

Full Length Research Paper

# Optimization of the industrial production of bacterial alpha amylase in Egypt. IV. Fermentor production and characterization of the enzyme of two strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens*

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Production of alpha amylase using amplified variants of *Bacillus subtilis* (strain SCH) and of *Bacillus amyloliquefaciens* (strain 267CH) was conducted in a bioreactor with multiprotein-mineral media. The time course of fermentation in a bioreactor revealed that the highest yield (about  $8 \times 10^4$  U/ml within 60 h) by strain SCH was obtained by applying: 3.5% initial starch, 2% additional starch after 19 h, 3 vvm aeration and 300 rpm agitation. The highest yield (about  $19 \times 10^4$  U/ml within 100 h) by strain 267CH was obtained by applying: 2.5% initial starch, 2% additional starch after 24 h, 3 vvm aeration, and 300 rpm agitation with the productivity after 60 h reaching only about  $14 \times 10^4$  U/ml. Production occurred in both the logarithmic and postlogarithmic phases of growth. Maximum consumption of starch and protein occurred during the first day of incubation. The optical density peak coincided with enzyme production peak in case of strain SCH and preceded that of enzyme production in case of strain 267CH. The alpha amylase produced by the two strains was shown to be of the liquefying and not the saccharifying type. Both enzymes liquefied starch to a dextrose equivalent of about 15 - 17 at 95°C hence they are classified among thermostable alpha amylases. They exhibited broad pH and temperature activity profiles. The optimum pH for activity was 4 - 7 for alpha amylase produced by strain SCH and 4 - 8 for alpha amylase produced by strain 267CH while the optimum temperatures for their activities were in the range 37 - 75°C at 0.5% starch and in the range 85 - 95°C at 35% starch.

**Key words:** Production kinetics, process modeling, fermentation, bacterial amylase, biotechnology.

## INTRODUCTION

The results of production of fermentation products aerobically in shake flasks usually cannot be extrapolated to indicate possible performance in a fermentor (Dewitt et al., 1989). Both physical and biological factors at play are quite different in a fermentor and in a shake flask. Moreover, controls on the reaction in a shake flask are

extremely limited while in a fermentor such controls are almost limitless. Accordingly, results obtained in a shake flask should be taken only as preliminary indicators of the conditions necessary for successful industrial production and must be verified in studies carried out in a fermentor. In this study a laboratory fermentor was used to verify shake flasks productivity of alpha amylase by two strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* and preliminary characterization of the enzyme produced by both strains was attempted in order to determine its

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industrial applicability.

## MATERIALS AND METHOD

### Bacterial strains

Amplified *B. subtilis* variant (strain SCH) and amplified *B. amyloliquefaciens* variant (strain 267CH) were used (El-Tayeb et al., 2000). They were subcultured and stored as reported by El-Tayeb et al. (2000).

### Media

Multiprotein-mineral media (El-Tayeb et al., 2001) composed of (g/100 ml): CaCl<sub>2</sub> anhydrous (0.011), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.0264), (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (0.132), (NH<sub>4</sub>)H<sub>2</sub>PO<sub>4</sub> (0.115), beef extract (0.9), peptone (1.5), yeast extract (1) and glycine (0.5). In case of strain SCH the initial starch concentration was either 2.5 or 3.5 while in case of strain 267CH the initial starch concentration was only 2.5%, the concentration of MgSO<sub>4</sub>·7H<sub>2</sub>O was increased to 0.264 and 0.1% theophylline monohydrate was additionally incorporated.

### Production of alpha amylase by the amplified strains SCH and 67CH in the fermentor

This was carried out in a 7.5 l laboratory glass fermenter (Laborferm, New Brunswick Scientific Co. Inc., Edison, New Jersey, USA). Unless otherwise indicated multiprotein-mineral media containing 2.5% starch were used. The inoculum was grown on the same media and used at the rate of 10% (v/v) of an overnight culture. The working volume was 3 L; agitation was carried out using Rushton impellers (49 mm diameter) which were positioned at three separate equal distances. Aeration was conducted using a multiorifice ring sparger which was controlled by a pressure regulator at 0.5 bar and a flow meter. The percent dissolved oxygen was monitored using dissolved oxygen analyzer (model DO50, New Brunswick Scientific Co.) while the pH was controlled by pH controller (model PH-22, New Brunswick Scientific Co.). Where indicated the pH was adjusted at 7.2 by 1.2 M NaOH and 0.6 M H<sub>2</sub>SO<sub>4</sub> and the temperature was adjusted at 37°C. Foam was suppressed by silicone oil using a foam controller (model AFP-101, New Brunswick Scientific Co.). Additional starch, if so required, was incorporated in the form of warmed gelatinized slurry in the least amount of sterile distilled water. Dry starch was sterilized before gelatinization in a hot air oven at 175°C for 40 min with the powder depth not exceeding 1 cm. The following parameters were monitored in samples drawn at different time intervals: extracellular alpha amylase activity was determined by starch-iodine method (El-Tayeb et al., 2000) while optical density, residual starch and/or its degradation intermediates were determined in terms of reducing sugars as described by Hyun and Zeikus (1985) and soluble proteins were determined as described by Lowry et al. (1951).

### Characterization of alpha amylase produced by the variants SCH and 267CH

Enzyme activity of the supernatant after removal of the bacteria by centrifugation at 3000 rpm for 20 min was tested for: optimum temperature and pH for activity, thermal stability, liquefying and saccharifying activity as well as the nature of the hydrolysis products of soluble starch.

The pH activity profile was determined by mixing 0.05 ml of diluted growth supernatant (containing 10<sup>3</sup> U/ml enzyme activity as

determined by starch-iodine method) in distilled water, 1 ml of 1% soluble starch (which was heated in a boiling water bath for about 30 min to become amenable for enzyme attack), and 1 ml of 20 mM phosphate buffer containing 10 mM NaCl and having different pH values ranging between 3 and 9. After incubation for 10 min at 37°C, the alpha amylase activity was assayed by reducing sugars method (El-Tayeb et al., 2001).

The temperature activity profile of the enzyme was determined by mixing 0.05 ml of diluted growth supernatant (containing 10<sup>3</sup> U/ml enzyme activity, as determined by the starch-iodine method, in 20 mM phosphate buffer, pH 6.9, containing 10 mM NaCl), 1 ml of 1% starch (prepared in 20 mM phosphate buffer, pH 6.9, containing 10 mM NaCl and heated in a boiling water bath for about 30 min) and 1 ml of the same buffer at various temperatures (ranging between 5 to 100°C). After incubation for 10 min, the reducing sugars released were assayed by the dinitrosalicylic acid method as referred to above.

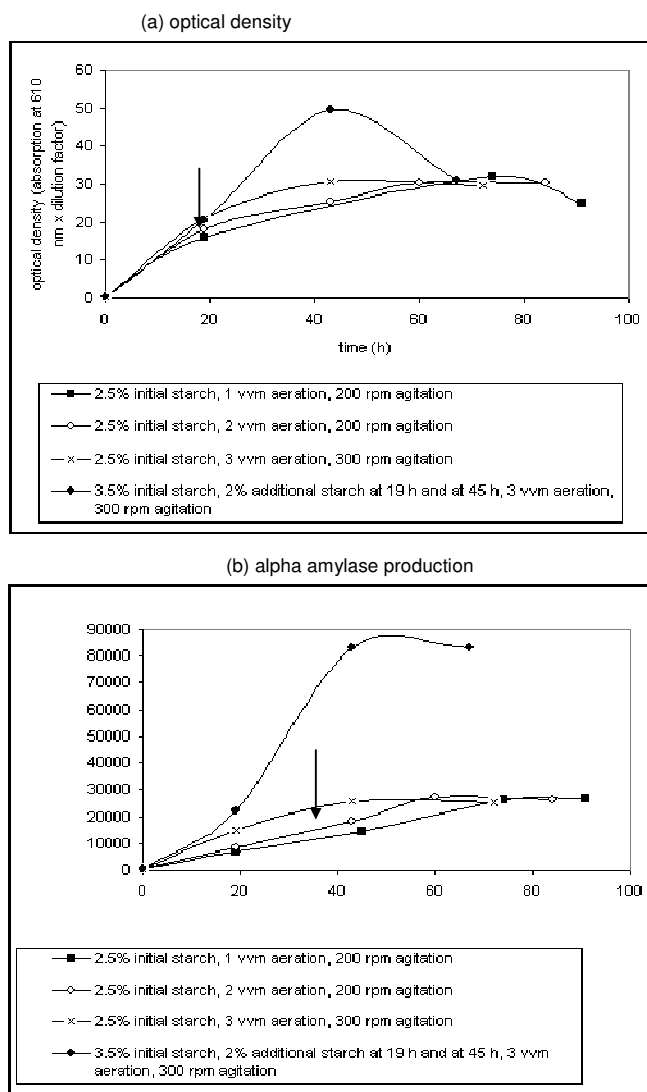
The thermal stability of the enzyme was determined by holding 5 ml of diluted growth supernatant (10<sup>3</sup> U/ml enzyme activity, as determined by starch-iodine method, in 20 mM phosphate buffer, pH 6.9, containing 10 mM NaCl) at various temperatures (37, 50, 70 and 90°C). Fifty microlitres were removed at different time intervals and added to 1 ml of 1% soluble starch (prepared in 20 mM phosphate buffer, pH 6.9, containing 10 mM NaCl and heated in a boiling water bath for about 30 min) and 1 ml of the same buffer. The reaction mixture was incubated at 37°C for 10 min and the reducing sugars released were assayed by the dinitrosalicylic acid method as described above.

The liquefying and saccharifying activities were examined by mixing 1 ml of diluted growth supernatant (containing 10<sup>3</sup> U/ml enzyme activity in 20 mM phosphate buffer, pH 6.9, containing 10 mM NaCl) and 20 ml of 1% soluble starch (prepared in 20 mM phosphate buffer, pH 6.9, containing 10 mM NaCl and heated in a boiling water bath for about 30 min). After incubation at 37°C, 2 ml samples were removed at different time intervals for determination of reducing sugars released by the dinitrosalicylic acid method and the dextrose equivalents as well as percentage of hydrolysis was then calculated (1 dextrose equivalent, DE, has the same reducing power as a 1% aqueous solution of pure dextrose and the quantitative hydrolysis of 1 g starch, by dilute HCl yields 1.11 g glucose (Peppler and Reed, 1987)).

The liquefying activity was determined by applying a method which simulates that described by Ostergaard (1982) with some modifications: 0.01 ml of 10<sup>5</sup> U/ml enzyme activity (as determined by starch-iodine method) of diluted growth supernatant (in phosphate buffer 20 mM, pH 6.9, containing 10 mM NaCl) was added to 3.5 gm maize starch in 10 ml of the same phosphate buffer and the temperature was elevated to 70, 75, 85, 95 or 105°C over a period of about 10 min and held there for 1 h. The slurry produced was autoclaved at 121°C for 10 min, cooled to the temperature of treatment, and a second enzyme addition (0.1 ml of growth supernatant diluted in phosphate buffer 20 mM, pH 6.9, containing 10 mM NaCl to contain 10<sup>5</sup> U/ml enzyme activity) was incorporated with further incubation for another 1 h at the specified temperature. The mixture was cooled to room temperature and the reducing sugars released were determined by the dinitrosalicylic acid method. The dextrose equivalents were then computed

### Qualitative detection of glucose and maltose in the hydrolysis products by paper chromatography

It was carried out on the hydrolysis products resulting from mixing 0.2 ml of diluted growth supernatant (containing 10<sup>3</sup> U/ml enzyme activity as determined by starch-iodine method) in phosphate buffer (20 mM, pH 6.9, containing 10 mM NaCl), and 1 ml of 2% starch



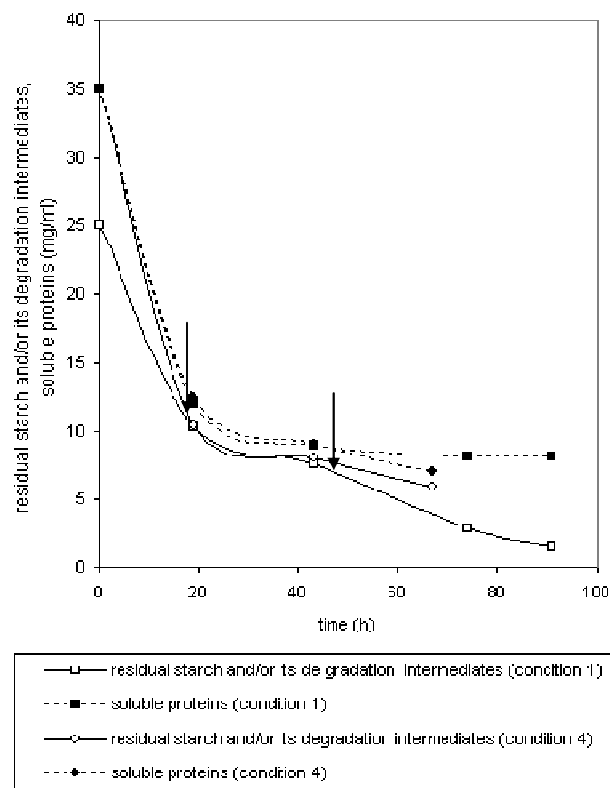
**Figure 1.** Time course of growth and of alpha amylase production by strain SCH under four different conditions in the fermentor with pH control.

(prepared in 20 mM phosphate buffer, pH 6.9, containing 10 mM NaCl). The volume was brought to 2 ml with the same phosphate buffer and the mixture was incubated for 40, and 140 min at 37°C. The reaction was stopped by heating in boiling water bath for 10 min and the hydrolysis products were developed by paper chromatography (Whatman filter paper No.1) at room temperature using n-butanol-pyridine-water (6:4:3) as developer. Sugars were detected by dipping method with silver nitrate-sodium hydroxide reagent (Hayashida et al., 1988).

## RESULTS

### Alpha amylase production in a fermentor

Production was carried out by both strains using the res-



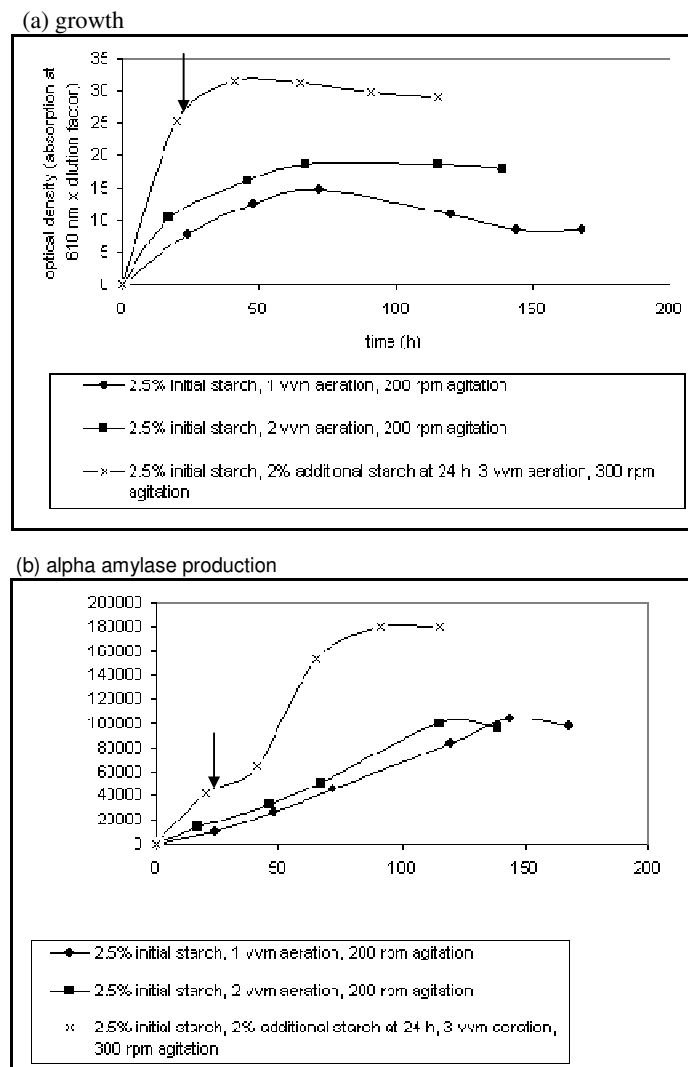
**Figure 2.** Time course of residual starch and/or its degradation intermediates and soluble proteins by strain SCH under two different conditions in the fermentor with pH control condition 1: 2.5% initial starch, 1 vvm aeration, 200 rpm agitation condition 4: 3.5% initial starch, 2% additional starch at 19 h and at 45 h, 3 vvm aeration, 300 rpm agitation.

pective multiprotein-mineral media with and without glycine. The fermentation was carried out under different conditions. Fermentation without pH control resulted in a drop in pH after 24 to 48 h to a less than 6 even in presence of glycine and alpha amylase production declined (data not shown). Accordingly glycine was omitted from the media and pH was controlled at 7.2 by a pH controller. Alpha amylase activity, optical density and in most cases residual starch and/or its degradation intermediates together with soluble protein concentrations were monitored at different time intervals.

### Production by strain SCH

The conditions applied were the following: Condition 1: 2.5% initial starch, 1 vvm aeration, 200 rpm agitation; Condition 2: 2.5% initial starch, 2 vvm aeration, 200 rpm agitation; Condition 3: 2.5% initial starch, 3 vvm aeration, 300 rpm agitation; Condition 4: 3.5% initial starch followed by 2% additional starch at hour 19 and again at hour 45, 3 vvm aeration, 300 rpm agitation.

The results are presented in Figures 1 and 2. In all



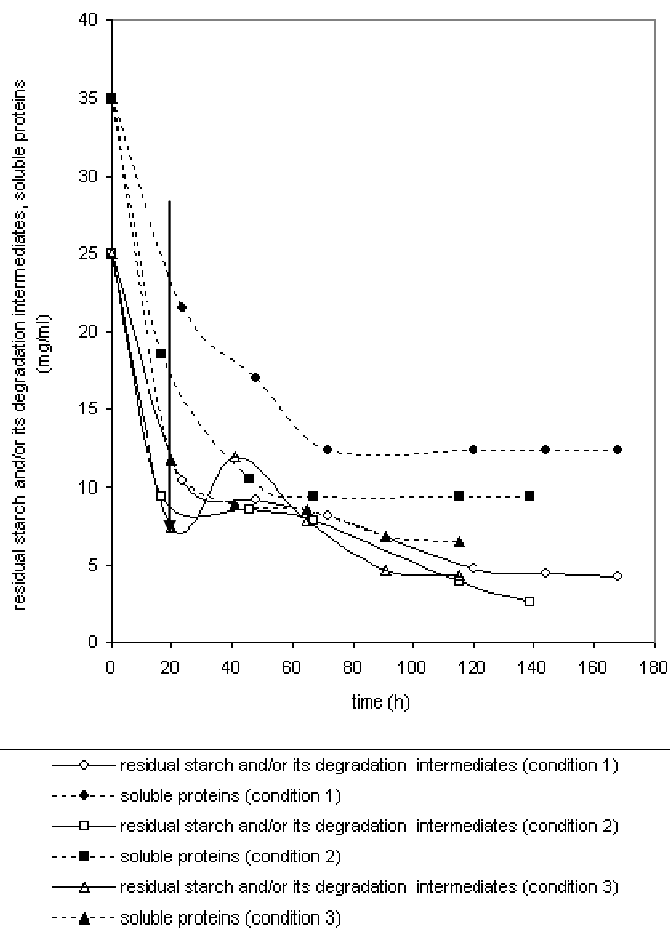
**Figure 3.** Time course of growth and of alpha amylase production by strain 267CH under three different conditions in the fermentor with pH control.

cases dissolved oxygen decreased to 0% saturation, 2 - 4 h after inoculation and remained there thereafter under all conditions (data not shown).

### Production by strain 267CH

The conditions applied were the following: Condition 1: 2.5% initial starch, 1 vvm aeration, 200 rpm agitation; Condition 2: 2.5% initial starch, 2 vvm aeration, 200 rpm agitation; Condition 3: 2.5% initial starch, followed by 2% additional starch at hour 24, 3 vvm aeration, 300 rpm agitation.

In all cases dissolved oxygen dropped to 0% saturation 2 - 4 h after inoculation and remained there for 72 - 96 h (condition 3) and for 96 - 120 h (condition 1 and 2) then



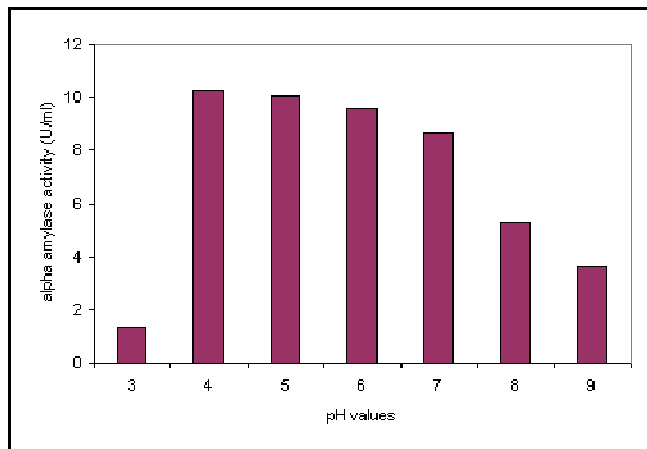
**Figure 4.** Time course of residual starch and/or its degradation intermediates and soluble proteins by strain 267CH under three different conditions in the fermentor with pH control. condition 1: 2.5% initial starch, 1 vvm aeration, 200 rpm agitation, condition 2: 2.5% initial starch, 2 vvm aeration, 200 rpm agitation, condition 3: 2.5% initial starch, 2% additional starch at 24 h, 3 vvm aeration, 300 rpm agitation

increased gradually. The results are shown in Figure 3 and 4.

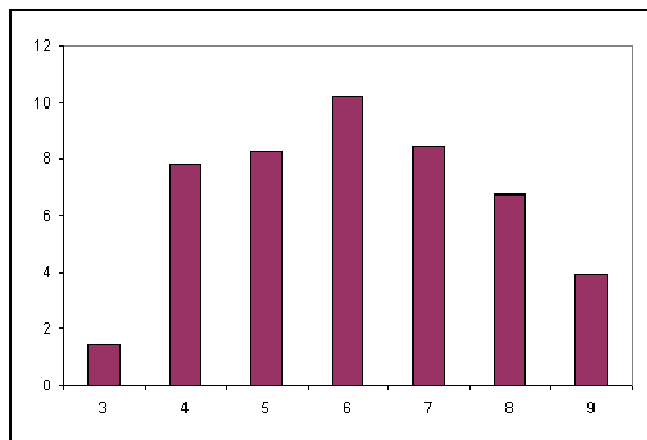
### Characteristics of alpha amylase produced by strains SCH and 267CH

The pH profiles for the two enzymes are illustrated in Figure 5 while the temperature profile and thermal stability are shown in Figures 6 and 7, respectively. The liquefaction and saccharification activity of each enzyme were examined by determining maximum hydrolysis ratio on soluble starch and the results are shown in Figure 8. The dextrose equivalent produced by each enzyme upon liquefaction of 35% maize starch was also determined at different temperatures (70, 75, 85, 95 and 105°C) and the results are presented in Figure 9. The action of both en-

(a) alpha amylase produced by strain SCH



(b) alpha amylase produced by strain 267CH



**Figure 5.** Optimum pH for alpha amylase activity produced by strains SCH and 267CH

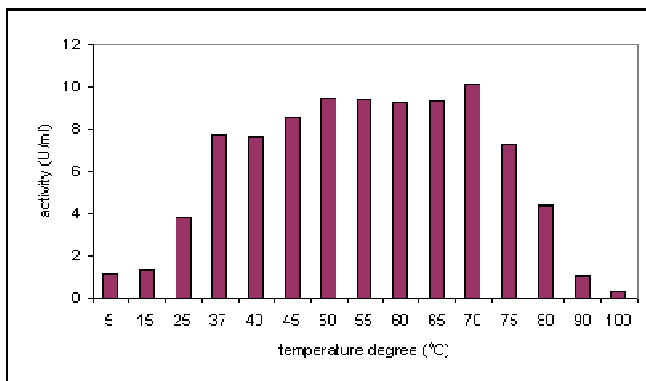
zymes on soluble starch was also examined by running the hydrolysis products on paper chromatography and the results are illustrated in Figure 10.

## DISCUSSION

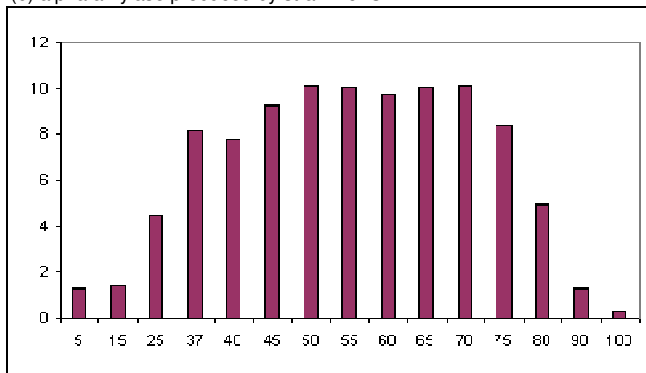
### Production of alpha amylase in a fermentor

With both strains batch fermentations were conducted at 37°C using multiprotein-mineral media containing 2.5% starch with or without the glycine as opposed to 5% starch in shake flask experiments (El-Tayeb et al., 2001) in order to avoid layering phenomenon that occurred after sterilization in the fermentor. In case of strain SCH only, the fermentation was also conducted at 3.5% starch concentration. Under uncontrolled pH, a drop in pH to less than 6 and an inability of the cultures to regulate

(a) alpha amylase produced by strain SCH



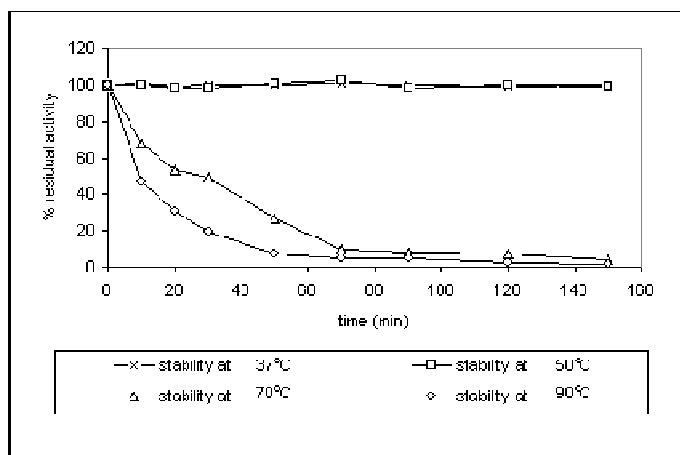
(b) alpha amylase produced by strain 267CH



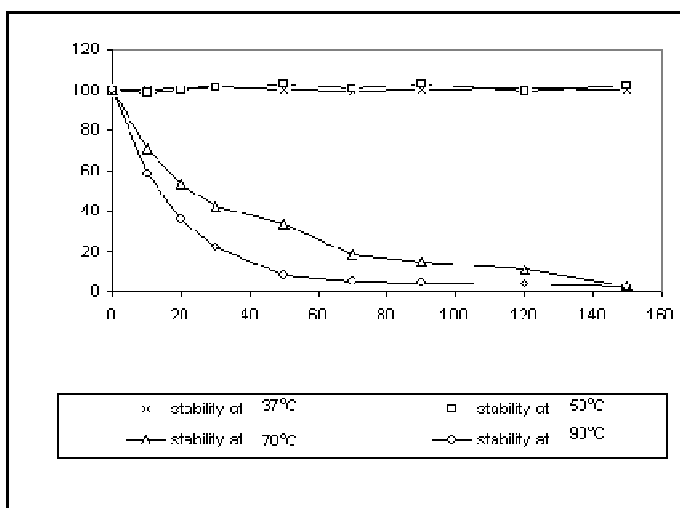
**Figure 6.** Optimum temperature for alpha amylase activity produced by strain SCH and 267CH.

their pH accompanied by a drop in alpha amylase activity were noted with both strains even when glycine was included in the medium. Glycine was included because it did not result in such drop in pH and alpha amylase in shake flask experiments in the same medium (El-Tayeb et al., 2001). This is interesting since the only change was the use of a fermentor rather than shake flasks. Since the main purpose of glycine was pH regulation (El-Tayeb et al., 2000), it was omitted in further experiments. Instead, the pH was controlled at 7.2 with a pH controller. In alpha amylase production, the pH profile and its role in production seem to be strain dependant. For example Priest and Sharp (1989) stated that depending upon the strain of *Bacillus* species being cultivated, fermentations are usually carried out at pH 7 and enzyme activity is lost if the pH falls below 6. On the other hand, Davis et al. (1980) showed that, in batch culture of *Bacillus stearothermophilus*, the pH increased to 8.2 after 3.5 h, with maximum alpha amylase activity reaching 790 U/ml but that when the pH was controlled at 7.2, amylase activity dropped to 470 U/ml after 3.5 h, although the biomass yield was the same.

(a) alpha amylase produced by strain SCH



(b) alpha amylase produced by strain 267CH



**Figure 7.** Thermal inactivation of alpha amylase produced by strains SCH and 267CH.

Where indicated the total reducing sugars consumption at different sampling points were calculated by subtracting the total residual reducing sugars after acid hydrolysis at the different sampling points in terms of glucose, from the initial starch concentration which was determined too in terms of glucose equivalents after acid hydrolysis. This approach made it possible to calculate sugar consumption rates ( $dS/dt$ ), a more indicative parameter than residual reducing sugars. Where indicated the data on biomass, alpha amylase and total reducing sugars consumption were smoothed (Mohammad et al., 2001) and then represented graphically and used for calculating process rates. Such smoothing enabled the computation of additional data points for biomass, alpha amylase and for total reducing sugars consumption as

well as the computation of the more descriptive process rates. This approach also enabled the monitoring of process parameters (biomass, alpha amylase, soluble proteins and, where indicated, residual starch and/or its degradation intermediates) at somewhat long time intervals up to 24 h, since with plot smoothing one would not necessarily need short sampling times.

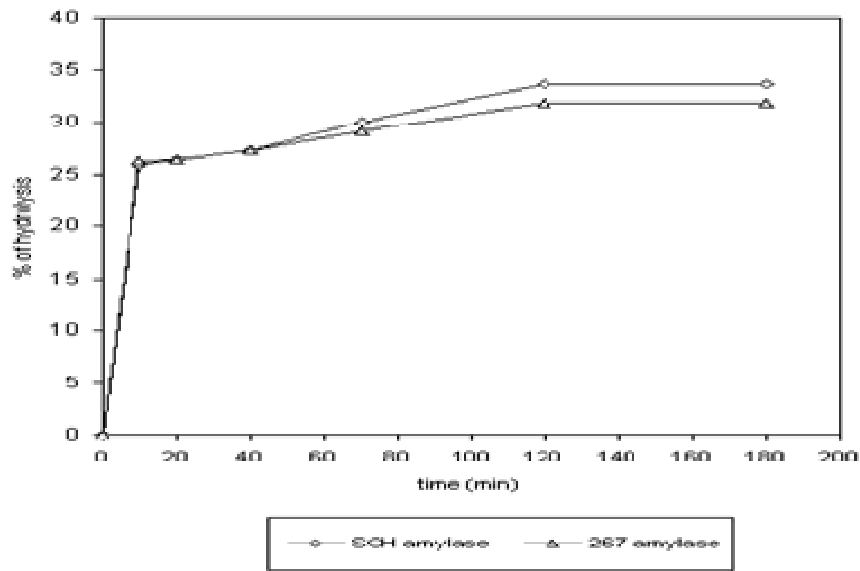
### Conditions for production by strain SCH

Three different fermentation conditions with regard to levels of aeration (1, 2 and 3 vvm) were tested with only two levels of agitation (200 and 300 rpm). In all three cases the initial starch concentration was 2.5%. The fourth experiment was identical with the highest aeration and agitation conditions tested, but the initial starch concentration was increased to 3.5% and two additional 2% starch increments were added at hours 19 and again at hours 45.

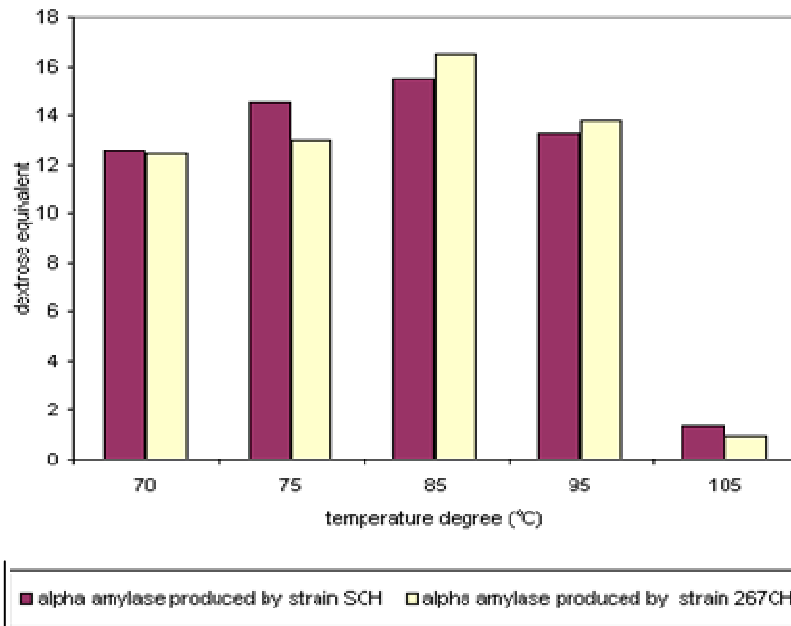
The results in Figure 1 showed that both biomass and alpha amylase production reach maxima within around 45 h under all conditions. In addition, this maximum remains somewhat constant upon further incubation except that under high starch concentration and high aeration and agitation (condition 4), a drop in biomass but not in alpha amylase is noted. While condition 4 produced a dramatic rise in both biomass and alpha amylase production – when compared to all 3 other conditions within the first 45 h – conditions 1 and 2 showed a slight continued rise in both biomass and alpha amylase production upon further incubation. When the first 3 conditions are compared we note that increased aeration and/or agitation increases alpha amylase production more pronouncedly than biomass.

Smoothed time plots were drawn in each case for biomass (optical density, X), enzyme production (P), and in the case of conditions 1 (lowest aeration and agitation) and 4 (highest aeration and agitation and higher starch concentration) also sugars consumption (S). The respective process rates  $dX/dt$ ,  $dP/dt$  and  $dS/dt$  were calculated from the smoothed plots and the results are shown in Figures 11 - 16. In all cases dissolved oxygen dropped to 0% saturation soon after growth began and remained there until the end of the experiment, indicating a degree of oxygen starvation even at high aeration and agitation rates (3 vvm and 300 rpm). Higher rates were not tested in order to avoid excessive foaming. Cantero (1990) found that the level of dissolved oxygen decreased quickly, practically to zero during the first 8 h of the fermentation of *B. subtilis*. He attributed this to the initial high substrate concentration.

By examination of the smoothed plots (Figures 11 and 13) there appears to be no substantial difference in biomass and enzyme productivity under conditions 1, 2 and 3 but that both parameters were significantly higher



**Figure 8.** Progress of hydrolysis of soluble starch (1%) by alpha amylase produced by strains SCH and 267CH



**Figure 9.** Comparison between the liquefaction efficiency (as dextrose equivalent) of alpha produced by strains SCH and 267CH at different temperatures. Substrate: 35% maize starch in phosphate buffer (20 mM, pH 6.9 containing 10 mM NaCl).

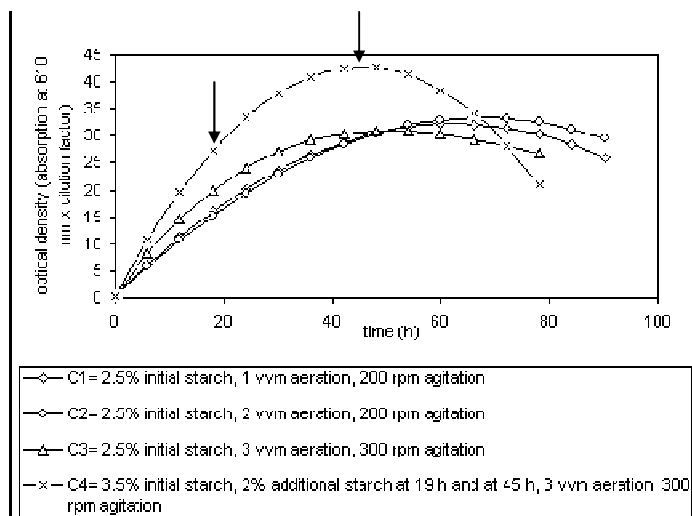
in condition 4. However, upon plotting  $dX/dt$  and  $dP/dt$  (Figures 12 and 14) it was shown that while condition 1 and 2 seemed to give more or less similar patterns, condition 3 showed substantially higher values than conditions 1 and 2 up to about 30 h of incubation. It is

thus concluded that increasing aeration rate alone does not enhance the rates of growth or enzyme production (within the limits tested) while simultaneous increase in both aeration and agitation rates (condition 3) substantially increased the rates of both growth and enzyme pro-

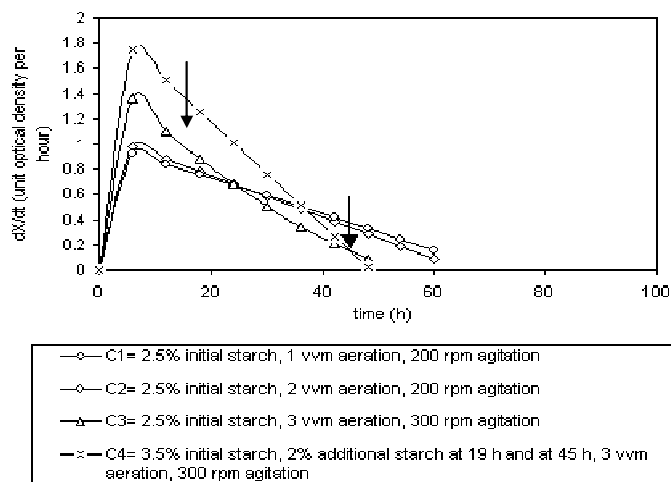


**Figure 10.** Paper chromatography of hydrolysis products of alpha amylase of strains SCH and 267CH. A: Chromatogram developed for hydrolysis products obtained after 40 minutes reaction time. B: Chromatogram developed for hydrolysis products obtained after 140 minutes reaction time. 1: Hydrolysis products by alpha amylase of strain SCH. 2: Hydrolysis products by alpha amylase of strain 267CH. 3: Glucose. 4: maltose.

duction. A somewhat similar trend was noted in condition 4 (modified starch addition regimen), with substantially higher values of  $dX/dt$  and  $dP/dt$ . Under this condition (much higher initial and "during-the-reaction" starch addition at still the higher aeration and agitation rates) enzyme production in fact increased 4 -fold (Figure 13) along with only about 50% increase in optical density (Figure 11). Both  $dX/dt$  and  $dP/dt$  showed similar trends: with  $dP/dt$  increasing 2.5 -fold and  $dX/dt$  increasing about 25%. Interestingly, under this condition the higher initial starch concentration showed a substantial effect on  $dX/dt$  while both the higher initial starch and the first starch addition stimulated enzyme productivity ( $P$  and  $dP/dt$ ). On the other hand the second starch addition had no influence on either biomass or enzyme production.



**Figure 11.** Smoothed plot of biomass formation by strain SCH under four different conditions in the fermentor.



**Figure 12.** Rate of biomass formation by strain SCH under four different conditions in the fermentor

Although it would appear that good growth could be sustained simply by increasing initial starch from 2.5 to 3.5%, an addition of 2% starch significantly stimulated enzyme production. Taking the results of  $P$  and  $dP/dt$  together (Figure 13 and 14) one may conclude that the main effect in condition 4 is due to the higher initial starch concentration and not the starch added during the reaction. However, with this strain it is probably best to use high initial starch concentration (higher growth and hence higher enzyme productivity potential) and to further induce enzyme productivity by an additional starch feed after about 15 - 20 h (sustained high level enzyme productivity).



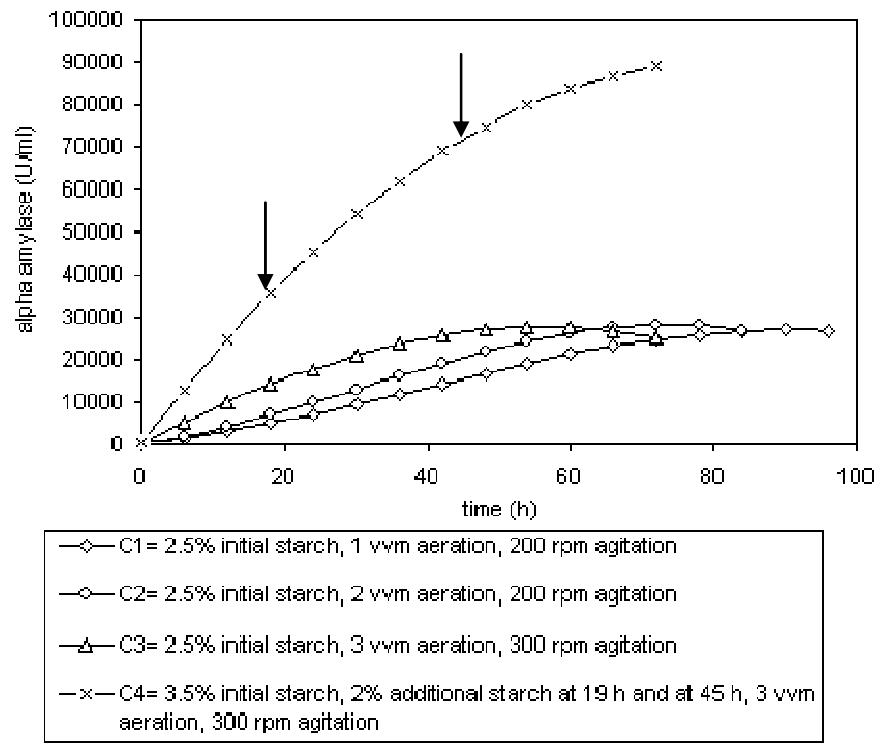


Figure 13. smoothed plot of alpha amylase production by strain SCH under four different conditions in the fermentor

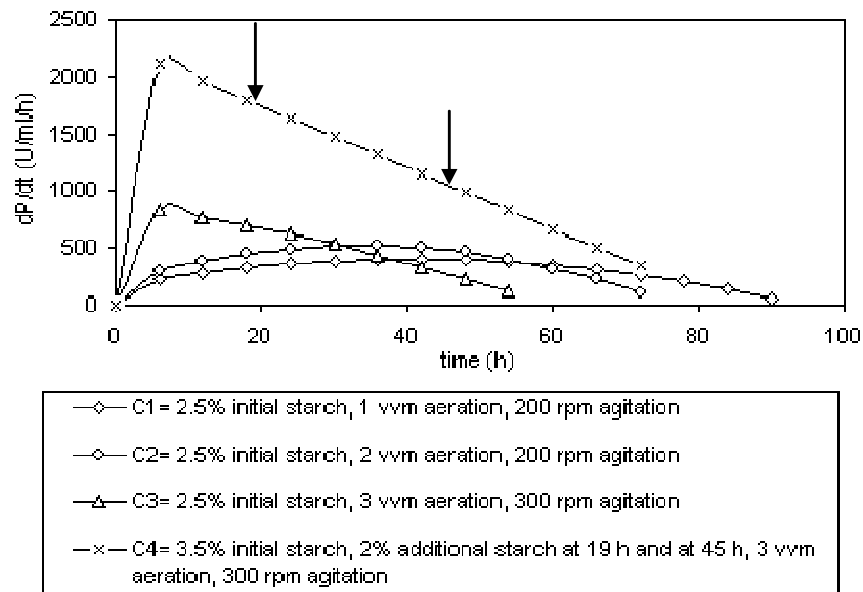
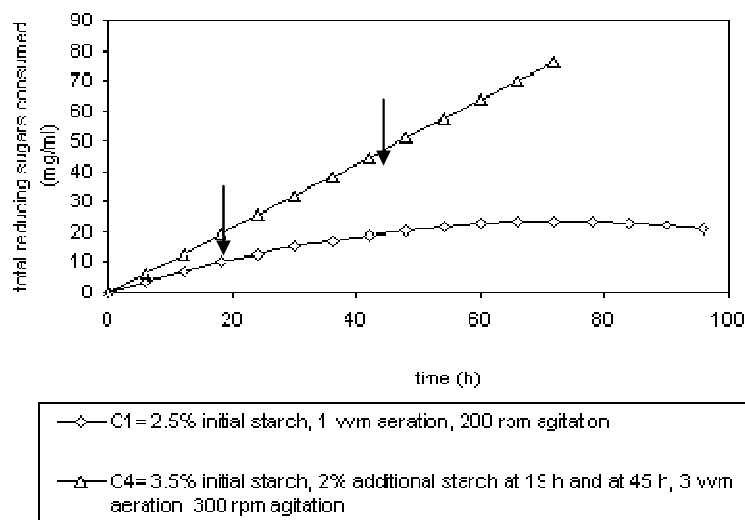


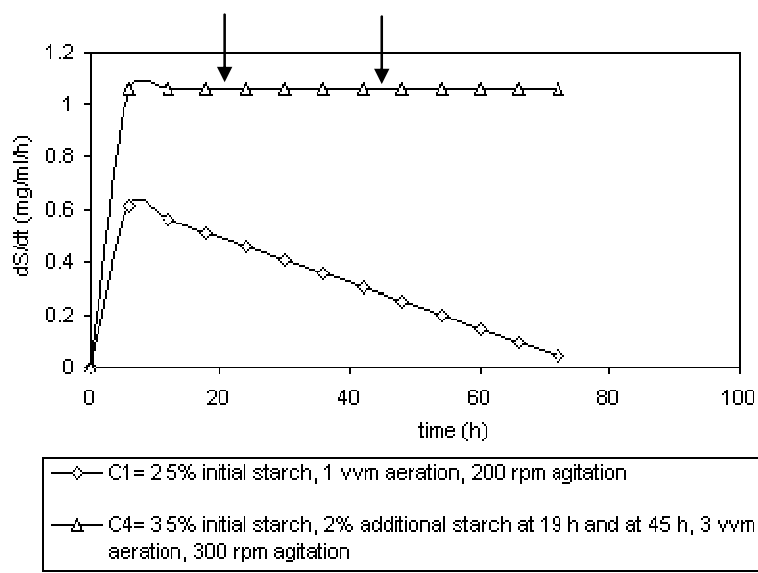
Figure 14. Rate of alpha amylase production by strain SCH under four different conditions in the fermentor

The approach of smoothing the plot proved useful both in clarifying subtle differences between different condi-

tions (through making available more time points) and also in making it possible to calculate more accurate



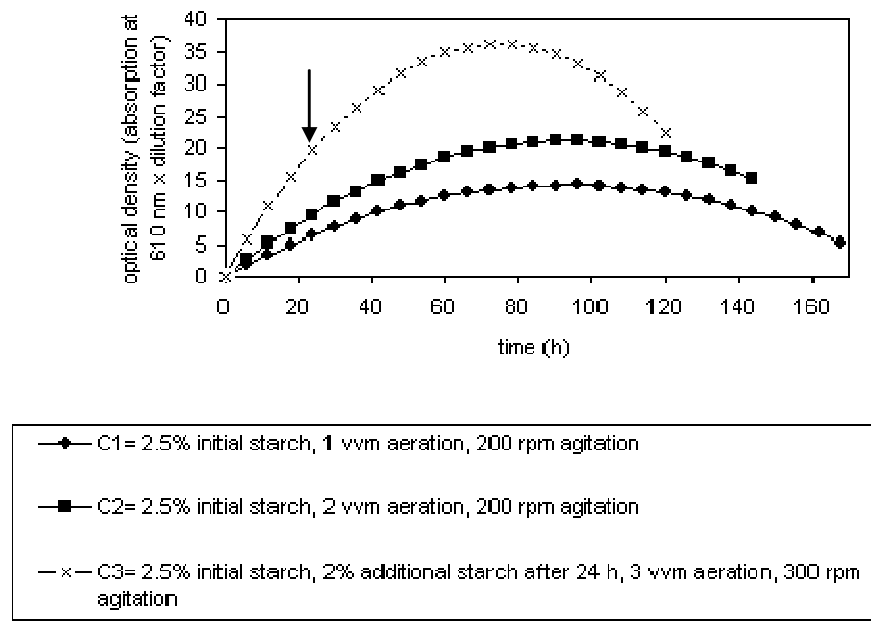
**Figure 15.** Smoothed plot of total reducing sugars consumption by strain SCH under two different conditions in the fermentor



**Figure 16.** Rate of total reducing sugars consumption by strain SCH under two different conditions in the fermentor

rates. Combining values of yields and rates enables one to determine the economics of changes in process conditions and of optimum harvest time. For example examining Figures 13 and 14 one finds that extending the process from 25 to about 50 h would probably be cost effective since it attains a doubling of enzyme yield and still a reasonably high rate of enzyme production. Similarly examining Figure 12 explains the drop in biomass yield after the second starch addition shown in Figure 11.

The smoothed plot of total reducing sugars consumption (Figure 15) showed that the sugars consumed under condition 4 exceeds that under condition 1 along the total incubation period. In addition the sugars consumption under condition 4 exhibited a linear pattern throughout the incubation period. This linearity in consumption probably resulted from on-line starch addition. Examining Figure 16, we note that under both conditions there was a rapid and sharp increase in sugar consumption rate with-



**Figure 17.** smoothed plot of biomass formation by strain 267CH under three different conditions in the fermentor

in the first 6 h followed by a constant high rate (condition 4) and an almost linear decline of rate (condition 1).

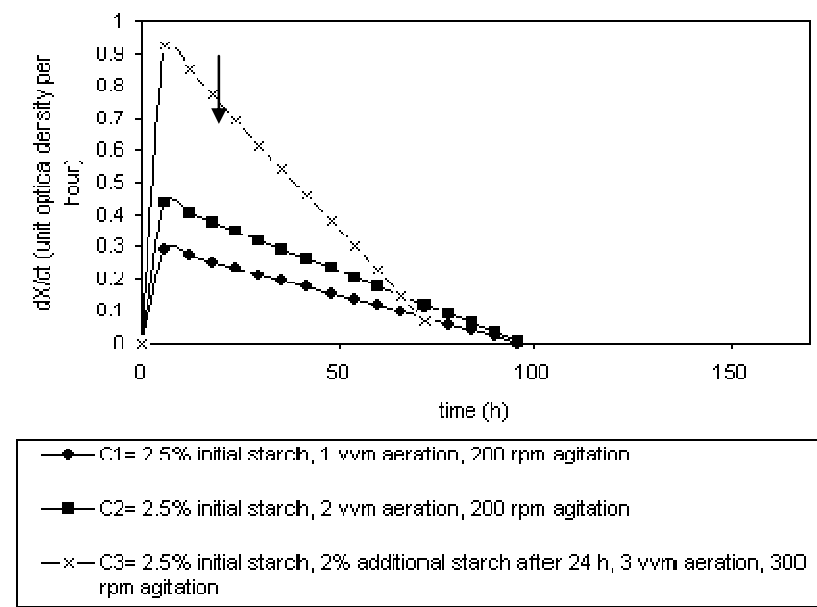
Figure 2 showed that soluble proteins and residual starch drop rapidly within the first 20 h then remain more or less constant afterwards – regardless of the conditions – with greater drop in residual starch than in soluble proteins upon further incubation. These were monitored on only the two extreme conditions: 1 and 4.

### Conditions for production by strain 267CH

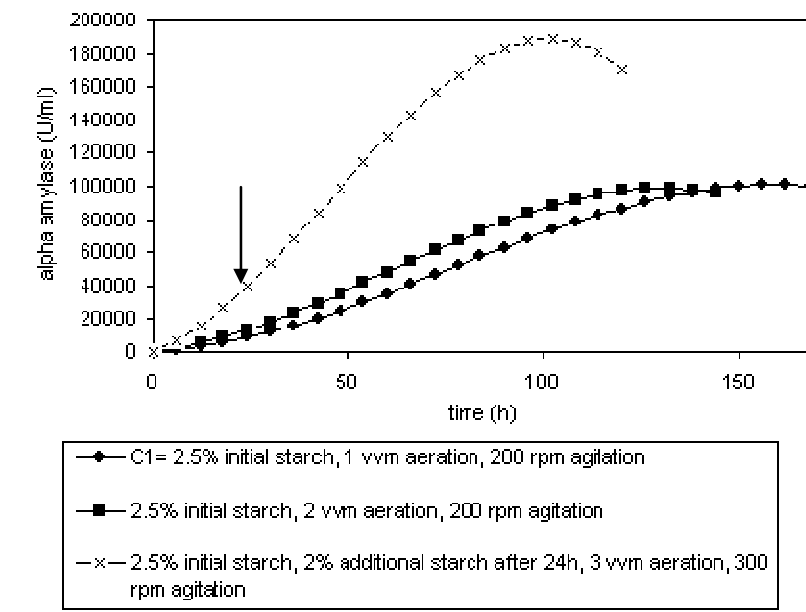
The conditions tested with strain 267CH took advantages of the results obtained with strain SCH. They were somewhat streamlined to emphasize the influence of during the process-addition of starch at the highest levels of oxygen supply (aeration and agitation). Two different fermentation conditions with regard to levels of aeration (1 and 2 vvm) were tested at a constant agitation of 200 rpm. In both cases, the initial starch concentration was 2.5%. In the third experiment, both aeration and agitation were increased to 3 vvm and 300 rpm respectively and an additional 2% starch was added at hours 24. In contrast to strain SCH, in all the three tested conditions, biomass maxima preceded the alpha amylase maxima (Figure 3). Biomass maxima occurred after either 24 h (condition 3) or after about 65 h (conditions 1 and 2) followed by a plateau in case of conditions 2 and 3 or a decline in case of condition 1. On the other hand, the alpha amylase maxima occurred after 90, 110 and 144 h

for conditions 3, 2 and 1 respectively (Figure 3). In all cases these enzyme maxima remained almost constant afterwards. In contrast to strain SCH the pattern of residual starch/intermediates and of residual proteins was different in spite of the general observation of rapid decline within the first 20 h (residual starch/intermediates) or within the first 40 h (residual soluble proteins) followed by either a small gradual decrease (residual starch/intermediates in conditions 1 and 2 only) or almost a plateau (residual soluble proteins). Here the residual starch/intermediates and residual proteins are inversely proportional to aeration and to both aeration and agitation (Figure 4) the on-line starch addition resulted in a peak in residual starch/intermediates. This is also different from the response with strain SCH where on-line starch addition resulted in a plateau followed by a slight decline for starch/intermediates. As was the case with strain SCH, in all cases dissolved oxygen dropped to 0% saturation soon after growth began and remained there for about 4 days (conditions 1 and 2) or about 3 days (condition 3), indicating a degree of oxygen starvation even at high aeration and agitation rates (3 vvm and 300 rpm). Higher rates were not tested in order to avoid excessive foaming.

Smoothed time plots were drawn in each case for biomass (optical density, X), enzyme production (P), and sugars consumption (S). The respective  $dX/dt$ ,  $dP/dt$ , and  $dS/dt$  were also calculated and the results are shown in Figure 17 - 22. Comparison of the three conditions reveals increased biomass production, enzyme produc-



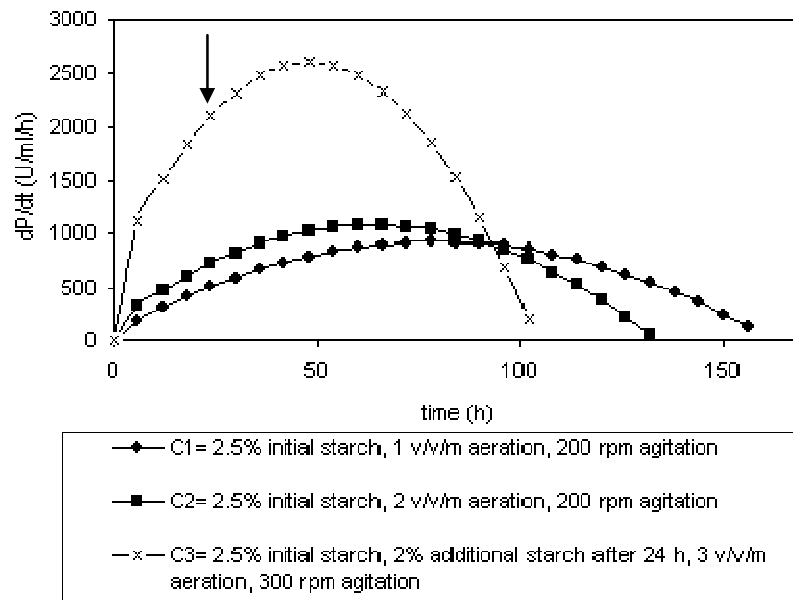
**Figure 18.** Rate of biomass formation by strain 267CH under three different conditions in the fermentor



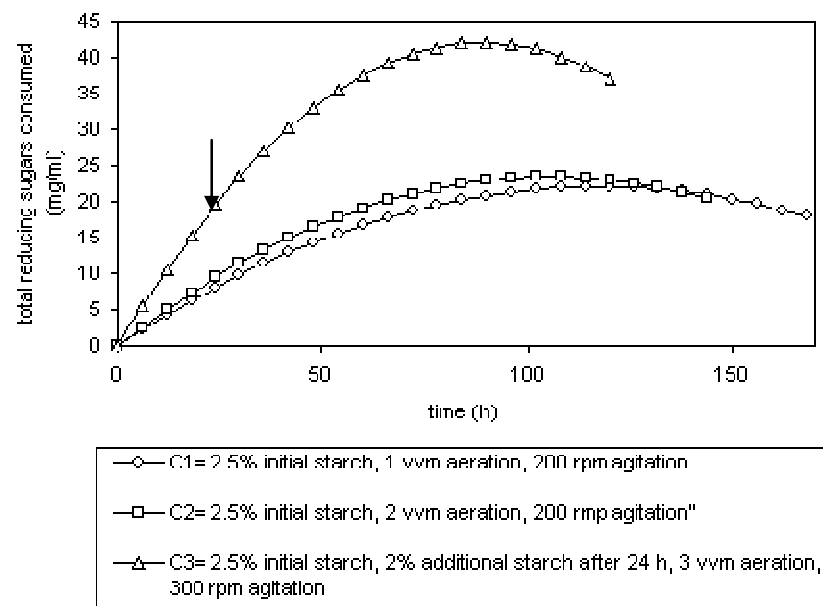
**Figure 19.** Smoothed plot of alpha amylase production by strain 267CH under three different conditions in the fermentor.

tion,  $dX/dt$  and  $dP/dt$  by increasing the rates of aeration and/or agitation (Figures 17 - 20). Looking closely at the results of conditions 1, 2 and the first phase (before starch addition) of condition 3, one note that at constant initial starch concentration, increased aeration alone (condition 2 compared to condition 1) produced only a

small effect on all the parameters ( $X$ ,  $P$ ,  $S$ ,  $dX/dt$ ,  $dP/dt$ , and  $dS/dt$ ) while increased aeration and agitation together resulted in a dramatic increase in all the six parameters (Figures 17 - 22). This result is qualitatively in line with observations made with strain SCH but the influence seems much more pronounced with strain



**Figure 20.** Rate of alpha amylase production by strain 267CH under three different conditions in the fermentor

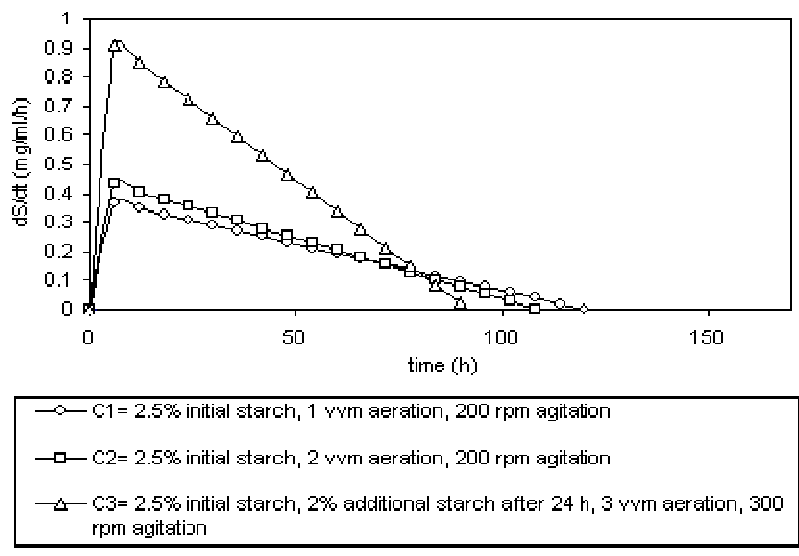


**Figure 21.** Smoothed plot of total reducing sugars consumption by strain 267CH under three different conditions in the fermentor.

267CH. Examining the influence of the second phase of condition 3 (2% additional starch at hour 24) we note that additional starch did not significantly influence biomass formation,  $dX/dt$ ,  $dP/dt$  or  $dS/dt$  although it resulted in sustaining the high  $dP/dt$  (hence increased enzyme productivity) and the high  $dS/dt$  (hence increased

substrate consumption) obtained in the first phase of the process as a result of simple increased aeration and agitation.

One may conclude that, with this strain it is best to apply high aeration and agitation (higher sugars consumption, higher growth and hence higher enzyme produc-



**Figure 22.** Rate of total reducing sugars consumption by strain 267CH under three different conditions in the fermentor

tivity potential) and to further maintain growth and induce enzyme productivity by an additional starch feed after about 20 - 25 h (sustained high level enzyme productivity).

The two strains thus responded somewhat differently to increased aeration, agitation and additional starch. Highest enzyme productivity by strain SCH was about  $8 \times 10^4$  U/ml within 60 h with 3.5% initial starch and 2% additional starch after 19 h and neither further incubation, nor additional starch could sustain higher productivity. On the other hand, productivity by strain 267CH was about  $14 \times 10^4$  U/ml within 60 h with only 2.5% initial starch and 2% additional starch after 24 h and the productivity was sustained by increased aeration/agitation and by the additional starch, reaching the highest level (about  $19 \times 10^4$  U/ml) in 100 h. This finding should be taken into consideration in examining the economics of production by both strains.

#### Characteristics of alpha amylase produced by the variants SCH and 267CH

The characteristics of alpha amylases produced by *B. subtilis* (SCH) and *B. amyloliquefaciens* (267CH) showed that both enzymes exhibited similar characteristics. The results may be summarized as follows:

- Broad pH and temperature optimum profiles for activity.
- Complete thermal stability up to 50°C and good thermal stability at higher temperatures up to 90°C.

- The enzyme is of the liquefying and not the saccharifying type: amyolytic action producing 15 - 20 DE at 70 to 95°C.
- Thermal activity is enhanced in the presence of high starch concentration (35%).

The result (a) could suggest that the product is a mixture of enzyme species while the results (c), (d) and (e) place the product in the category of thermostable liquefying alpha amylases.

Both enzymes showed higher activity over a wide pH range extending from pH 4 to 8 (Figure 5) with pH optima of 4 - 7 (strain SCH) and of 6 (strain 267CH). A difference may be noted between the enzymes produced by the two strains. The enzyme produced by strain 267CH seem to show a tendency to "peak" at pH 6 while that of strain SCH appears to act more or less equally at pH 4 - 7. This difference may prove exploitable in industrial production and/or application. Reports on bacterial alpha amylase with broad pH profiles are not uncommon (Saito, 1973; Medda and Chandra, 1980). Bacterial alpha amylase activity extending over the pH 3 - 9.5 with an optimum at pH 6 was reported by Windish and Mhatre (1965). Similarly, the temperature activity profile of both enzymes do not show obvious differences. The activity extends over a temperature range of 25 to 80°C (Figure 6) with optimum temperature ranging between 37 and 70°C (at 0.5% starch) for both enzymes. However, this optimum increased to 85 - 95°C at 35% starch (Figure 9). Gerhartz (1990) reported that the bacterial alpha amylase from *B. subtilis* and *B. amyloliquefaciens* has an optimal temperature of 70 to 85°C while "hyper" thermostable bacterial alpha amylase showed a temperature optimal at

90 to 105°C.

On the other hand, thermal inactivation profiles of both enzymes showed that the enzyme was nearly unaffected at 37 and at 50°C. Increasing the temperature to 70 and 90°C resulted in about 50% reduction in activity after 30 and 10 min respectively (Figure 7). Gerhartz (1990) reported that the bacterial alpha amylases from *B. subtilis* and *B. amyloliquefaciens* are inactivated at 95°C while "hyper" thermostable bacterial alpha amylases were inactivated at 120°C. Pepler and Reed (1987) reported that about 80% reduction in alpha amylase activity of *B. amyloliquefaciens* and about 68% reduction in alpha amylase of *B. licheniformis* were obtained in 30 min at 70°C at pH 6.5 - 7 in the absence of the substrate. They stated that such bacterial alpha amylases are quite heat-stable as compared to fungal and cereal alpha amylases. They further reported that this heat stability is well suited to the liquefaction of starch at temperatures exceeding the gelatinization temperature.

Both enzymes hydrolyzed soluble starch to not more than 35% (Figure 8) indicating that they are of the liquefying type according to the classification proposed by Windish and Mhatre (1965). The pattern of hydrolysis by the enzymes of both strains was rather sharp: starch was hydrolyzed to about 26% within the first 10 min. The hydrolyzed products produced were not so much attacked by the enzyme where not more than 8% increases in the hydrolysis were obtained in the following 110 min. After 120 min there was no further increase in the percent hydrolysis. This pattern of hydrolysis gives additional evidence that the enzymes are of the liquefying and not the saccharifying type. In industrial processes starch liquefaction is carried out at a pH around 6.5 with a starch concentration between 30 and 40%. Generally the alpha amylase of *B. amyloliquefaciens* is added at the beginning of the heating cycle. The temperature is raised to 80°C and held there for 1 h (heating without the use of enzymes resulted in a colloidal solution which is extremely viscous at higher starch concentration and can not be handled in an industrial plant). The temperature is then briefly raised to 140°C under pressure. After cooling to 85°C more enzyme is added and the liquid is held at this temperature for another 1 h (Ostergaard, 1982). In examining the liquefaction efficiency of alpha amylases of *B. subtilis* and of *B. amyloliquefaciens* used in our study at different temperatures (70, 75, 85, 95 and 105°C) the following conditions were applied: 35% raw maize starch, pH 6.9, at the first liquefaction step; the slurry produced was heated at 121°C in the autoclave for 10 min then cooled to the specified temperature before the incorporation of more enzyme. The results showed the efficiency of both enzymes in the liquefaction of starch to an acceptable dextrose equivalent of 14 to 17 (Figure 9). The optimum liquefaction temperature was 85 - 95°C. There is a slight noticeable difference between the enzymes produced by the two strains in this respect, with

that of strain 267CH showing higher stability at the upper limits of the temperature range. The application of 35% maize starch rather than the usual 0.5% soluble starch more realistically reflects industrial processes for hydrolysis of starch by alpha amylase.

Paper chromatography of soluble starch hydrolysis products by the two enzymes showed small amounts of glucose and maltose and higher amounts of higher maltosaccharides at all reaction periods tested (Figure 10). This provides another indication that both enzymes are of the liquefying type since the saccharifying enzymes would produce mostly glucose and maltose from starch (Chiang et al., 1979; Matsuzaki et al., 1974).

Both enzymes thus appeared promising for industrial application since industrial liquefaction of starch is usually performed at 80 - 95°C. The liquefaction hydrolysate can be subsequently modified by saccharifying enzymes as a step in production of either dextrose or high fructose corn syrup, an important commodity in the international market. The higher activity observed at low pH further supports them as viable candidates for industrial application since the use of such enzymes reduces the need for pH adjustment for the saccharification of reaction following liquefaction.

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