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Enhanced amylase production by *Bacillus subtilis* using a dual exponential feeding strategy

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Abstract A recombinant *Bacillus subtilis* strain (ATCC 31784) harboring the plasmid pC194 with a thermostable α -amylase gene was cultured in a 22-l B. Braun Biostat C fermenter. Traditional batch operations suffer from low cell mass and protein productions because a high initial glucose concentration causes substrate inhibition and also product inhibition due to acetate accumulation. An exponential fed-batch strategy to prevent these inhibitions was developed in this work. The host strain is auxotrophic for phenylalanine, tyrosine and tryptophan. Due to low solubilities of tyrosine and tryptophan in the feed stream, tyrosine and tryptophan were dissolved separately in ammonia water to form a second feed stream. By dual feeding both streams at different exponential feed rates, a high cell density of 17.6 g/l and a final α -amylase activity of 41.4 U/ml and the overall biomass yield of 0.39 g cell/g glucose were achieved.

Keywords Fermentation · Dual feeding · Exponential fed-batch culture · *Bacillus subtilis*

Nomenclature

F	Volumetric feed rate of feed stream 1 containing glucose (l/h)
F_1	Volumetric feed rate for feed stream 1 (l/h)
F_2	Volumetric feed rate for feed stream 2 (l/h)
S	Glucose concentration in the broth (g/l)
S_0	Glucose concentration in the feed (g/l)
S_{N0}	Concentration of the nutrient other than glucose (e.g., ammonium sulfate) in the feed (g/l)

S_N	Initial concentration of the nutrient other than glucose (e.g., ammonium sulfate) in the fermenter for a batch run (g/l)
t	Time (h)
V_0	The broth volume when feed starts (l)
V	The broth volume at time t (l)
X_0	The cell mass concentration when feed starts (g/l)
X	The cell mass concentration in the broth at time t (g/l)
$Y_{X/S}$	Biomass yield on glucose (g/g)
Y_{X/S_N}	Biomass yield on nutrient (g/g)
μ	Specific growth rate (h^{-1})

Introduction

With the development of genetic engineering, *Bacillus subtilis* is becoming an increasingly attractive host for cloning. The advantages of *B. subtilis* such as high secretion level and non-pathogenic safe (GRAS—generally recognized as safe) status for non-antibiotic strains have made it suitable for the production of heterologous enzymes [1, 2].

High-density cultivation of cells may be the most effective method to obtain high concentrations of heterologous products. Fed-batch fermentation is used in high-density recombinant cell fermentation, as a fed-batch strategy can attain a high cell density by avoiding substrate inhibition and accumulation of inhibitory metabolites such as acetic acid [3]. Accumulation of acetic acid can be caused by the following factors: an anaerobic condition, a high glucose concentration, or a high specific growth rate [4]. In fed-batch fermentation, the concentration of glucose or the specific growth rate is normally used in controlling the addition of the substrate (glucose in this work) in order to maintain a stable, low concentration of the substrate throughout the culture.

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For recombinant *B. subtilis*, there are only a few published papers on high cell density cultures. Park et al. [3] used a glucose sensor with an adaptive control strategy to control the glucose concentration at 1 g/l during fed-batch culture. The recombinant product in their work was an intracellular enzyme. Martinez et al. [5] used an exponential fed-batch culture, and the results were unsatisfactory due to the characteristics of their *B. subtilis* strain. The lower the specific growth rate, the higher the cell density and the lower the heterologous protein production in their case. A final cell density of about 40 g/l was achieved by controlling the specific growth rate at 0.05 h⁻¹ during the exponential feeding phase. Vuolanto et al. [6] reported a cell mass of 56 g/l for the fed-batch culture of recombinant *B. subtilis* BD170 in a semi-defined medium with glucose and yeast extract feeding. During the fed-batch phase, glucose concentration in the growth medium was maintained constant (1–2 g/l) by on-line glucose monitoring and by manually controlling the glucose feed rate.

In this work, the exponential fed-batch culture method was chosen, since it had been applied successfully in high cell density culture of recombinant *Escherichia coli* [4, 7–10].

Model development

The relatively simple algorithm for exponential fed-batch is described below: If the specific growth rate μ is constant, Eq. 1 is valid.

$$XV = X_0 V_0 e^{\mu t} \quad (1)$$

Next, assume that the glucose amount in the broth is kept very low [4],

$$\frac{d(SV)}{dt} = 0 \quad (2)$$

The mass balance equation for glucose is

$$\frac{d(SV)}{dt} = FS_0 - \frac{\mu XV}{Y_{x/s}} \quad (3)$$

Substituting Eqs. 1 and 2 into Eq. 3 gives

$$F = \frac{\mu X_0 V_0 e^{\mu t}}{S_0 Y_{x/s}} \quad (4)$$

In this work, the following characteristics of the exponential fed-batch culture were assumed.

1. There is a quasi-steady state on glucose concentration and it is essentially kept zero. Akesson et al. [11] reported that the saturation constant in Monod equation for exponential fed-batch is 0.01 g/l, which helps us to explain that cell can grow at a specific growth rate of 0.12 h⁻¹ at an essentially zero glucose concentration.

2. μ can be maintained at an essentially constant value below the specific growth rate that will cause the accumulation of acid by-products or reduction of α -amylase production. A dilution rate of 0.12 h⁻¹ during the fed-batch culture was the threshold for accumulation of acetate according to a report by Snay et al. [12]. In this work, μ is set to 0.12 h⁻¹ to avoid the accumulation of acetate at higher specific growth rate. If μ is set too low, the cell growth will be too slow. Apart from this, CO₂ production per glucose consumption increases as the specific growth rate decreases, so biomass yield decreases with the decrease of the specific growth rate. Due to the drawbacks above, no study was conducted for the specific growth rate below 0.12 h⁻¹ [5].
3. The lower the specific growth rate, the lower the specific oxygen uptake rate. Since the specific growth rate is below the maximum specific growth rate, the specific oxygen uptake rate is relatively low, and thus the dissolved oxygen (DO) is relatively in excess.
4. The biomass yield on glucose is essentially constant.

There are three types of media: defined, complex and semi-defined. Defined media are generally used to obtain high cell density, as the nutrient concentrations are known and can be controlled during the culture. Whether a defined medium or semidefined medium is applied to exponential fed-batch culture depends on the strain. Some recombinant strains cannot grow well in a defined medium, so a semidefined medium is needed in order to boost product formation [13].

Balanced nutrients in the fermentation broth are essential for high cell density cultures. Overfeeding of mineral ions can produce a high ionic strength that may affect product formation and cell growth. High solute concentration reduces the maximum DO concentration in the medium and lowers the rate of oxygen transfer to the medium [14, 15].

In order to make the feed medium simple, some nutrients such as mineral ions are supplied as much as possible in the starting medium instead of the feed medium [16]. For the other nutrients in the feed, a balanced feed medium should be developed. The equation for the other nutrient concentrations in the feed is as follows:

$$\frac{dS_N}{dt} = \frac{F}{V} (S_{N0} - S_N) - \frac{\mu X_0 V_0 e^{\mu t}}{V Y_{X/S_N}} \quad (5)$$

Substituting Eq. 4 into Eq. 5, Eq. 6 is obtained.

$$\frac{dS_N}{dt} = \frac{\mu X_0 V_0 e^{\mu t}}{V} \left(\frac{S_{N0} - S_N}{Y_{X/S} S_0} - \frac{1}{Y_{X/S_N}} \right) \quad (6)$$

Since the other nutrients are not the limiting substrate, assume that the nutrient concentration in the fermenter is constant

$$\frac{dS_N}{dt} = 0 \quad (7)$$

Equation 7 leads to Eq. 8.

$$S_{N0} = \frac{Y_{X/S}}{Y_{X/S_N}} S_0 + S_N \quad (8)$$

S_N values are the initial concentration of nutrients in a typical medium for a batch run. S_N equals to 2.5 g/l for $(\text{NH}_4)_2\text{SO}_4$, and 0.25 g/l for MgSO_4 . For phe, trp and tyr, S_N equals to 0.05 g/l.

Materials and methods

Strain and medium

Bacillus subtilis (ATCC 31784) carrying the plasmid pC194 was used throughout this work. Plasmid pC194 contains a thermostable α -amylase gene from *Bacillus stearothermophilus*. The plasmid also codes resistance to the antibiotic chloramphenicol (Cm). The host strain is amylase negative *B. subtilis* 1A289, which does not produce homologous amylase. It produces and secretes heterologous thermostable α -amylase into the medium. The genotype for the host strain is phe-tyr-trp-sacA [17]. The recombinant strain was maintained as spores at 4°C on plates of LB agar having the following composition: NaCl 10 g/l, yeast extract 5 g/l, tryptone 10 g/l, and agar 15 g/l.

Culture conditions

The structural instability of the strain can be eliminated by the plasmid evolution method [18]. Inoculum was prepared in the LB medium containing 10 mg/l chloramphenicol (10 g/l chloramphenicol dissolved in 100% ethanol and stored in a -20°C freezer) overnight. The inoculum volume was equal to 3% of the initial culture volume in the bioreactor. The culture was carried out in the minimum defined medium containing 10 mg/l chloramphenicol at 37°C in a 22-l (15-l working volume) B. Braun bioreactor (Biostat C, B. Braun Biotech International, GmbH, Melsungen, Germany) at a stirring rate of 400–1,100 rpm and aeration rate of 1.0–2.0 VVM to maintain the DO concentration greater than 20% of air saturation. A 5% (v/v) antifoam 289 (Sigma) solution was used for elimination of foaming.

A defined medium was used for the batch phase of the cultures. The minimum defined medium consisted of I: $(\text{NH}_4)_2\text{SO}_4$ 2.5 g/l, K_2HPO_4 5 g/l, KH_2PO_4 5 g/l, sodium citrate 1 g/l, MgSO_4 0.25 g/l; II: CaCl_2 0.1 g/l; III: MnSO_4 0.01 g/l; IV: FeSO_4 0.01 g/l; V: ZnSO_4 0.002 g/l; VI: Glucose 4.5 g/l; VII: phe, tyr, trp 50 mg/l each; and VIII: chloramphenicol 10 mg/l. I–VII were autoclaved separately, and VIII was filter sterilized. For the exponential feed phase, feed stream 1 contained 500 g/l glu-

cose, 135 g/l $(\text{NH}_4)_2\text{SO}_4$, 4 g/l MgSO_4 , 8.35 g/l phenylalanine, and 10 mg/l chloramphenicol. Feed stream 2 contained 17 g/l tyrosine and 17 g/l tryptophan dissolved in 14.4% (w/v) ammonia water. Feed streams 1 and 2 were fed to the fermenter at the same time with the specific growth rate set to 0.12 h⁻¹ after the depletion of glucose. The automatic control of dual feeding was implemented using the LabVIEW software (National Instruments, Austin, TX, USA).

The effect of the initial $(\text{NH}_4)_2\text{SO}_4$ concentration on cell growth and α -amylase production for *B. subtilis* was studied in shake flask cultures at 37°C. The initial concentrations of ammonium sulfate in minimum medium were varied from 2.5 to 50 g/l. α -Amylase concentration was assayed after 10 h incubation. The effect of initial amino acid concentrations of phe, trp and tyr, and the effect of initial sodium acetate concentration were also studied separately in shake flask cultures at 37°C. The concentrations for phe, trp and tyr were varied from 0.05 to 1 g/l in the minimum medium simultaneously. α -amylase concentration was assayed after 10 h incubation. In the sodium acetate study, its initial concentrations in the minimum medium were varied from 0 to 3 g/l by adding sodium acetate to the medium. α -Amylase concentration was assayed after 13 h incubation.

Biomass assay

Cell concentration was measured with a spectrophotometer by optical density at 660 nm and was converted to dry cell weight per liter of broth (1 OD₆₆₀ unit = 0.45 g/l based on experimental results).

Glucose assay

Glucose concentration was determined using a glucose assay kit from Sigma (Kit No. 510).

α -Amylase assay

Amounts of 0.5 ml enzyme solution and 0.5 ml starch (1%) in 0.1 M NaAC (pH 6.0) were mixed and allowed to react at 40°C for 30 min. DNS reagent (2 ml) [19] was added to stop the reaction and the sample was incubated at 100°C for 10 min (DNS reacted with the reducing sugars released by the α -amylase). The control was a test tube with inactivated enzyme solution (boiled) or with enzyme added after incubation and the DNS reagent. Reducing sugar amount was determined by comparing absorbance at 540 nm of the assay solution to a standard curve of glucose solutions (1–10 mg/ml). One unit of α -amylase activity was defined as the release of 1 μmol reducing sugar from the soluble starch per minute.

Acetic acid assay

Acetic acid concentration was determined using an acetic acid assay kit (E0148261) from R-Biopharm Inc. (Marshall, MI, USA).

Results and discussion

Effects of ammonium sulfate concentration on cell growth and α -amylase production

Ammonium concentration must be kept low since higher levels inhibit growth for *E. coli* [6, 20]. Since yield coefficients for different nutrients can be dependent on many parameters such as the specific growth rate, cultivation temperature, culture broth osmolarity, pH, nutrient concentration, and product formation, the yield coefficient data obtained from experiment may not be the optimal yield coefficient for the exponential fed-batch culture. Overfeeding of nutrients other than glucose can result in the accumulation of very high nutrient concentrations at a high cell density. Hence, there is a need to study the effect of high nutrient concentrations on cell growth and amylase production. $(\text{NH}_4)_2\text{SO}_4$ was of particular interest because of its aforementioned inhibition effect. $(\text{NH}_4)_2\text{SO}_4$, 2.5 g/l, in the minimal medium was used as control. As shown in Fig. 1, 50 g/l $(\text{NH}_4)_2\text{SO}_4$ caused a lag phase for cell growth and inhibited α -amylase production, while 20 g/l $(\text{NH}_4)_2\text{SO}_4$ did not show any inhibition.

Effects of amino acid concentration on cell growth and α -amylase production

It was reported that high concentration of amino acids could inhibit cell growth [21]. To investigate this, 0.05 g/l phe, trp and tyr in the minimum medium was used as control. Figure 2 shows that up to 1 g/l phe, trp and tyr did not inhibit cell growth or amylase production. Due to the solubility limitation of tyrosine, no test was done for concentrations greater than 1.5 g/l.

Effects of sodium acetate concentration on cell growth and α -amylase production

The effect of the initial sodium acetate concentration on cell growth and α -amylase production was studied in a minimum medium in shake flasks. It was reported that the pH of the medium would affect the dissociation of sodium acetate. The lower the pH in the medium, the higher the concentration of protonated acetate, and so the higher the inhibition [22]. From Fig. 3, it can be seen that addition of 0.3 g/l acetate caused a long lag phase for growth, and 1 g/l acetate seriously inhibited cell growth. Acetate inhibited amylase production as specific amylase activity decreased with the increase of acetate concentration.

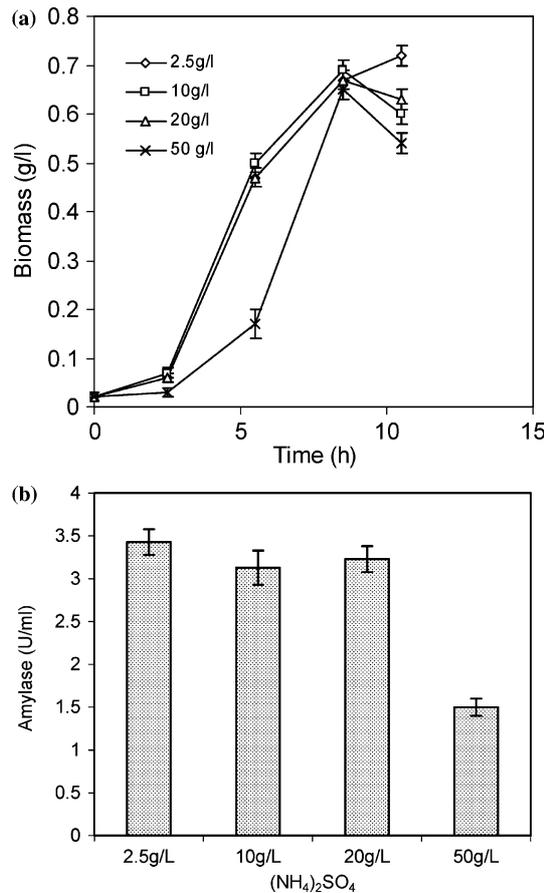


Fig. 1 a Effect of initial concentration of ammonium sulfate on cell growth. open diamond 2.5 g/l, open square 10 g/l, open triangle 20 g/l, multi 50 g/l. b Effect of initial concentration of ammonium sulfate on α -amylase production

Exponential fed-batch culture with dual feeding

The initial fermenter liquid volume was 6 l. The reservoir for feed stream 1 was 1 l and 250 ml for feed stream 2, respectively. The feed rates for feed streams 1 and 2 are expressed as follows:

$$F_1 = \frac{\mu X_0 V_0 e^{\mu t}}{Y_{x/s} S_0} \quad (9)$$

$$F_2 = \frac{F_1}{4} \quad (10)$$

The feed rate ratio for feed streams 2 to 1 at any time was 1:4, which was the ratio of the feed volume of feed stream 2 to that of feed stream 1. The total feed rate equals to feed rate of feed stream 1 plus feed rate of feed stream 2. The nutrient yield coefficients for $Y_{x/\text{phe}}$, $Y_{x/\text{tyr}}$, $Y_{x/\text{trp}}$, $Y_{x/(\text{NH}_4)_2\text{SO}_4}$ and $Y_{x/\text{MgSO}_4 \cdot 7\text{H}_2\text{O}}$ were 24, 48, 48, 1 and 53, respectively. They were determined in nutrient-limited batch cultures. For glucose, $Y_{x/s}$ was given a constant value of 0.4 $\text{g}_{\text{DCW}}/\text{g}_{\text{GLUC}}$ as determined by Martinez et al. [23]. The yield coefficients above and $S_0 = 500$ g/l were inserted into Eq. 8 to calculate the

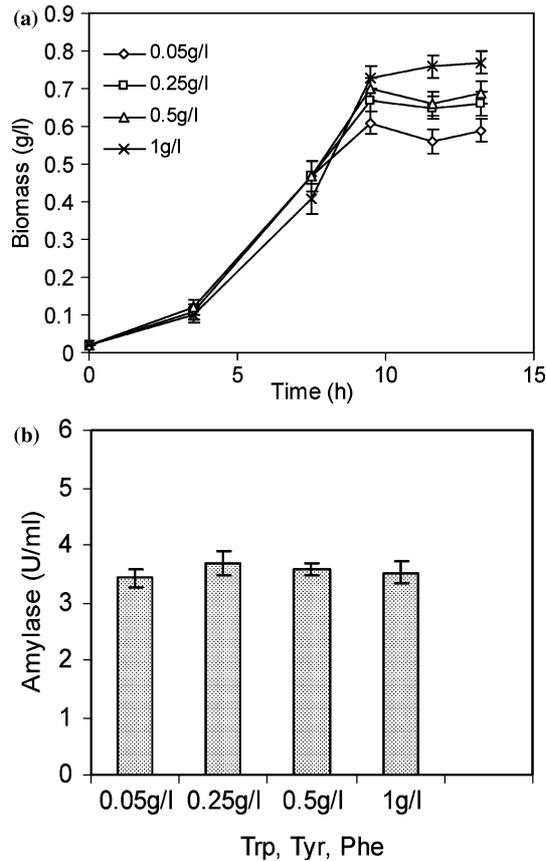


Fig. 2 **a** Effect of initial concentrations of trp, tyr and phe on cell growth. The same initial concentration was used for the three amino acids. *open diamond* 0.05 g/l, *open square* 0.25 g/l, *open triangle* 0.5 g/l, *multi* 1 g/l. **b** Effect of initial concentrations of trp, tyr and phe on α -amylase production

concentrations of nutrients other than glucose in the feed before splitting the feed into two. For ammonium sulfate, it was mentioned earlier that $S_N = 2.5$ g/l. Equation 8 yields $S_{N0} = 202.5$ g/l calculated from the data above. This ammonium sulfate concentration was for a single feed stream situation. In this work, feed stream 1 contained 135 g/l ammonium sulfate. The deficit of 67.5 g/l was made up by the NH_4OH in 14.4% (w/v) (equivalent to 4.1 mol/l) ammonia water in feed stream 2. Ammonium water of 4.1 mol/l is equivalent to 271 g/l ammonium sulfate in terms of the N amount. Because the feed stream 2 flow rate was only 1/4 of that of feed stream 1, 271 g/l ammonium sulfate in feed stream 2 is equivalent to 67.8 g/l ammonium sulfate in feed stream 1. Similarly, the calculated values for tyrosine and tryptophan from Eq. 8 should be multiplied by 4 to obtain their concentrations in feed stream 2. The calculated concentration values of nutrients for feed streams 1 and 2 are shown in the Materials and Methods section.

In this work, the cells grew in the batch mode for about 9.3 h after inoculation until glucose was depleted. At this time, the cell density was 1.8 g/l, then the two feed streams were initiated at the same time at

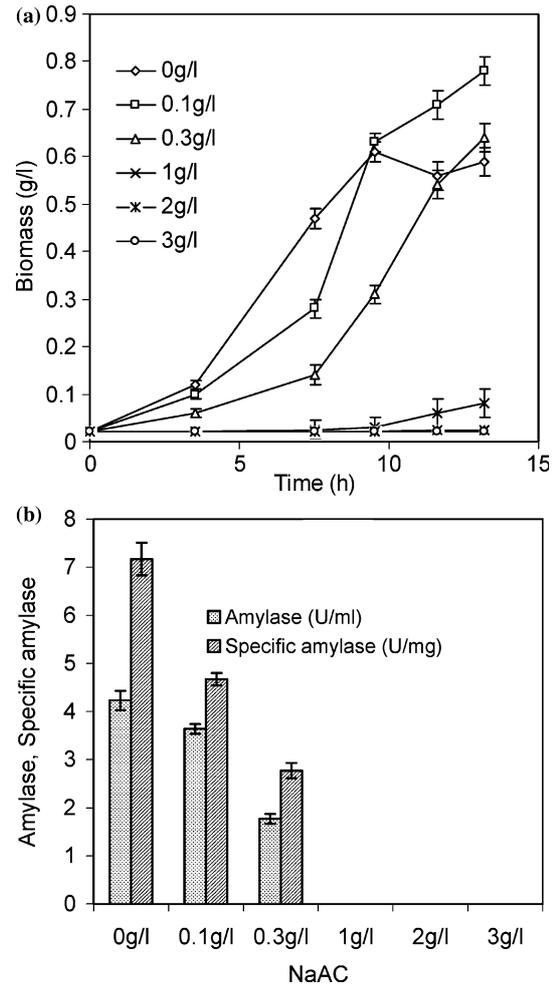


Fig. 3 **a** Effect of initial concentration of sodium acetate on cell growth. *open diamond* 0 g/l, *open square* 0.1 g/l, *open triangle* 0.3 g/l, *multi* 1 g/l, *asterisk* 2 g/l, *open circle* 3 g/l. **b** Effect of initial concentration of sodium acetate on α -amylase production

different exponential rates. When DO in the broth was below 20% that was the critical value for oxygen limitation [11, 24, 25], dual exponential feedings were stopped. If pure oxygen is supplied to the fermenter to keep DO at above 20%, a higher cell density can be expected. The cell density, glucose concentration, acetate concentration, α -amylase concentration and specific α -amylase concentration plotted as a function of time are shown in Fig. 4. In the fed-batch experiment, acetate accumulated in the batch phase was reassimilated in the beginning of the fed-batch phase [26].

Figure 4 shows that the final cell density reached 17.6 g/l and α -amylase concentration reached 41.4 U/ml. The specific amylase activity was 2.97 U/mg. The overall biomass yield was 0.39 g cell/g glucose that is consistent with the literature report [23]. For the batch culture with the initial glucose concentration of 8 g/l, the final cell density was only 2.3 g/l, and the α -amylase concentration was 1.5 U/ml. The specific amylase activity was 0.65 U/mg. The corresponding biomass

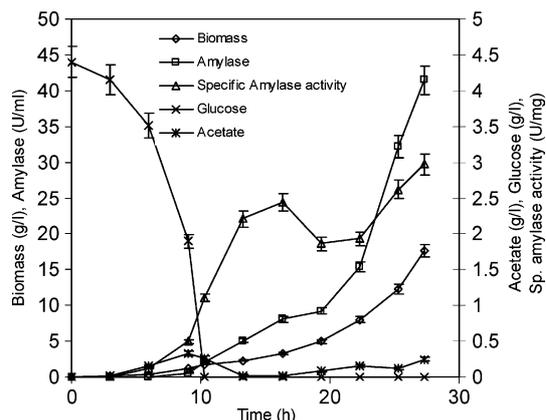


Fig. 4 Exponential fed-batch culture with dual feeding. open diamond biomass, open square amylase, open triangle specific amylase activity, × glucose, asterisk acetate

yield was 0.28 g cell/g glucose. So dual exponential fed batch culture can achieve higher biomass yield, higher specific amylase activity, amylase concentration and cell density compared to batch culture. It is known that amylase production is depressed by a high glucose concentration. Exponential fed-batch culture can maintain an essentially zero glucose concentration resulting in enhanced amylase production in addition to a high cell density.

For exponential fed-batch fermentation with pulse feeding of amino acids when there is a DO rise, cells will endure starvation that is not good. Less amino acids were needed in dual exponential fed-batch than in exponential fed-batch with pulse feeding when there was a DO rise due to the lower yield coefficients at higher amino acid concentration [4]. For yeast, ethanol is an inhibitory metabolite at high glucose concentration or high specific growth rate. For *E. coli*, it has the same inhibitory metabolite acetate as *B. subtilis*. The dual exponential feeding strategy presented in this work can also be applied to high density cultures of yeast and *E. coli* that also encounter the amino acid solubility limitation.

Conclusions

Experimental results demonstrated that a concentration of 20 g/l ammonium sulfate in the broth did not inhibit cell growth or α -amylase production. High phe, trp and tyr concentrations did not inhibit cell growth or α -amylase production. Due to low solubilities of tyrosine and tryptophan in feed stream 1, tyrosine and tryptophan were taken out and dissolved in 14.4% (w/v) ammonia water to form feed stream 2. By dual feeding the two streams at different exponential feed rates, the cells grew exponentially and a high final cell density and α -amylase concentration were achieved compared to the batch results.

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