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Full Length Research Paper

Optimization of the industrial production of bacterial α-amylase in Egypt. V. Analysis of kinetic data for enzyme production by two strains of *Bacillus amyloliquefaciens*

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A kinetic study was conducted for α-amylase production process in shake flasks by the wild type strain of *Bacillus amyloliquefaciens* (strain 267) and in the fermentor by the amplified variant of the same organism (strain 267CH). α-Amylase was produced concurrently with growth up to about 72 h, after which it continued without increase in biomass and, in the case of strain 267 even after biomass declined. Application of logistic, Luedeking-Piret and the modified Luedeking-Piret mathematical models to the kinetic data revealed that α-amylase production in both cases occurred through both growth- and non-growth associated mechanisms and that the amount of enzyme produced through non-growth associated mechanism exceeded that produced through growth associated mechanism by 3.5 and 2.3 fold by strains 267 and 267CH, respectively. Although with both strains substrate consumption continued even after growth leveled, the application of the model revealed that the major portion of substrate consumption occurred during growth but that a considerable amount was also consumed after maximum growth was reached, mainly for enzyme production. In the bioreactor, increasing aeration from 1 to 2 vvm increased the overall specific growth rate, the production rate, the specific production rate, and the specific substrate consumption rate and also shortened the time necessary for maximum production of both biomass and enzyme. The increase in biomass either by prolonging the incubation time or by increasing aeration was accompanied by an increase in enzyme production. However, even after maximum biomass was reached, enzyme production still continued to increase, under both conditions.

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

INTRODUCTION

Modeling microbial systems has become a popular means of aiding in the understanding of how the system operates, and/or predicting how it would perform under different sets of conditions. Kinetics of α-amylase production has been reported in a number of reports (Baig et al., 1984; Pazlarova et al, 1984; Ponzo and Weigand, 1991; Uchiyama and Shioya, 1999; Vortruba et al., 1984; Yoo et al., 1988). The Logistic model has been used to describe biomass formation (Moraine and Rogovin, 1966). The most widely used kinetic model for product formation in biotechnology processes is the Luedeking–Piret model (Zeng, 1995). This model assumes that product formation may be attributed to growth–associated and/or non growth–associated mechanisms. The non growth-associated mechanism is often considered to be conditional on maintaining the functions of cells. Substrate consumption for the fermentation processes can be described by applying the modification of Luedeking and Piret model (Weiss and Ollis, 1980). In this communication the last three mathematical models were applied to experimental results of the batch culture of α-amylase production in shake flasks and in the fermentor.

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MATERIALS AND METHODS

Kinetics of α-amylase production by *B. amyloliquefaciens* strains 267 (in shake flasks) and 267CH (in the fermentor)

Batch culture experiments were conducted in shake flasks using strain 267 and in a 7.5 l New Brunswick Scientific Laborferm bioreactor using the amplified variant 267CH. In shake flasks, an inoculum was prepared by inoculating 50 ml nutrient broth in 250 ml Erlenmeyer flask at a rate of 10% from an overnight culture. After incubation at 37°C for 24 h in a reciprocating water bath, the growth obtained was adjusted to $O.D_{610}$ of 0.818 and used to inoculate 100 ml of mutiprotein-mineral medium contained in 250 ml Erlenmeyer flask at a rate of 10% (El-Tayeb et al., 2000). The flasks were incubated in a reciprocating water bath shaker for 6 days at 37°C. In the bioreactor, 3 l multiprotein-mineral medium containing only 2.5% starch instead of 5% were inoculated at the rate of 10 % v/v of an overnight culture in the same media developed in shake flasks. Agitation was adjusted at 200 rpm, aeration at either 1 or 2 vvm, pH at 7.2, temperature at 37°C and foam was suppressed by silicone oil using a foam controller (model AF P-101, New Brunswick Scientific Co.) (El Tayeb et al., 2002).

In both shake flasks and in the fermentor, samples were withdrawn at different time intervals for determining: growth as dry weight using a calibration curve constructed between optical density and dry weight, α-amylase activity and residual substrate in terms of total reducing sugars. Methods for analysis were described previously (El Tayeb et al., 2001).

Manipulation of experimental data and mathematical modeling

The experimental data from shake flasks and from the fermentor were smoothed by computing the equations of the best fit plots followed by calculating additional data points. These best fit plots were either polynomial or exponential and the correlation coefficient was close to 1. The smoothed data obtained were applied for computing additional kinetics data points.

The logistic model was used for describing biomass formation, the Luedeking-Piret model was used for describing α-amylase production and the modified Luedeking-Piret model was used for describing substrate consumption. All three models are unstructured and non-segregated.

Biomass: logistic model

$$dX/dt = \mu X (1.0 - X/X_{max}) \quad [1]$$

Where, X is the biomass (mg/ml) at time (t), μ and X_{max} are the specific growth rate (h^{-1}), and the maximum attainable biomass (mg/ml), respectively. The integrated form using $X_o = X$ (t=0) gives a sigmoidal variation X (t) that may empirically represent both an exponential and a stationary phase:

$$X(t) = \frac{X_o e^{\mu t}}{1.0 - (X_o/X_{max})(1.0 - e^{\mu t})} \quad [2]$$

The Logistic equation [1] has been used to evaluate μ (Moraine and Rogovin, 1966), rearranged form of equation [2] produces equation [3]

$$\mu t = \ln [(X_{max}/X_o) - 1.0)] + \ln[X^-/(1 - X^-)] \quad [3]$$

Where $X^- = X/X_{max}$, X_o = initial biomass concentration, t = time (h). When $\ln [X^-/(1.0 - X^-)]$ is plotted against time, a straight line is obtained with a slope equals μ.

$$\text{The intercept} = \ln [(X_{max}/X_o) - 1.0] \quad [4]$$

Product: Luedeking-Piret model

Luedeking-Piret model was developed originally for the formation of lactic acid by *Lactobacillus delbrueckii* (Luedeking and Piret, 1959).

$$dP/dt = nX + m(dX/dt) \quad [5]$$

Where n and m are the non-growth ($U.mg^{-1}.h^{-1}$) and growth ($U.mg^{-1}$) associated constants respectively and dP/dt ($U^{-1}.ml^{-1}.h^{-1}$) is the production rate throughout the earlier exponential phase. The constant n may be evaluated from the stationary phase data (dX/dt = 0)

$$n = \frac{(dP/dt) \text{ at stationary phase}}{X_{max}} \quad [6]$$

Integration of equation [5] using equation [3], gives an equation with two initial concentrations (X_o, P_o), a final condition X_{max} and three parameters (μ, n and m)

$$P_{(t)} = P_o + m.X_o \{e^{\mu t}/[1.0 - (X_o/X_{max})(1.0 - e^{\mu t})] - 1.0\} + n.X_{max}/\mu.\ln[1.0 - (X_o/X_{max})(1.0 - e^{\mu t})] \quad [7]$$

When $\{P_{(t)} - P_o - n.X_{max}/\mu.\ln[1.0 - (X_o/X_{max})(1.0 - e^{\mu t})]\}$ is plotted against $X_o \{e^{\mu t}/[1.0 - (X_o/X_{max})(1.0 - e^{\mu t})] - 1.0\}$, the slope of the straight line produced equals m.

Specific production rate dP/dt/X ($U.mg^{-1}.h^{-1}$) can be computed from equation [5].

The amount of product produced by growth associated P_m, (U/ml) and the non-growth associated, P_n, (U/ml) mechanisms were calculated by equations [8] and [9] respectively.

$$P_m = m.X_o \left[\frac{e^{\mu t}}{1.0 - (X_o/X_{max})(1.0 - e^{\mu t})} 1.0\right] \quad [8]$$

$$P_n = n.(X_{max}/\mu) \ln \{1.0 - (X_o/X_{max})(1.0 - e^{\mu t})\} \quad [9]$$

Substrate consumption: Modified Luedeking-Piret model

The substrate balance for the fermentation may be written by applying the modification of Luedeking and Piret as follows (Weiss and Ollis, 1980)

$$\frac{dS}{dt} = -\frac{1}{Y_x}\left(\frac{dX}{dt}\right) - \frac{1}{Y_p}\left(\frac{dP}{dt}\right) - K_e X \quad [10]$$

growth product maintenance

Where Y_x, Y_p are the yield coefficients of biomass and product respectively and where K_e is the specific maintenance rate that is the substrate used to support cell activity even in the absence of growth.

From equations [5] and [10] we deduce:

$$dS/dt = -\alpha(dX/dt) - \beta X \quad [11]$$

Where α and β are constants for growth associated ($mg.mg^{-1}$) and non-growth associated substrate consumption ($mg.mg^{-1}.h^{-1}$) respectively. Constant β can be calculated from the stationary phase

Table 1. Kinetic parameters (overall reaction)[1] for biomass formation, α-amylase production and substrate consumption by strains 267 (in shake flasks[2]) and 267CH (in the fermentor[3]).

Parameter	Strain 267	Strain 267CH	
		1 vvm	2 vvm
X_{max} (at hr) ($mg.ml^{-1}$)	6.07 (90)	6.18 (120)	7.14 (108)
μ (hr^{-1})	0.087	0.0644	0.073
P_{max} (at hr) ($U.ml^{-1}$)	4.3×10^4 (144)	9.9×10^3 (144)	9.9×10^3 (120)
dP/dt ($U.ml^{-1}.hr^{-1}$)	298.8	684.6	823.9
(dP/dt)/X ($U.mg^{-1}.hr^{-1}$)	212.8	443.3	473.3
m ($U.mg^{-1}$)	1,880.8	5,161.8	4,903.0
P_m ($U.ml^{-1}$)	9,863	29,044	32,095
n ($U.mg^{-1}.hr^{-1}$)	49.2	110.8420	115.337
P_n ($U.ml^{-1}$)	34,515	72,134	69,193
(dS/dt)/X ($mg.mg^{-1}.hr^{-1}$)	0.429	0.173	0.200
α ($mg.mg^{-1}$)	4.542	2.486	2.598
β ($mg.mg^{-1}.hr^{-1}$)	0.034	0.013	0.010

[1]Except for X_{max} and P_{max}; [2]medium, multiprotein-mineral medium, initial starch concentration, 5%; [3]medium, multiprotein-mineral medium without glycine; starch concentration, 2.5%; pH, 7.2; agitation, 200 rpm. In both strains, incubation temperature 37°C.

X_{max} = maximum biomass; μ = specific growth rate; P_{max} = maximum enzyme production; dP/dt = production rate; (dP/dt)/X = specific production rate; m = constant for growth associated production; n = constant for non-growth associated production; (dS/dt)/X = specific substrate consumption rate; α = constant for growth associated substrate consumption; β = constant for non-growth associated substrate consumption.

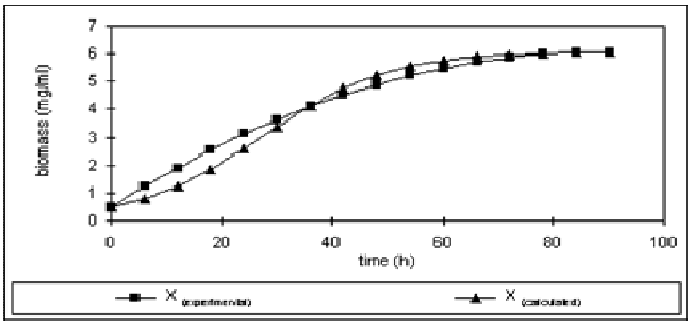

Figure 1. Time course of experimental and calculated biomass by strain 267 in shake flasks (5% starch).

$(dX/dt) = 0$

$$\beta = \frac{(dS/dt) \text{ at stationary phase}}{X_{max}} \quad [12]$$

Substituting equation [2] in equation [10] and integrating gives:

$$S_o - S_{(t)} = \alpha (X - Xo) + \beta (X_{max}/\mu) \ln (1.0 - (X_o/X_{max}) (1.0 - e^{\mu t}) \quad [13]$$

Where S_o refers to the initial substrate concentration. When $\{(S_o - S_{(t)}) - \beta . (X_{max}/\mu) \ln (1.0 - (X_o/X_{max}) (1.0 - e^{\mu t})\}$ is plotted against (X-Xo), the slope of the straight line produced equals α.

The specific substrate consumption related to growth metabolic activity, S_α, (mg/ml), and to non-growth metabolic activity, S_β, (mg/ml) are given in equation 14 and 15 respectively.

$$S_\alpha = \alpha X_o \left(\frac{e^{\mu t}}{1.0 - (X_o/X_{max}) (1.0 - e^{\mu t})} - 1.0\right) \quad [14]$$

$$S_\beta = \beta (X_{max}/\mu) \ln (1.0 - (X_o/X_{max})(1.0 - e^{\mu t}) \quad [15]$$

RESULTS

The kinetic parameters of the production process in shake flasks (strain 267) and in the fermentor (strain 267CH) were obtained by applying Logestic equation (for biomass formation), the Luedeking-Piret equation (for α-amylase production) and the modified Luedeking-Piret equation (for substrate consumption). These are presented in Table 1 for strains 267 and 267CH. The fitting of the description of the process by the models applied was checked by comparing the experimental data to the data calculated by simulating the model equations. Figures 1, 2 and 3 and Figures 4, 5 and 6 show good fitting of calculated and experimental data for all parameters for strain 267 and strain 267CH respectively. The yields of enzyme and of the biomass in terms of substrate consumption for strain 267 are calculated and are presented in Figure 7 showing that while enzyme yield increases by time, biomass yield decreases by time. Similar observations are noted for strain 267CH at both aeration regimens tested (Figure 8).

The increment of enzyme produced as related to increment of biomass produced by strains 267 and 267CH were also calculated and plotted in Figures 9 and 10, res-

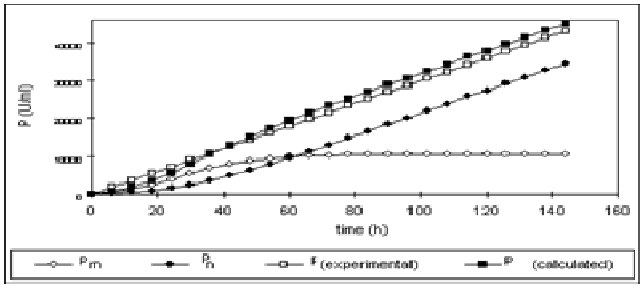

Figure 2. Time course of Pexperimental, Pcalculated, Pm and Pn by strain 267 in shake flasks (5% starch).

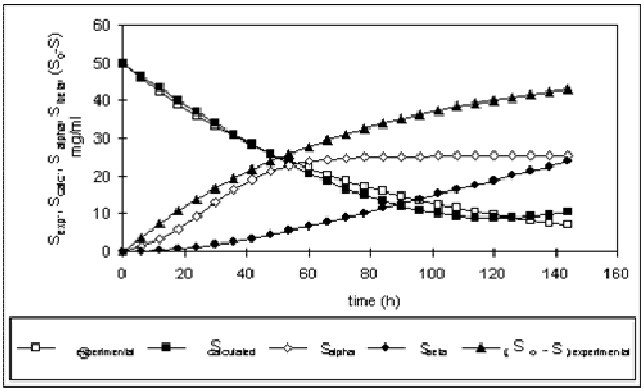

Figure 3. Time course of $S_{experimental}$, $S_{calculated}$, S_{alpha}, S_{beta} and $(S_o-S)_{experimental}$ by strain 267 in shake flasks (5% starch).

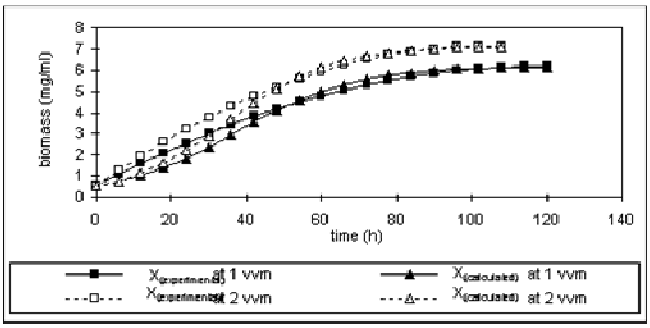

Figure 4. Time course of experimental and calculated biomass by strain 267CH in the fermentor under conditions of 1 and 2 vvm aeration (2.5% starch, 200 rpm agitation).

pectively. The plots showed that enzyme production paralleled biomass formation until a certain point where enzyme production continued while biomass formation leveled. In case of strain 267, at another point in the process enzyme production continued even though biomass declined.

Application of the models for distinguishing enzyme production associated with growth and with non-growth mechanisms produced values of Pm and Pn. These values were plotted for strain 267 and 267CH and the re-

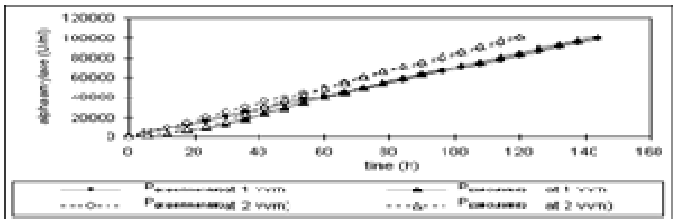

Figure 5. Time course of experimental and calculated alpha amylase production by strain 267CH in the fermentor under conditions of 1 and 2 vvm aeration (2.5% starch, 200 rpm agitation).

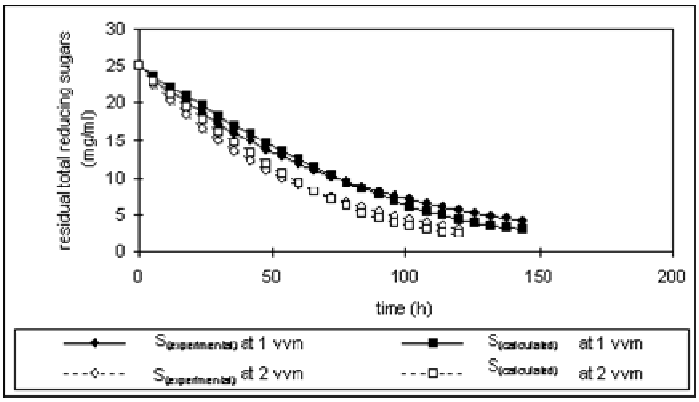

Figure 6. Time course of experimental and calculated residual total reducing sugars by strain 267CH in the fermentor under conditions of 1 and 2 vvm aeration (2.5% starch, 200 rpm agitation).

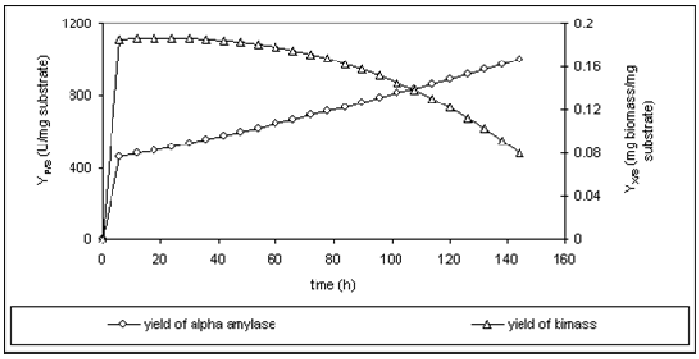

Figure 7. Time course for yields of biomass and alpha amylase with respect to substrate consumption in shake flasks by strains 267 (5% starch).

sults are presented in Figures 2 and 11, respectively. Growth associated enzyme production always preceded and exceeded non-growth associated enzyme production as long as growth was rapid (logarithmic) but it leveled when growth slowed down while non growth associated enzyme production continued. Similarly, application of the models for determining substrate consumption associa-

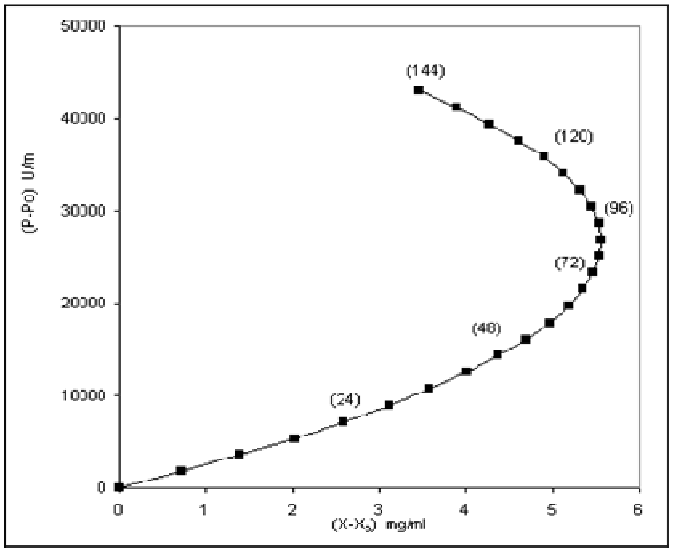

Figure 8. Time course for yields of biomass and alpha amylase with respect to substrate consumption in the bioreactor at 1 and 2 vvm aeration (2.5% starch, 200 rpm agitation).

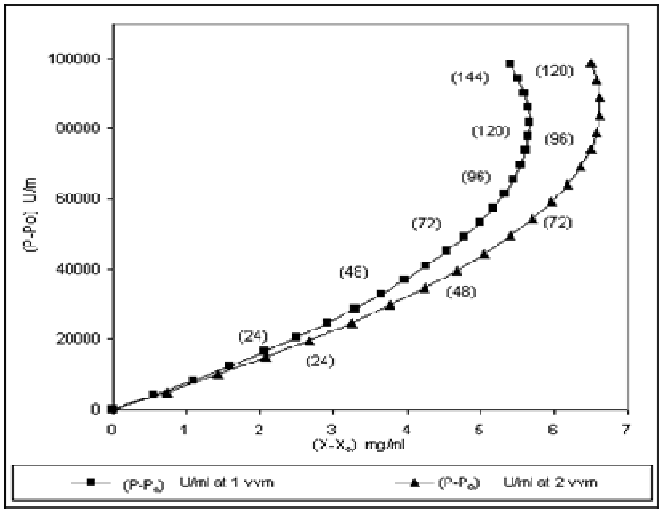

Figure 9. Relationship between biomass formed and alpha amylase produced by strain 267 in shake flasks (5% starch). Values between brackets are the incubation time in hours.

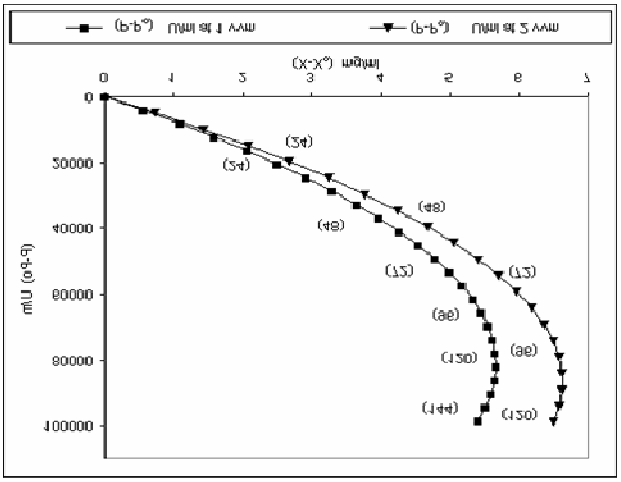

Figure 10. Relationship between biomass formed and alpha amylase produced by strain 267CH in the fermentor under conditions of 1 and 2 vvm aeration (2.5% starch, 200 rpm agitation).

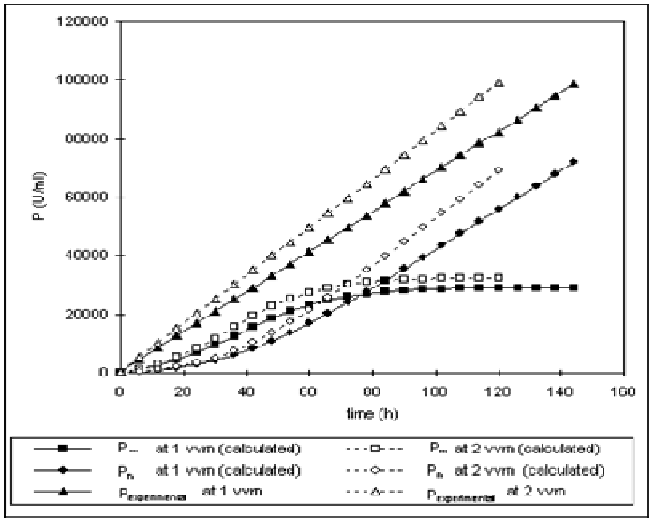

Figure 11. Time course of Pm, Pn, and Pexp. by strain 267CH in the fermentor under conditions of 1 and 2 vvm aeration (2.5% starch, 200 rpm agitation).

ted with growth and with non growth mechanisms produced values of S_α and S_β. These values were plotted for strains 267 and 267CH and the results are shown in Figures 3 and 12, respectively. Growth associated substrate consumption exceeded non-growth associated substrate consumption as long as logarithmic growth continued but it leveled when growth slowed down while non-growth associated substrate consumption continued to increase to reach the maximum value of S_α in case of strain 267, but remained considerably lower than S_α in case of strain 267CH by the end of incubation at both aeration levels.

DISCUSSION

Plotting the calculated data obtained from the model against the experimental data (Figures 1, 2, 3, 4, 5 and 6) one can conclude that, under the operating conditions the model described well the fermentation process with respect to biomass formation, α-amylase production and substrate consumption. For production in shake flasks by strain 267, α-amylase production was growth and non-growth associated although the non-growth associated production (P_n) exceeds that of growth associated production (P_m) by 3.5 fold (Figure 2). This emphasis that the main production occurred in the post-logarithmic growth

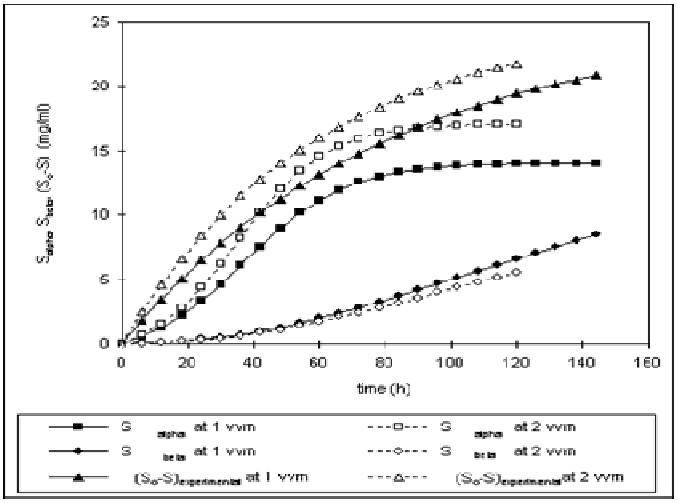

Figure 12. Time course of Salpha, Sbeta and (So-S)experimental by strain 267CH in the fermentor under conditions of 1 and 2 vvm aeration (2.5% starch, 200 rpm agitation).

phase (from 60 to 144 th h). The kinetic data showed also that substrate consumption (total reducing sugars consumption) was due to both growth and non-growth metabolic activities (S_α and S_β respectively) but at X_{max} (60 h) S_α exceeds S_β by about 2.5 fold (Figure 3) while at P_{max} (144 h) S_α equals S_β. This was due to the fact that after X_{max} is reached there was no longer an increase in S_α while S_β continued to increase parallel to P_n reaching its maximum at P_{max}. This indicates that both growth and production contribute to substrate consumption. After X_{max} was reached the concentration of substrate consumed (hence its amount, since the volume is practically constant) was about 14 mg/ml while that consumed from zero time up to X_{max} was about 28 mg/ml (Figure 3) with about 8 mg/ml remaining at the end of the fermentation. The biomass showed a significant decline after X_{max} was reached (Figure 9) and accordingly one may conclude that further substrate consumption (about 14 mg/ml) mainly served enzyme production, which became biomass independent.

A striking observation is that there was a significant decline in biomass after X_{max} was reached, although the organism under study is a sporulating one, while production still continued during this period. This was obvious upon plotting ($P-P_o$) against ($X-X_o$) (Figure 9). Roychoundhury et al. (1989) using a strain of *B. amyloliquefaciens* showed that while α-amylase synthesis and secretion began during the early part of the growth phase, the bulk of the enzyme activity was detected during the death phase. They further reported that during the death phase, the specific enzyme formation rate was linearly proportional to the specific cell lysis rate indicating the role of cell lysis in the increase in enzyme activity during this phase. We should take into consideration that biomass determination by either dry weight or by optical density does not necessarily correspond with viable count (hence reproduction of cells) in a sporulating culture where the dry weight or optical density of vegetative, sporangial and spore populations are not comparable.

For production in the fermentor by strain 267CH, the kinetics study was carried out at 1 and 2 vvm aeration levels. At the higher aeration rate there was an increase in X_{max} (which also shifted to an earlier time) along with an increase in specific growth rate (μ), this increase in biomass was not accompanied by an increase in P_{max} but shifted the time of P_{max} to an earlier time (120 h at 2 vvm instead of 144 h at 1 vvm aeration). Concurrently both the production rate (dP/dt) and specific production rate [(dP/dt)/X] increased (Table 1). As was observed in shake flasks with strain 267, α-amylase production at both aeration levels was related to both growth- and non-growth associated mechanisms (P_m and P_n) but P_n exceeds P_m by only about 2.3 fold (3.5 fold in shake flasks). At the time of X_{max}, S_α exceeded S_β by 3 fold (1 vvm) and by 4 folds (2 vvm). However in contrast to the results in shake flasks with strain 267, when P_{max} was reached here (144 h at 1 vvm and 120 hrs at 2 vvm), S_α still exceeded S_β (Figure 12). At 2 vvm, the total amount of substrate consumption (S_o - S) was higher than that at 1 vvm (Figure 12), probably as a result of increased specific substrate consumption rate {(dS/dt)/X} than that at 1 vvm (Table 1) and most of the substrate was consumed during the period up to X_{max}. Upon plotting ($P-P_o$) against ($X-X_o$) it was noted that, the increase in biomass by time or by an increase in aeration was accompanied with an increase in production (Figure 10) and as the culture entered the stationary phase the rate of production showed a further increase in slope.

The results of kinetic analysis of the experimental data reveal a common pattern for both strains in shake flasks and in a fermentor. These could be summarized as follows:

(i) Enzyme production is both growth and non-growth associated but P_n is always higher than P_m

(ii) Substrate consumption is both growth and non-growth associated but at the time of X_{max}, S_α is higher than S_β and this difference is more obvious at the higher aeration rate in the fermentor. At the time of P_{max} (144 h), however S_α and S_β became equal in the case of strain 267 grown in shake flasks suggesting that both growth and enzyme production contribute to substrate consumption, with substrate consumption becoming mainly due to enzyme production after X_{max}, has been attained. This is further confirmed by the fact that P_m too becomes constant after X_{max}. On the other hand with strain 267CH in the fermentor S_α remains considerably higher than S_β at the time of P_{max} (120 or 144 h) at both aeration rates (1 and 2 vvm respectively). Here too, while both growth and enzyme production contribute to substrate consumption the latter becomes mainly due to enzyme production after X_{max} has been attained and this is also further confirmed

by the fact that P_m too becomes constant after X_{max}. This suggests that the metabolic pattern for both strains is similar regardless of use of shake flasks or fermentors.
(iii) After X_{max}, has been attained, X declines in shake flasks than in the fermentor.
(iv) A general observation is that while biomass yields reach their maxima at early incubation then become nearly constant and ended by a pronounced (strain 267 in shake flasks) or slight (strain 267CH in the fermentor) decrease, α-amylase yields increase gradually in a linear manner to reach their maxima at the end of incubation.

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