative.

A.1.3. Interpolation

To make the time intervals homogeneous, Newton's fundamental interpolating formula (Perry and Green, 1986) with divided differences was used:

$$\begin{aligned} f(x) \approx\ & f(x_0) + (x - x_0) f[x_0, x_1] + \\ & (x - x_0)(x - x_1) f[x_0, x_1, x_2] \\ & + \cdots + (x - x_0)(x - x_1) \cdots \\ & (x - x_{n-1}) f[x_0, x_1, x_2, \cdots, x_n] + E_n(x), \end{aligned} \tag{A.8}$$

where the error term $E_n(x)$ can be computed from,

$$E_n(x) = \frac{1}{(n+1)!} f^{(n+1)}(\varepsilon)\pi(x), \tag{A.9}$$

with,

$$Min\{x_0 \cdots x_n, x\} < \varepsilon < Max\{x_0 \cdots x_n, x\}$$

and,

$$\pi(x) = (x - x_0) \cdots (x - x_n). \tag{A.10}$$

A.1.4. Goodness-of-fit

After obtaining the differentiated data, the parameter set, θ, was estimated for a selected kinetic model. The estimated parameters were then passed through the χ^2-test to determine the *goodness-of-fit* (Bevington and Robinson, 1994). The model equations were integrated to obtain estimates, $y_\theta(x_i)$, for the experimental data, y_i, at the measurement times. Next, the χ^2 value was calculated from

$$\chi^2 = \frac{1}{\nu}\sum (y_i - y_\theta(x_i))^2 \qquad \text{(A.11)}$$

and the degree of freedom ν from,

$$\nu = N - m$$

where N is the number of data points and m is the number of parameters in the model.