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A study on the α-amylase fermentation performed by *Bacillus amyloliquefaciens*

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Abstract

α-Amylase fermentation with soluble starch as the limiting substrate was performed by *Bacillus amyloliquefaciens*. We investigated the influence, of the presence of α-amylase, on the hydrolysis of different starch concentrations. Both the initial and average rates for the hydrolysis of starch under different initial conditions were compared and calculated. The time for the completion of starch hydrolysis was also obtained. Effects of kinetic factors, such as pH of 5.5, 6.3, 7.0 and temperature of 30 °C and 37 °C, on the performance of α-amylase fermentation were studied. The influence of the dynamic variables, such as agitation speed and aeration rate, on this fermentation were also evaluated and are discussed. The variations of cell mass, α-amylase activity, and reducing sugar with regard to the step changes of aeration rates during a batch fermentation are also discussed. The optimal conditions of these variables have been determined. © 1997 Elsevier Science S.A.

Keywords: *Bacillus amyloliquefaciens*; α-amylase; Substrate; Starch; Hydrolysis; Aeration rate

1. Introduction

α-Amylase (E.C. 3.2.1.1) is a very important industrial enzyme in many food and textile processes. It can be secreted either extracellularly or intracellularly by different species of microorganisms. It breaks down the α-1,4 glucosidic bonding of linear amylose and branching amylopectin which are the major building blocks of starch. The viscosity of starch solutions can thus be reduced using α-amylase. It is often applied in the industrial process of starch liquefaction. For industrial applications, α-amylase may in some cases be immobilized to promote the operating efficiency of the process. The immobilization methods for starch conversion have been studied by Linko et al. [1] and Srivastava [2].

In the presence of α-amylase, starch can be degraded to limit dextrin of α-1,6 branches, linear-chain oligosaccharides, maltose, and glucose. However, the formation of glucose and maltose is rather slow since they are the final basic units which cannot be further degraded by α-amylase. Whenever α-amylase is secreted from the producing cells, it is soon adsorbed onto the surface of starch particles, with maximum adsorption being achieved in 2 h [3]. Somers et al. [4] studied the activity of α-amylase from two different species, *Bacillus subtilis* and *Bacillus licheniformis*. The enzyme adsorption kinetic parameters of the α-amylase enzyme onto the starch substrate were estimated. Bacterial α-amylase can be produced from different *Bacillus* species and also from engineered *E. coli* [5,6]. An engineered *Bacillus licheniformis* was screened by Bajpai and Sharma which can produce high-temperature resistant α-amylase [7]. Ca-Alginate immobilized cells for continuous production of α-amylase have also been studied [8].

In addition to the *Bacillus* species often used for α-amylase production, *Aspergillus oryzae* was also used to produce α-amylase under the continuous mode of operation. The metabolic regulation of the glucose uptake by this *Aspergillus* species was investigated [9]. The morphology as well as the physiology of the *Aspergillus oryzae* during the α-amylase batch fermentation was also studied [10]. Sudo et al. [11] also used the species of *Aspergillus*, by submerged culture, to produce α-amylase. French et al. [12] proposed a simple two-stage recovery method to efficiently release a recombinant *Streptomyces thermoviolaceus* α-amylase from the periplasm of *E. coli*. The fraction recovery method they developed has the potential for scale-up as the scale of 5.0 l being performed up to then. *Pseudomonas*, with a membrane recycle bioreactor, was used to convert starches. Simultaneous production and purification were involved in one single step [13]. Shiba et al. [14,15] used recombinant *Saccharomyces cerevisiae* to produce α-amylase. A fuzzy controller was applied to regulate glucose and ethanol concentrations

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during fed-batch fermentation so that the expression of α-amylase can be improved. Thermostable α-amylase formed by *Saacharomonospora viridis* can hydrolyze starch to obtain rather high level of maltose. The mechanism of starch hydrolysis by α-amylase was also examined.

Yoo et al. [16] used maltose and glucose as substrates for α-amylase fermentation. They investigated the reaction kinetics of the fermentation. Maltose was found to have no induction on the excretion of α-amylase and glucose showed catabolite repression on the formation of α-amylase, which has been a very common behavior observed in many fermentation systems.

The effect of different operational factors such as pH and temperature on the activity of α-amylase and also the influence of polyalcohols and carbohydrates on the thermostability of this enzyme has been studied [17,18]. The kinetics using glucose as a substrate for α-amylase fermentation has also been discussed. Factors affecting the production of α-amylase fermentation and also the enhancement and the optimization of the production have all been investigated and reported in the literature [19–22]. Kole and Gerson [23] found in their work that ammonium concentration shows a significant effect on the growth yield of both bacteria and yeast. Accordingly, they provided an ammonium controlled fed-batch fermentation for the production of α-amylase.

The effect of yeast extract on the growth of cell mass and the formation of amylase without pH control was studied by Alam et al. [24]. Gandhi and Kjaergarrd [25] found that proper supply of CO_2 would stimulate the formation of amylase and also increase the enzyme activity while overfeeding of CO_2 would inhibit the growth of the α-amylase producing cells, *Bacillus subtilis*. The continuous production of α-amylase by *Bacillus amyloliquefaciens* has been carried out by immobilizing the cells in an anionic exchange resin (Rohm and Haas Amberlite XE-352) [26].

The rheological characteristics of a pseudoplastic starch suspension formed during α-amylase fermentation were measured. The oxygen transfer coefficient was evaluated [27] and discussed. The operations under different programmings of mixer revolution rates, therefore different oxygen transfer rates, were discussed in terms of power consumption. Cheese whey can be a carbon source for α-amylase production. The medium and culture were both optimized by Pajpai et al. [28] By applying a high aeration rate of 2.2 vvm, very high α-amylase yield can be achieved from the optimized medium. Milner et al. [29] found that higher aeration rates were good to the yield of α-amylase. However, a foaming problem resulted. They also discussed that the foaming problem and therefore the amount of added antifoam could be reduced from the control of dissolved oxygen tension (DOT). Nevertheless, the yield of enzyme may not be benefited thereby.

Studies of the effects of aeration on α-amylase fermentation form the basis of this article. The related kinetic variables were also studied and starch, rarely used in the study of α-amylase fermentation, was used as the sole substrate in this work. How the α-amylase affected the hydrolysis of the starch is also discussed.

2. Materials and methods

2.1. Microorganism

Bacillus amyloliquefaciens (ATCC 23350, CCRC 10268) was obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan. *Bacillus amyloliquefaciens* was kept by slant cultures in a 4 °C refrigerator and transferred to another new agar plate every 2 weeks.

2.2. Medium and cultures

The medium composition used for batch fermentation experiments was yeast extract, 1.0 g l^{-1}; K_2HPO_4, 9.0 g l^{-1}; KH_2PO_4, 2.0 g l^{-1}; $(NH_4)_2SO_4$, 5.0 g l^{-1}; sodium citrate, 1.0 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 g l^{-1}; $CaCl_2 \cdot 2H_2O$, 0.01 g l^{-1}; $FeSO_4 \cdot 7H_2O$, 0.045 g l^{-1}; $MnSO_4 \cdot 7H_2O$, 0.001 g l^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.001 g l^{-1}.

Cells were precultured twice before each fermentation. Preculture was carried out in a shaker bath (Hotech Instruments Corp., Model 903, Taipei, ROC) using a working volume of 100 ml. Each preculture lasted for around 10 h. 2 ml (2% (v/v)) of seed culture was inoculated for each batch of cell culture. Temperature was kept at 37 °C and the shaking speed was set at 110 rpm.

2.3. Analytical methods

2.3.1. Cell density

Cell concentration was determined by measuring the absorbance of the sample at 550 nm with a UV/VIS spectrophotometer (UV 160A, Shimadzu Co., Kyoto, Japan). A calibration curve was obtained for linear correlation of the measured absorbance with the dry cell weight per unit volume of sampled broth. Filter paper containing cells from centrifugation was placed in an 80 °C oven till the weight no longer changed. The dry cell weight was thus obtained. A proportion factor of 0.23 was used to convert the measured absorbance to cell density. The value of optical density (O.D.) for the maximum range of linear calibration is 0.45. Cell suspensions with an O.D. value of higher than 0.45 were properly diluted before measurement.

2.3.2. Reducing sugar

Concentration of reducing sugar was measured by the DNS method [30]. Sugars with a functional group of CHO have reducing power and can thus convert 3,5-dinitrosalicylic acid of the DNS reagent into 3-amino-5-nitrosalicylic acid. The reaction was carried out in boiled water for 5 min. This was then diluted with deionized water and placed in cool running water to stop the reaction.

2.3.3. Starch

A 0.1 N KI–I_2 solution was prepared and 5 ml of this was mixed with 5 ml 1 N HCl and deionized water was added to 100 ml. 0.1 ml of cell-free fermentation broth was added to 1 ml of KI–I_2–HCl solution. The resultant solution was then diluted with 10 ml of deionized water. The absorbance of the prepared sample solution was measured at the UV wavelength of 550 nm. A linear correlation below O.D. value of around 0.2 or equal to the starch concentration of around 60 g l^{-1} was observed. For starch concentrations higher than 60 g l^{-1}, proper dilution was required before the measurements.

2.3.4. α-Amylase

One unit of α-amylase activity is defined as the reducing sugar formation rate by 0.5 ml enzyme solution to hydrolyze 0.1 mg (1% w/v) starch in 10 min under the reaction conditions of 37 °C, pH 7.0, and a shaking speed of 50 rpm. In other words, the α-amylase activity is measured by the hydrolysis rate of starch. The reaction can only occur when α-amylase is present. The extent of reaction can be measured by the liquefying power, or saccharifying power, or dextrinizing power [31]. The enzyme activity was measured by its dextrinizing power in this work.

1% Phosphate buffered starch solution was used as the substrate of this enzymatic reaction for the assay of enzyme activity. The reagent is I_2–KI solution (0.5%:5%). 5 ml of 1% starch solution was incubated in a 37 °C water bath. 0.5 ml sampled enzyme solution was added to the starch solution. The intensity of the blue color of the I_2–KI solution diminished corresponding to the decreasing starch concentration. After 10 min, 5 ml of 0.1 N HCl was pipetted into the reaction solution. 0.5 ml of such solution was mixed with 5 ml KI–I_2 reagent and the absorbance of the solution was measured at the UV wavelength of 580 nm.

2.4. Shake flask cultures

Bacillus amyloliquefaciens was cultured in a 250 ml shake flask with a working volume of 150 ml for each batch experiment. The shaker bath was set at 37 °C with a shaking speed of 110 rpm. The initial pH was 7.0. Samples were taken every hour and measured for optical density. They were then centrifuged to obtain a cell-free culture broth for assays of reducing sugars and enzyme activity.

2.5. Fermentor studies

Fermentations were carried out in a 2.5 l fermentor (MBF-250, Eyela Co., Japan) with a working volume of 1.7 l. The agitator was composed of three impellers at equal distance in vertical axis. The impellers were the type with six flat blade turbines. The diameter of the central portion of the impeller was 30 mm and each of the turbine was 11 mm × 8 mm. Maximum power of the agitator was 40 W. The inoculum of *Bacillus amyloliquefaciencs* from the double precultures was 2% (v/v). Starch was used as the major carbon source of the fermentations and different initial starch concentrations were used for different batches.

The fermentation operating conditions were normally set at 37 °C, 300 rpm, 0.5 l min^{-1} (0.3 vvm) of aeration. The fermentation pH was controlled at 7.0 by automatically feeding with 1.0 N NaOH solution. Diluted antifoaming agent (KM-70, Hsin-Yu Co., Tokyo, Japan) was added when the foaming occurred since the foaming effect would be harmful to the α-amylase activity. Samples were taken regularly for the measurement of cell density. The cell-free broth from centrifugation was then prepared for assays of starch concentration, reducing sugar concentration, and α-amylase activity. The whole system for the batch fermentation is shown in Fig. 1.

3. Results and discussion

Very few articles refer to using starch as the only carbon source. However, starch, a rather important, cheap, and abundant natural product, is found widely in many crops, especially, cereals and potatoes. It can be a very good raw material carbon source for α-amylase fermentation. Fermentations using different kinds of saccharides as well as starch as the substrate for the excretion of α-amylase have been compared in our previous work. Starch was then chosen as the best substrate based on several aspects such as specific cell growth rate, enzyme activity level, specific enzyme activity level, specific enzyme formation rate, etc. However, how the presence of α-amylase affects the hydrolysis of starch has not yet been studied. The effect from regulating aeration rate on the excretion of α-amylase has not been investigated either. Work according to these two considerations is therefore included in this article. A study on the decay rate of this enzyme is also discussed as part of this work, and a comparison of the effect of different kinetic factors as well as the dynamic factors is also made.

3.1. α-Amylase thermal stability

In our previous work, α-amylase showed very good thermal stability. Among different temperatures ranging from 30, 35, ..., 80 °C, the enzyme appeared more stable at 65 °C. When the enzyme was placed at a temperature higher than 65 °C, it was rapidly deactivated. If it was boiled in hot water for only a few minutes, the activity was irreversibly destroyed. However, when stored at 0 °C, the enzyme activity was retained for quite a long time as shown in Fig. 2. In other words, the enzyme stability can be well maintained for at least 180 h. Nevertheless, it can also be observed from Fig. 2 that as the enzyme was placed at 37 °C, activity decayed quickly. Although the α-amylase fermentation was normally carried out at 37 °C which is optimal for cell physiology and growth, α-amylase activity appeared to decay at this temperature.

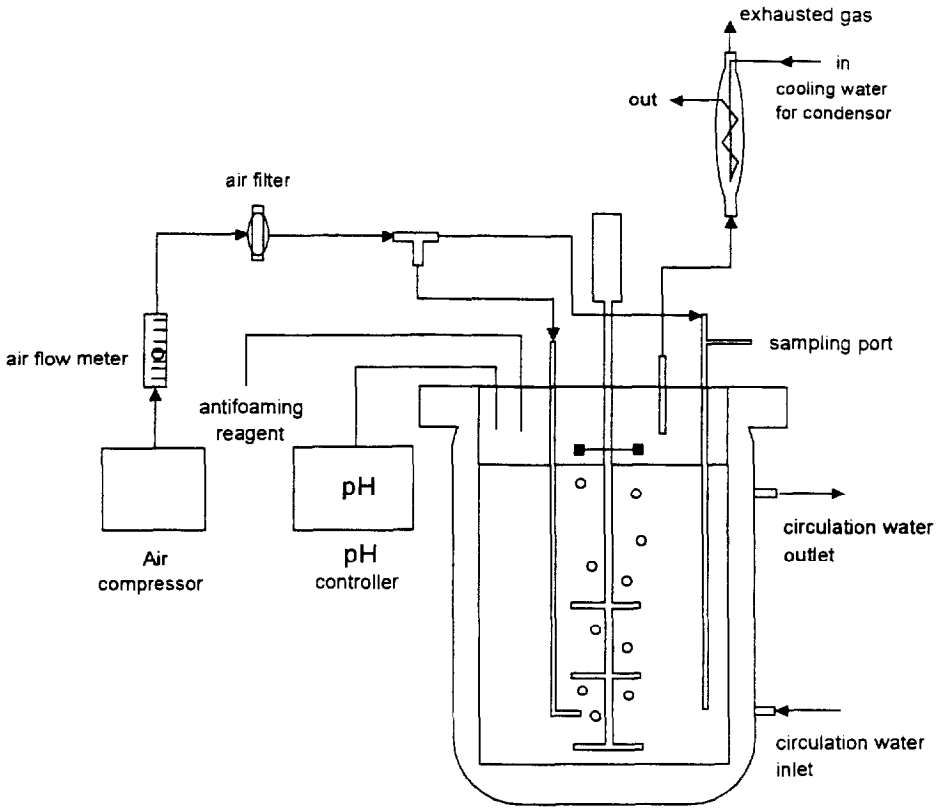

Fig. 1. The fermentation system.

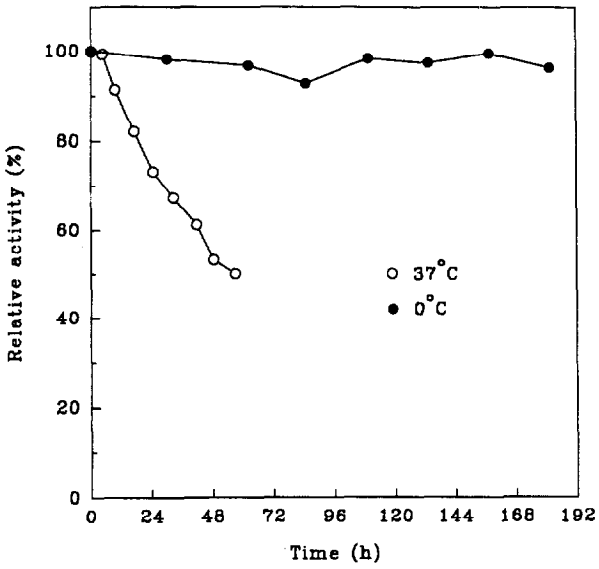

Fig. 2. Temperature effect on α-amylase stability.

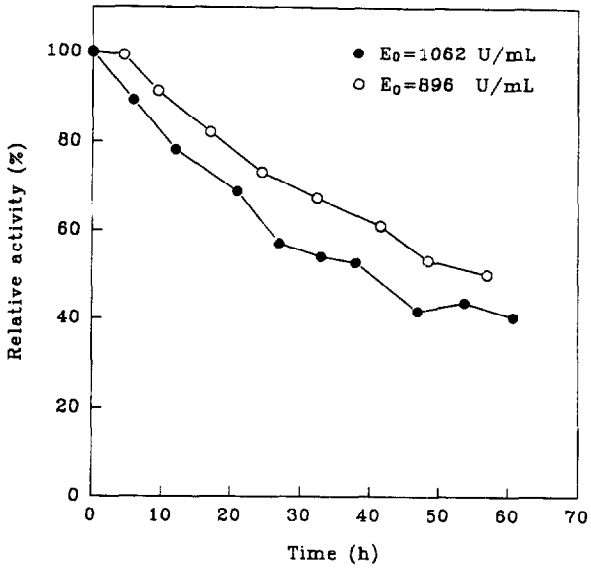

Fig. 3. Decay of α-amylase activity at different initial enzyme activities at 37 °C.

3.2. Decay rate of α-amylase

Deactivation of α-amylase under different initial enzyme activity levels at 37 °C is shown in Fig. 3. It should be noted that relative activity instead of absolute activity was used in the figure for better observation of the extent of decay. The initial enzyme activities were 896 U ml^{-1} and 1062 U ml^{-1}, respectively. The decay behavior of α-amylase can be described by an expression of first order from theoretical analysis. The decay rate of this enzyme at 37 °C was found to be dependent on initial enzyme activity with the higher initial activity decaying faster. The decay rate also depended

on the initial starch substrate concentration as discussed in the following section.

3.3. Effect of α-amylase on the hydrolysis of starch

To understand how the presence of α-amylase affects the hydrolysis of starch, diluted α-amylase was added to the starch solution. In Fig. 4, the starch solution was very stable at room temperature without adding any α-amylase. As α-amylase with very dilute activity of 31.7 U ml^{-1} was presented, the hydrolysis soon occurred and starch disappeared rapidly. The conclusion can be made that the hydrolysis of starch occurs even under a very low activity level of α-amylase.

The rate of enzymatic hydrolysis of the starch was also governed by the initial concentration of starch substrate. As the starch concentration was increased, the hydrolysis rate increased accordingly. Fig. 5 is the hydrolysis of different initial starch concentrations at the existence of low activity level of α-amylase. The rate of hydrolysis was very fast and for starch substrate concentrations lower than 100 g l^{-1}, the reaction can be completed within 6 h.

Different enzyme activity levels (E_o) of 31.7 U ml^{-1} and 15.8 U ml^{-1} for the enzymatic reaction at different initial starch concentrations (S_o) of 10 g l^{-1} and 42 g l^{-1} were compared. It was observed that at the enzyme level of 15.8 U ml^{-1} and initial starch concentration of 42 g l^{-1}, the average reaction rate was comparably much slower, which corresponded to a much longer reaction time. However, the initial rate for starch hydrolysis was only slightly higher. Normally, higher enzymatic reaction rates are obtained from higher initial substrate concentrations and higher initial enzyme activity. It appears that the higher initial starch concentration of 42 g l^{-1} was sufficient to conquer the opposite effect from the lower initial enzyme activity of 15.8 U ml^{-1}. Both the initial rate and average rate of hydrolysis with values

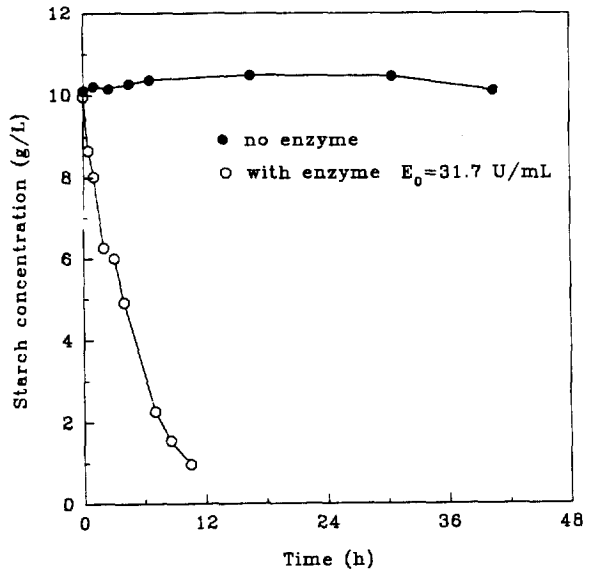

Fig. 4. Comparison of starch hydrolysis with and without α-amylase.

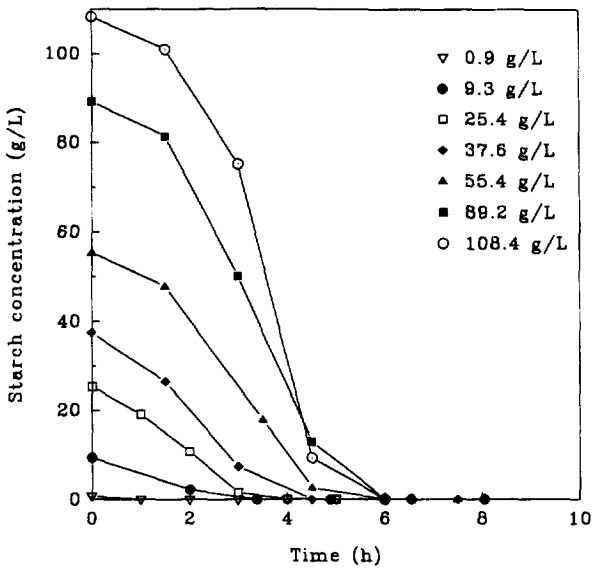

Fig. 5. Starch hydrolysis at different initial starch concentrations.

of 1.13 g l^{-1} h^{-1} and 0.81 g l^{-1} h^{-1} respectively were higher for the condition of $E_o = 15.8$ U ml^{-1} and $S_o = 42$ g l^{-1} than for $E_o = 31.7$ U ml^{-1} and $S_o = 10$ g l^{-1} h (1.11 g l^{-1} h^{-1} and 0.59 g l^{-1} h^{-1} respectively).

3.4. Comparison of shake flask and fermentor cultures

The fermentations from both the shaker culture and the fermentor are compared in Fig. 6(a)–(d). Initially, provided with 9.2 g l^{-1} of starch substrate, the growth of cells was almost the same before the first 18 h, after which a faster growth rate occurred and a higher cell density was reached in the fermentor. The hydrolysis rate of starch was observed to be almost the same and the reaction was rapidly completed, in the first 3 h. Therefore, the disappearance of starch is from hydrolysis through the existence of enzyme rather than by consumption of the cells.

The α-amylase activity increased almost at the same rate in both systems initially. After 18 h, however, excretion rates became different and a much higher maximum enzyme level was reached in the fermentor. The enzyme stability was also better maintained and the activity decayed slower in the fermentor as can be realized from the well maintained operation conditions. Comparing Fig. 6(a) and 6(c), the excretion of α-amylase appears to be largely growth associated.

The reducing sugar concentration increased within the first 6 h due to the hydrolysis of starch. At this stage, the cell physiology can be enhanced by the hydrolysis of starch caused by the enzyme. The starch was degraded by the enzyme so that edible sugar was obtained. The formation of edible sugar can then supply the growth of cells and therefore the further excretion of α-amylase. Continued formation of α-amylase can further degrade the α-1,4 bonding of the different saccharides and therefore stimulate the growth of cells as well as the excretion of α-amylase. However, the cells need edible sugar as the carbon source to grow. Therefore,

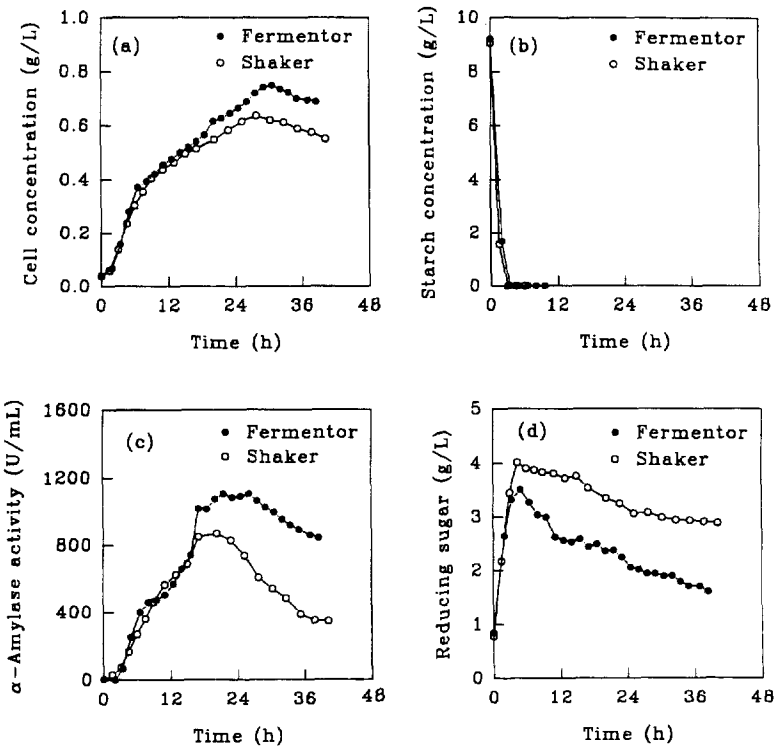

Fig. 6. Comparison of the results from shake culture and fermentor. (a) Cell concentration ($g\,l^{-1}$), (b) starch concentration ($g\,l^{-1}$), (c) α-amylase activity ($U\,ml^{-1}$), (d) reducing sugar concentration ($g\,l^{-1}$).

when the edible sugar was formed, it would then be consumed by the growing cells. When the cells grew to a certain population density for the utilization of more reducing sugars and the breakage of the sugars gradually slowed down, then the reducing sugar concentration gradually decreased. A faster consumption rate of the sugar occurred in the fermentor due to the faster growth of cells. Nevertheless, It can be observed from the profile of the reducing sugar that the sugar concentration first increased due to the hydrolysis of starch and then decreased due to the faster sugar consumption and slower or even no hydrolysis of the saccharides with molecular weight distribution.

3.5. Agitation

The fermentations were carried out at the same initial conditions but with different agitation speeds of 220, 300, 400 rpm, respectively (Fig. 7(a)–(d)). It can be seen from the figures that the agitation speed did not affect the fermentation performance very much. The degradation of starch concentration and the formation of reducing sugar were nearly the same at different agitations. As reported above, hydrolysis can occur even at very low levels of enzyme, so the disappearance of starch should be expected to be almost the same. However, a slightly difference can still be observed for the formation and consumption of sugar. The consumption of sugar at the speed of 400 rpm was slightly more than that at 300 rpm. The consumption rate at the speed of 220 rpm was the slowest among these agitations. Therefore, different agitations seemed to provide different distribution and transportation of air and nutrients to the cells. As a result, a faster growth of cells occurred at 400 rpm, and therefore, a corresponding higher formation rate of α-amylase was observed. Nevertheless, it seemed that the best stability of both cell density and enzyme activity was maintained at the agitation speed of 220 rpm. The better maintenance of enzyme activity might be caused from the mild agitation so that the enzyme was not damaged. However, the better maintenance of cell density at stationary phase might be due to the smaller population so that the utilization of nutrients was still sufficient to maintain the cells alive.

The maximum cell density as well as maximum α-amylase activity level resulting from different agitation speeds were not significantly different. The maximum cell densities, for the agitation speeds of 220 rpm, 300 rpm, and 400 rpm, were 0.72 $g\,l^{-1}$, 0.75 $g\,l^{-1}$, and 0.77 $g\,l^{-1}$, respectively. The corresponding maximum enzyme activities were 1165 $U\,ml^{-1}$, 1106 $U\,ml^{-1}$, and 1162 $U\,ml^{-1}$. However, the cell density was slightly higher with higher agitation speed. Therefore, agitation speed in the range of 220 to 400 rpm only affected the cell growth rate and enzyme excretion, rather than the maximum concentrations.

3.6. Aeration

The excretion of α-amylase was growth associated, therefore α-amylase is a primary metabolite which is formed under sufficient aeration conditions. The aeration was supplied at the rate of 0.5 l min^{-1} (0.3 vvm) and 1.0 l min^{-1} (0.6 vvm), respectively. The results are shown in Fig. 8(a)–(d). Fer-

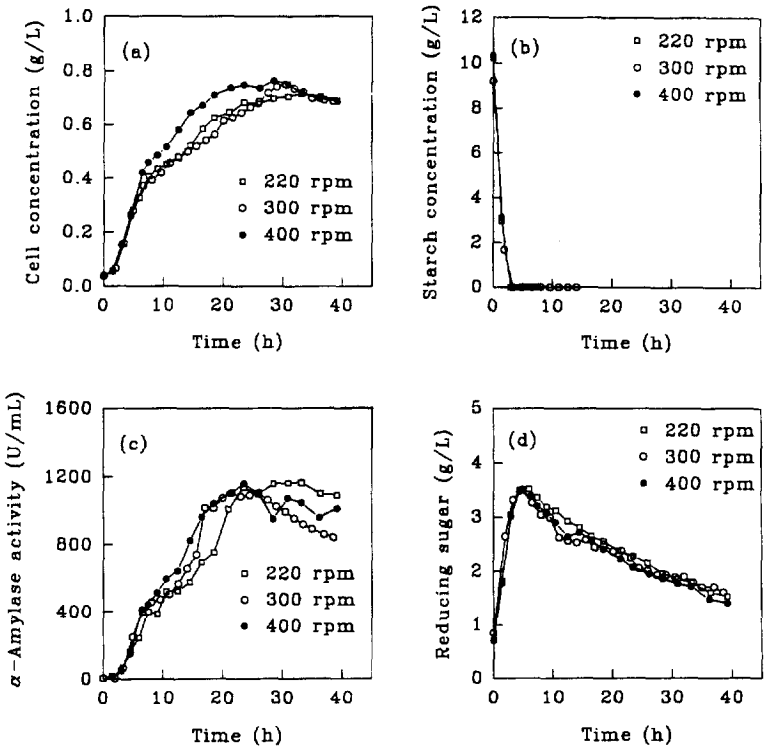

Fig. 7. Effect of agitation speed on the α-amylase fermentation. (a) Cell concentration ($g\,l^{-1}$), (b) starch concentration ($g\,l^{-1}$), (c) α-amylase activity (U ml^{-1}), (d) reducing sugar concentration ($g\,l^{-1}$).

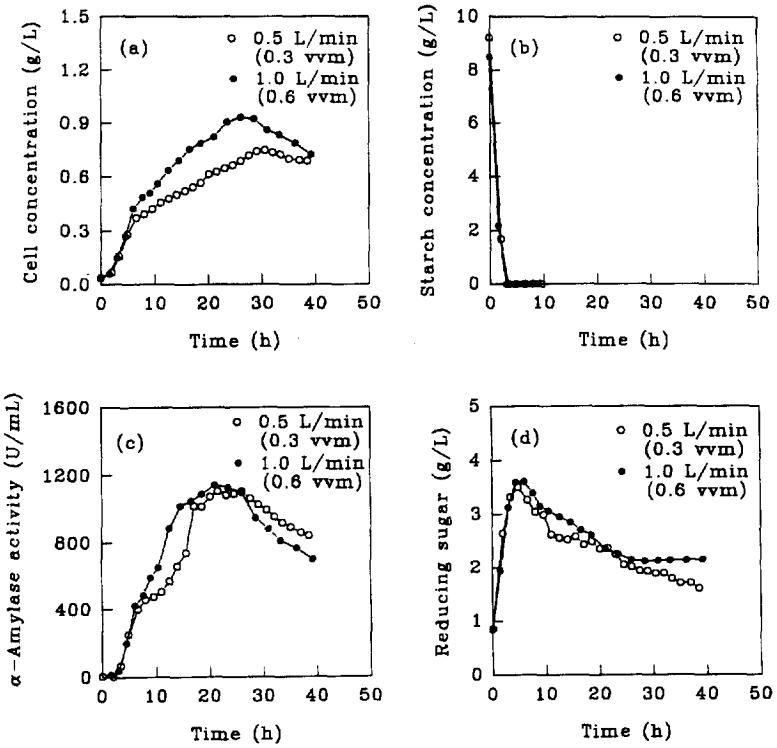

Fig. 8. Effect of aeration rate on the α-amylase fermentation. (a) Cell concentration ($g\,l^{-1}$), (b) starch concentration ($g\,l^{-1}$), (c) α-amylase activity (U ml^{-1}), (d) reducing sugar concentration ($g\,l^{-1}$).

mentations were performed with a working volume of 1.7 l. The growth of cells was much faster at 0.6 vvm of aeration and a corresponding higher cell mass was therefore obtained. However, significant cell lysis occurred by the final stage of the fermentation at this aeration rate while a better maintenance of cell density was observed for 0.3 vvm of aeration. It was therefore assumed that the cell lysis might be caused from the insufficient air supply to each individual cell since

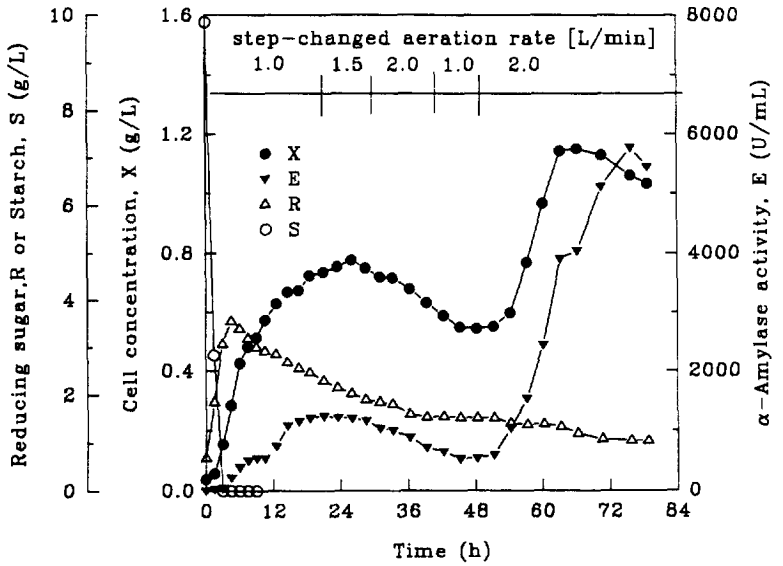

Fig. 9. A schedule of step-changed aeration rate for the α-amylase fermentation.

higher cell density was formed at the higher aeration of 0.6 vvm and such higher aeration may still not be enough to provide the required air to such higher density of cells.

However, the starch hydrolysis was still very fast and, therefore, the starch concentration was diminished very quickly. Different aerations did not show much effect on the hydrolysis of starch. As for the formation and consumption of reducing sugar, still not much difference was observed under these two aeration rates. However, before 20 h, higher reducing sugar was caused from the aeration of 0.6 vvm. This can be realized from the higher cell density by this higher aeration rate, therefore higher excreted α-amylase stimulates the breakdown of more saccharides into reducing sugars. It should also be assumed that the formation of sugar was still enough to provide for the consumption of sugar by the high density of cells. After 20 h, the sugar concentration was leveled off and less sugar was consumed at aeration of 0.6 vvm due to the cell lysis at the final stage of the fermentation. This was coincident with the occurrence of cell lysis, as the viability and activity of cells dropped due to insufficient oxygen utilization, the ability to consume the sugar diminished accordingly.

The enzyme excretion rate was somehow higher at the aeration of 0.6 vvm and the maximum α-amylase activities under both aeration conditions were almost the same. However, the enzyme stability was better maintained at the smaller aeration of 0.3 vvm. If the comparison was made between Fig. 8(a) and 8(c), the growth-associated type of behavior would still be concluded for the two aerations. Before reaching maximum cell density, faster cell growth rate corresponded to faster enzyme formation rate at 0.6 vvm. As cell lysis occurred, less cell stability was observed at the final stage, which corresponded to a less stable enzyme activity. Therefore, the enzyme activity decreased faster at the aeration rate of 0.6 vvm than of 0.3 vvm. This might be due to the fact that more foaming occurred and also there was insufficient air for each cell to produce α-amylase at this higher aeration rate, as a result, the enzyme activity was damaged.

Operating the fermentations at different aeration rates did influence the enzyme activity, therefore. Hence, subsequent fermentation was designed with a schedule of step-changed aeration rates as shown in Fig. 9. Initially, the aeration was set at 0.6 vvm, until around 21 h (i.e. 0–21 h), the aeration rate was then increased to 1.5 l min^{-1} (0.9 vvm) during 21–28.5 h for observing the influence of such step change on the excretion of α-amylase. As the fermentation further proceeded, it was found that the enzyme activity decreased slightly and cell lysis occurred, so the aeration was further increased to 2.0 l min^{-1} (1.2 vvm) during 28.5–39.3 h and it was expected that the cell lysis could be prevented for the excretion of enzyme to be continued. However, the enzyme level still decreased. It might be possible that the cell physiology was not yet readily adapted to this adjustment or the aeration was too much and was damaging to the cells. It was assumed that the latter situation was true, so the aeration was again adjusted, in the opposite direction this time, to 0.6 vvm from 39.3 h to 45.3 h. The cell lysis still continued and the α-amylase activity level still decreased. So, the aeration was once again brought back to 1.2 vvm from 45.3 h until the end of the fermentation. Then, a second growth phase with a higher growth rate than the first growth phase was observed. The α-amylase activity also increased accordingly with a nearly associated type. A much faster enzyme formation rate can be observed at this stage. A maximum enzyme activity of 5772 U ml^{-1} could be achieved from such an aeration rate supply schedule. During this fermentation, all the other conditions were kept the same, it is only the aeration rate that was different from the other batches. Therefore, the schedule with increased aeration rate has demonstrated a positive influence on the excretion of enzyme and its activity level.

It can be concluded from the above results that with the aeration rate of less than 2.0 l min^{-1}, the growth of cells as

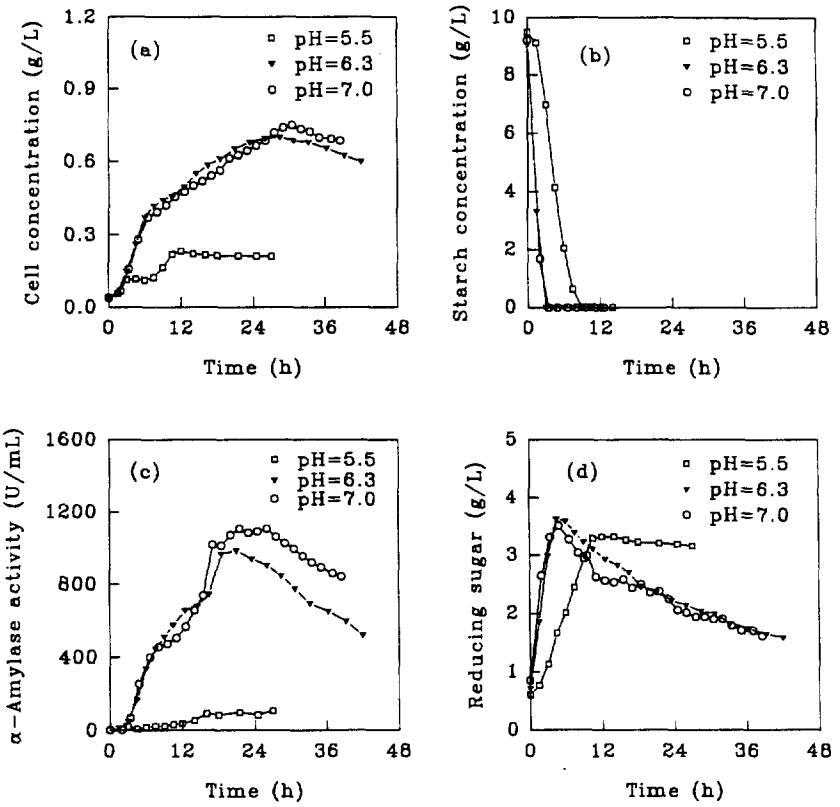

Fig. 10. pH effect on the α-amylase fermentation. (a) Cell concentration (g l^{-1}), (b) starch concentration (g l^{-1}), (c) α-amylase activity (U ml^{-1}), (d) reducing sugar concentration (g l^{-1}).

well as the excretion of α-amylase were both limited by the insufficient supply of oxygen to such aerobic fermentation. With the aeration rate of 2.0 l min^{-1}, α-amylase can be excreted to a very high level. Therefore, the overall performance of the previous batches was limited by insufficient oxygen.

3.7. pH

The effect of pH on the performance of the α-amylase fermentation was studied and the results are shown in Fig. 10(a)–(d). The fermentations at the controlled pH of 5.5, 6.3, 7.0 were carried out, respectively. The cell growth for pH of 6.3 and 7.0 was almost the same, but a higher cell density at the final stage of the fermentation was observed at the pH of 7.0. The cell mass from pH of 5.5 was comparatively much lower than the other pH conditions. Consequently, a correspondingly much lower enzyme activity level was observed for the condition of pH = 5.5. The enzyme activity at pH = 7.0 is higher than that at pH = 6.3. The major influence is from the effect of the pH on the enzyme activity itself. It was found that a slower hydrolysis of starch occurred at pH = 5.5, and, as a result, the reducing sugar increase was slower than the others. The cell growth was also slower under this condition, therefore the reducing sugar was not consumed as significantly as the others. The formation and consumption schedule of the reducing sugar for the other two conditions is almost the same.

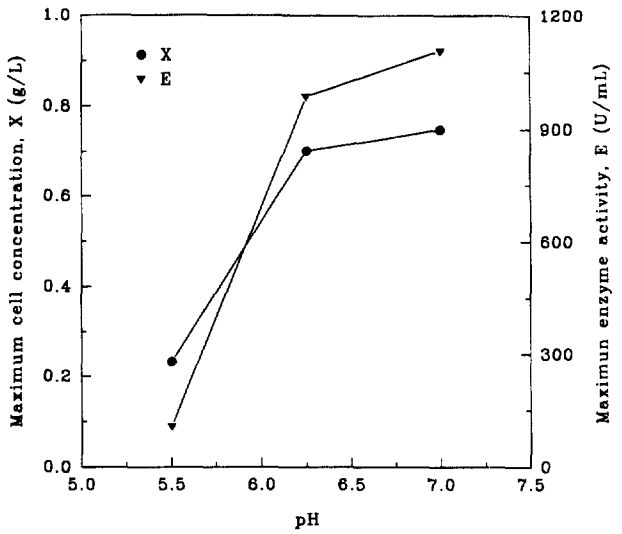

Fig. 11. Effect of pH on maximum cell density and maximum α-amylase activity.

The maximum cell mass and maximum α-amylase are compared under these three pH conditions in Fig. 11. It was found that the maximum of both cell mass and enzyme activity appeared increasing with increasing pH while much less difference was observed between pH values of 6.3 and 7.0.

The α-amylase activity was assayed by the hydrolysis of starch. Since starch solution is hydrophobic, it can have more affinity toward the enzyme under a hydrophobic environ-

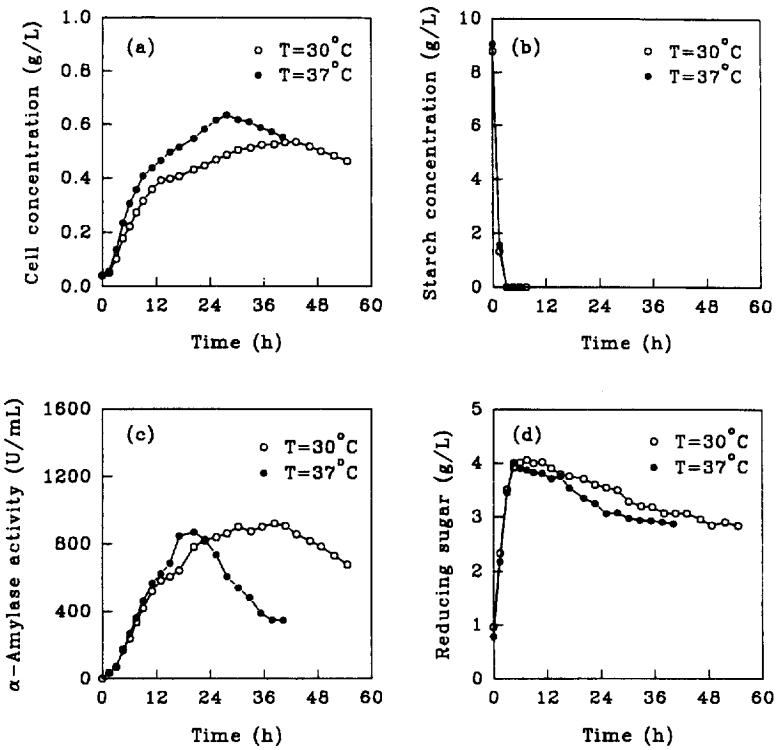

Fig. 12. Temperature effect on the α-amylase fermentation. (a) Cell concentration (g l^{-1}), (b) starch concentration (g l^{-1}), (c) α-amylase activity (U ml^{-1}), (d) reducing sugar concentration (g l^{-1}).

ment. For either the acidic or basic pH range, the enzyme is more hydrophilic and therefore is more difficult to react with the hydrophobic starch substrate. As a result, the enzyme activity would be lower at either the acidic or basic pH. Hence, higher α-amylase activity was obtained at a neutral pH 7.0. This can explain why and how different pH affected the α-amylase activity.

3.8. Temperature

The fermentations were compared for 30 °C and 37 °C, respectively. Fig. 12(a)–(d) is provided to illustrate the results. Higher cell density can be seen in the fermentation at 37 °C and therefore a slightly lower concentration of reducing sugar was observed at this temperature. However, the starch substrate was degraded at the same rate for fermentations at these two temperatures. The α-amylase activity showed a large variation for these two temperatures. A slightly higher enzyme level was obtained at 30 °C. However, maximum enzyme activity at 30 °C occurred at a time later than that at 37 °C. The enzyme activity and therefore the enzyme stability was also better maintained at the temperature of 30 °C.

It can be concluded that the optimal temperatures for cell growth and enzyme activity are different. It was found that 37 °C is better for cell growth while 30 °C is, on the other hand, better for enzyme excretion. Hence, it might be suggested that the fermentation is operated at 37 °C during the growth phase and followed by a change to 30 °C for the stimulation of α-amylase excretion.

4. Conclusions

The activity of α-amylase decayed fast when placed at 37 °C, however the activity can be well maintained for more than 180 h at 0 °C. The rate for starch hydrolysis is governed by both initial starch concentration and initial enzyme activity. A higher initial rate of 1.13 g l^{-1} h^{-1} and higher average rate of 0.81 g l^{-1} h^{-1} for starch hydrolysis were obtained at the condition of $E_o = 15.8$ U ml^{-1} and $S_o = 42$ g l^{-1}. The hydrolysis of starch solution was initiated in the presence of even sparse amounts of α-amylase activity. Fermentation carried at 37 °C favored the growth of cells while 30 °C favored the excretion of α-amylase. A pH of 7.0 was the best pH for both cell growth and enzyme excretion. Higher aeration rate definitely stimulated the excretion of α-amylase activity. The agitation speed did not show significant effect on the performance of the fermentations. However, at the speed of 220 rpm, better stability of both cell density and enzyme activity was maintained.

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