Journal of Scientific & Industrial Research
Vol. 75, August 2016, pp. 480-486

Optimization of fed-batch cultivation strategy for extracellular α-amylase production by *Bacillus amyloliquefaciens* in submerged culture

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Received 2 December 2015; revised 10 April 2016; accepted 3 June 2016

Alpha amylase (E.C. 3.2.1.1) is one of the most important starch-degrading enzymes and widely applied in pharmaceutical, food, feed, detergent, textile and bio refinery industries. The present work was focused on optimization of α-amylase production using the bacteria strain *Bacillus amyloliquefaciens* in submerged cultivation system. The results showed that, the optimal soluble starch concentration for maximal enzyme production of 1950 μkat.L^{-1} was 20 g.L^{-1}. Compared to shake flask, bioreactor batch cultivation yielded higher volumetric enzyme production of 4125 μkat.L^{-1} (about 1.84-folds higher than the value obtained in shake flask). In addition, fed-batch cultivation with two feeding strategies (constant feeding and increased feeding) was also designed to improve the production process. The maximal volumetric enzyme production of 8160 μkat.L^{-1} was obtained in increased feeding strategy after 36 hours of cultivation. This value was almost 98 and 18% higher than the enzyme produced in batch and constant feeding fed-batch cultivations, respectively.

Keywords: *Bacillus amyloliquefaciens,* Submerged culture, α-amylase, Bioreactor, Batch cultivation, Fed-batch cultivation.

Introduction

Amylases are a vast group of industrially important microbial enzymes, which have many applications in the fields of starch processing industry, bread making, brewing, detergent industry, pharmaceutical industry as well as paper and textile manufacture[1,2]. Amylases are widely distributed in animals, plants, fungi, plants and unicellular prokaryotes and eukaryotes, and they share about 30% of the world industrial production of microbial enzymes[3]. α-Amylase, an endo-amylase; EC 3.2.1.1, catalyzes the hydrolysis of the α-1,4-glucosidic linkages in the linear amylose0 chain in starch, resulting in the production of poly- and oligosaccharides of varying length and sugars. Recently, α-amylase has been reported to have a great potential in textile, feed, food, and bio refinery industries[4]. Several microbial species have been extensively studied for the production of α-amylase; e.g. *Aspergilli, Rhizopus* sp., *Bacilli,* and *Brevibacterium linens*[4-8]. Among these strains, *Bacillus amyloliquefaciens* is one of the most reliable species used in the industrial production of α-amylase[8]. The production of α-amylase is generally carried out either by submerged (SMF) or solid-state fermentation (SSF)[2,4,9]. Although SSF has been applied for the economical and efficient production of certain microbial enzymes[8,10],however, the industrial production of α-amylase is mainly carried out by SMF. Previously, we reported soluble starch as the best carbon source for α-amylase production by*B. amyloliquefaciens* compared to glucose and maltose[11]. Fed-batch cultivation is characterized by the longer production time with minimum effects of substrate inhibition and/or catabolite repression[12]. Although fed-batch cultivation for industrial enzymes production has been intensively investigated[13-16], however, little information is available at present in applying this cultivation strategy for the production of α-amylases. The present work aimed to optimizeα-amylase production process by *B. Amyloliquefaciens* susing different cultivation modes. At first, the effect of soluble starch on the growth kinetics and production of α-amylase was studied in shake-flask. Afterwards, the kinetics of cell growth and enzyme production were evaluated under batch cultivations in

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shake-flask level as well as in bioreactor level. Based on batch data, different feeding strategies were applied to increase the production of α-amylase.

Material and Methods

Microorganism

The α-amylase producing strain *Bacillus amyloliquefaciens* NRRL B-14396 was kindly provided in a lyophilized by the Northern Regional Research Laboratory (NRRL), US Department of Agriculture, and Peoria, Illinois, USA.

Bacteria were initially activated in LB broth and were subsequently cultivated on LB agar medium of the following composition ($g.L^{-1}$): beef extract, 5.0; peptone, 10.0; yeast extract, 5.0, NaCl, 5.0; and agar 20.0. The pH of medium was adjusted to 7.0 before sterilization. After 24 hours, the arisen colonies were collected in 50% glycerol culture and stored in 2 ml cryogen vials at -80°C to prepare the master cell bank (MCB). Another working cell bank (WCB) was prepared subsequently to be used in experimental work. Each experiment was started with inoculation of 50 ml LB broth in 250 ml Erlenmeyer flask with 1 ml of bacterial suspension (obtained from frozen WCB culture after thawing at 25°C for 10 min). The inoculated flasks were incubated on rotary shaker at 200 rpm and 30°C (Multitron, Infors AG., Bottmingen, Switzerland). After 24 hours, the grown cells were used to inoculate either shake flask or bioreactor in a final concentration of 3% (v/v).

Production medium and cultivation conditions

The initial enzyme production medium was composed of ($g.L^{-1}$): $(NH_4)_2PO_4$, 5.0; yeast extract, 1.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; sodium citrate, 1.0; $CaCl_2$, 0.1; $FeSO_4.7H_2O$, 0.1 and $MnSO_4.H_2O$, 0.1. The carbon source of this medium was in the form of potato soluble starch and was added to culture medium at different concentrations. In case of shake flask, cultivations were carried out in 250 mL Erlenmeyer flaks (50 mL working volume) and incubated at 30°C in temperature controlled shaker at 200 rpm (Multitron, Infors AG, Bottmingen, Switzerland). All bioreactor experiments were carried out in a 3-L stirred tank bioreactor Bioflow III (New Brunswick Scientific Co., New Brunswick, NJ, USA) with a working volume of 2-L. Agitation was performed using two 4-bladded Rushton tubrines of the following dimensions: di(impeller diameter) = 65 mm; dt(tank diameter) = 135 mm; di/dt = 0.48. Agitation speed was set at 600 rpm and the aeration was adjusted to 1.5 $v.v^{-1}.min^{-1}$. The pH of the culture was determined continuously using sterilizable pH electrode (Mittler-Toledo AG, Switzerland). In all bioreactor cultures, the pH was controlled and was kept constant at 7.0 using continuous feeding of 2N Ammonium hydroxide and 1N Phosphoric acid. Dissolved oxygen in the culture was also determined during the cultivation using polar graphic electrode (In gold, Mittler-Toledo AG, Switzerland). Foaming was controlled using (Hi/Lo foam sensors) and was cascaded to antifoam pump connected to the antifoam agent (Silicon antifoam A, Sigma-Aldrich, USA). In case of fed-batch culture, the initial working volume was reduced to 1.5 L. A concentrated soluble starch solution, which was previously prepared and separately sterilized, was used for the feeding at the designated feeding rate.

Sample preparation and cell Growth determination

Samples in the form of 2 flasks (each containing 50 mL) or 10 mL in the case of bioreactor culture were taken at different time intervals during the cultivation. 1 ml of fresh sample was used for biomass determination by measuring the optical density at 600 nm using spectrophotometer after proper dilution in distilled water. One OD600 was equivalent to 0.235 g (cell dry weight). The fermentation broth was separated from the cells using cooling centrifuge at 5000 rpm. The supernatants were frozen immediately at -20°C for further use in enzyme determination.

Enzyme assay

α-Amylase saccharifying activity was determined by the method of Bernfeld using soluble starch as enzyme substrate as described in Stellmach[17]. Briefly, 0.5 mL of diluted enzyme in 0.05 M acetate buffer (pH 4.9) was firstly incubated for 3 minutes at 25°C with 0.5 mL of 1% starch solution. The hydrolysis reaction was stopped by the addition of 1 mL of colour developing agent containing dinitrosalisylic acid. The tube containing the reaction mixture was heated for 5 minutes in a boiling water bath and was then cooled immediately in an ice bath. After addition of 10 mL distilled water, the optical density of the reaction mixture was measured at 540 nm. The enzymatic activity is expressed in Katal unit, which is defined as the amount of activity that produces one mole of reducing group per second[18]. Maltose, in a concentration ranging between 0.2 and 2 $mg.L^{-1}$ was used as a standard.

Statistical analysis

Data were analysed with the help of SPSS 9.0 and the results were given as mean ± SD of three separate

experiments replicates. The mean comparison between different evaluated parameters was performed using ANOVA one-way analysis of variance. Statistical significance was defined when $p< 0.05$.

Results and Discussion

Effect of different starch concentrations on cell growth and enzyme production

This experiment was conducted to evaluate the effect of substrate concentration, i.e. starch, on cell growth and enzyme production by *B. amyloliquefaciens*. The cultivation was carried out in shake flasks containing 50 mL of the cultivation medium with different concentrations of starch ranging from 0.0 to 30 $g.L^{-1}$. The flasks were inoculated and incubated for 24 h as previously described in the Materials and Methods section. The obtained results (Fig. 1) showed clearly that initial starch concentration has a positive effect on cell growth as well as enzyme production. The cell dry weight increased significantly with increasing starch concentration and reached its maximal (2.82 $g.L^{-1}$) at 25 $g.L^{-1}$ starch. Afterwards, the cell dry weight decreased to 2.64 $g.L^{-1}$ at 30 $g.L^{-1}$. Concomitantly, α-amylase production increased with increasing starch concentration up to 20 $g.L^{-1}$, where the maximal enzyme volumetric production was obtained (1950 $\mu Kat.L^{-1}$). However, increasing starch concentration above 20 $g.L^{-1}$ resulted in a significant increase in volumetric enzyme production, where the enzyme production decreased by about 29% at 30 $g.L^{-1}$ (1395 $\mu Kat.L^{-1}$). Additionally, the maximal enzyme specific yield of 706.1 $\mu Kat.g^{-1}$ cells was obtained at 20 $g.L^{-1}$ of starch. These results are in good agreement with those obtained previously by our group[4], where 20 $g.L^{-1}$ of starch was the best suitable concentration allowing the maximal volumetric and specific production of α-amylase with a newly isolated *B. polymyxa*.

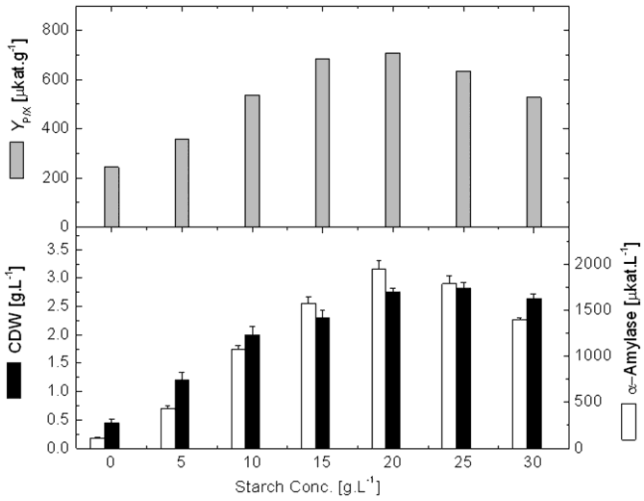

Fig. 1—Effect of different starch concentrations on cell growth and α-amylase production by *B. amyloliquefaciens* (Data were taken after 24 hours cultivation).

Kinetics of cell growth and α-amylase production in shale flasks and3-Lbioreactor under batch conditions

The parameters of cell growth and enzyme production have been followed with cultivation time in order to better understand the kinetics of cell growth and α-amylase production. From the results obtained (Fig. 2), it can be clearly observed that the cell growth increased with the a growth rate of 0.127 $g.L^{-1}.h^{-1}$, and reaching the maximal cell growth (2.65 $g.L^{-1}$) at 20 h of cultivation. After 20 h, the cell growth started to decrease gradually for the rest of cultivation (2.47 $g.L^{-1}$ at 32 h). Meanwhile, the α-amylase volumetric production increased with increasing cultivation time and parallel to cell growth, where the maximal volumetric production of 2245 $\mu Kat.L^{-1}$ was obtained at 24 h. However, after 20 h, and due to entering the

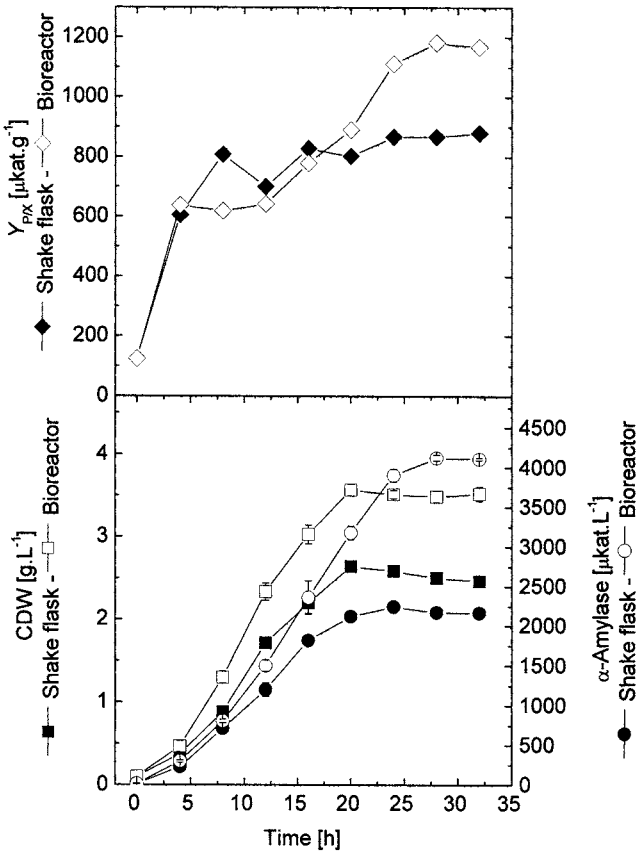

Fig. 2—Kinetics of cell growth, volumetric and specific α-amylase production, DO change, pH profile of *B. amyloliquefaciens* cultivated in shake flask and bioreactor cultures.

stationary cell growth phase, the volumetric production of enzyme did not increase. Additionally, the pH medium was decreased initially form 6.9 to 5.7 at 8 h, and then started to increase again to the end of the cultivation. The results for the specific productivity showed that at 20 h (maximal cell growth), the enzyme specific productivity reached 802.5 $\mu Kat.L^{-1}$. However, further increase in specific productivity can be attributed to the decrease in cell growth after entering the stationary phase. Based on the above results, the kinetics of cell growth and α-amylase production were followed in 3-L bioreactor to further develop and investigate the effect of scaling up the cultivation process. The bioreactor with a working volume of 2-L was inoculated and run as described in Materials and Methods. The agitation speed was set to 600 rpm, the aeration was adjusted to 1.5 $v^{-1}.v^{-1}$.min and the pH was kept constant at 7.0 during the cultivation. From the results obtained (Fig. 2), it can be observed that cell growth and enzyme production profiles run similarly to those obtained in shake flask cultivation. It can be seen that cell clearly grew exponentially with a growth rate of 0.174 $g.L^{-1}.h^{-1}$, which is about 37% higher than the growth rate in shake flask culture. Under bioreactor cultivation conditions, the maximal cell growth of 3.57 $g.L^{-1}$ was reached at 20 h of cultivation, which was about 35% higher than the maximal cell growth obtained in shake flask cultivation (2.65 $g.L^{-1}$ at 20 h). From the data of dissolved oxygen, it can be shown that DO% decreased during the growth phase as a result of high oxygen consumption as well as cellular activities, reaching a minimal DO% of 64.3% at 20 h. After entering the stationary phase, the DO% started to increase again gradually. Similarly to shake flask cultivation, the volumetric and specific enzyme production increased with cultivation time up to 28 h, where they reached their maximal (4125 $\mu Kat.L^{-1}$ and 1181.9 $\mu Kat.g^{-1}$ cells). Thus, it can be concluded that bioreactor cultivation resulted in a significant increase in both volumetric and specific productivities of α-amylase in comparison to shake flask cultivation. The maximal volumetric productivity increased by 83.7% from that obtained in shake flask cultivation (2245 $\mu Kat.L^{-1}$ at 24 h), while the specific productivity increased by about 34.4% from that obtained in shake flask cultivation (879.3 $\mu Kat.L^{-1}$ at 32 h). Such increase in productivities can be attributed to the better cultivation conditions in terms of better oxygenation and mixing, as well as pH control in bioreactor. Such increased cellular growth and productivities in bioreactor cultivation when transferred from shake flask level has been previously investigated for the production of different primary as well as secondary metabolites[4,19,20].

Kinetics of cell growth and α-amylase production in 3-Lbioreactor under fed-batch conditions

The aforementioned results in bioreactor batch cultivation clearly showed that the optimized conditions in the bioreactor resulted in a significant increase in both cell growth as well as volumetric and specific productivities. Based on these results, further improvement in the bioreactor cultivation was carried out using fed-batch strategies. The bioreactor working volume was initially reduced to 1.5 L. The bioreactor was then inoculated and run as a typical batch cultivation for the 1st 20 h, where the above results showed that cells started after 20 h to enter the stationary phase. Therefore feeding was started at 20 h for about 12 h, with both constant- and increased-rate feeding.

Constant-rate feeding

In this experiment, the feeding started at 20 h of cultivation and the feeding rate was set to 1 $g.L^{-1}.h^{-1}$ for the whole fed-batch phase. Results in Figure 3 clearly showed that feeding has a significant effect on the parameters of cell growth, α-amylase production and the overall cultivation time. After inoculation, the 1stbatch phase (20 h) proceeded as a typical batch phase, where the cell growth increased exponentially with a growth rate of 0.182 $g.L^{-1}.h^{-1}$, which is more or less the same as the previous bioreactor batch results. The α-amylase production reached 3412 $\mu Kat.L^{-1}$ at 20 h. On the other hand, feeding starch with a constant rate (1 $g.L^{-1}.h^{-1}$) for 12 h, significantly influenced the kinetics of cell growth and enzyme production. Although the cell growth rate decreased during feeding phase from 0.182 to 0.151 $g.L^{-1}.h^{-1}$, however, cells continued to grow exponentially, reaching a maximal cell growth of 5.54 $g.L^{-1}$ by the end of the feeding phase, which corresponds to a 48.1% increase than the cell growth before feeding. Concomitantly, the α-amylase production rate increased from 169.9 $\mu Kat.L^{-1}.h^{-1}$ before feeding to 253.5 $\mu Kat.L^{-1}.h^{-1}$ during feeding phase, resulting in a significant increase in the volumetric productivity of the enzyme reaching a maximum of 6910 $\mu Kat.L^{-1}$ by the end of the feeding process. Looking into DO% data, it can be clearly observed that during feeding phase, the DO% continued to decrease reaching a

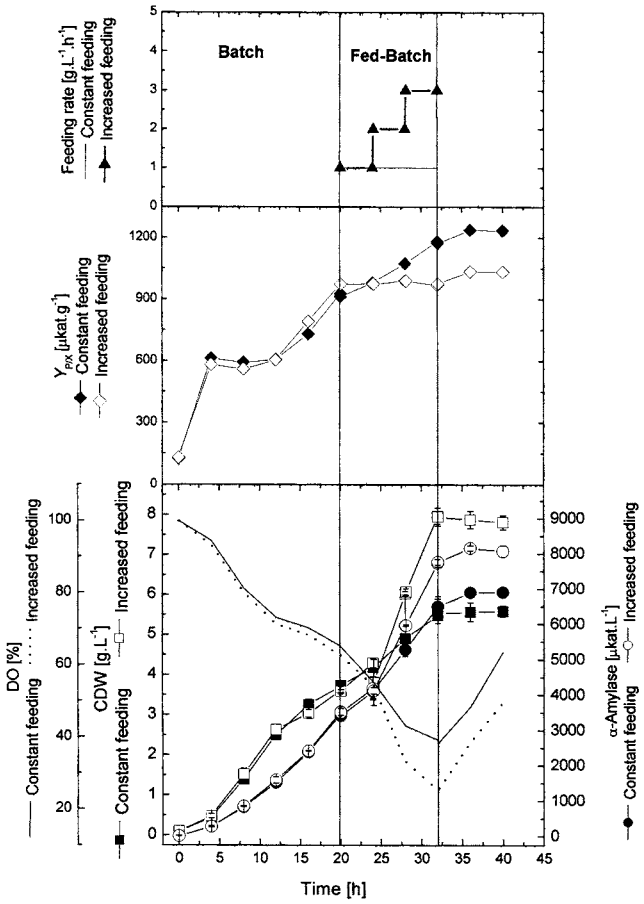

Fig. 3—Kinetics of cell growth, DO change, volumetric and specific α-amylase production of *B. amyloliquefaciens* cultivated in Fed-batch bioreactor culture. The pH was set constant at 7 during the cultivation process. Feeding was carried out using soluble starch solution between 20 h and 32 h with eitherconstant feeding rate of 1 $g.L^{-1}.h^{-1}$ or an increased feeding rate between 1-3 $g.L^{-1}.h^{-1}$.

minimum of 38.2% by the end of the feeding. In comparison to the minimal value of 64.9% recorded before the feeding, it can be concluded that the availability of starch through substrate feeding allowed the cells further to grow and promoted the higher volumetric production of the enzyme. Our obtained results are in good agreement with those previously reported by Naili*et al.*[21], who reported about58.4% increase in α-amylase produced by *Aspergillus oryzae* S2 under fed-batch cultivation as compared to batch cultivation. Moreover, the intermittent feeding of sucrose has been found to increase the production of β-fructofuranosidase by *Aspergillus japonicas* by 20% than batch culture[13].

Increased-rate feeding

In this experiment, and similar to the constant-rate feeding experiment, the feeding started at 20 h of cultivation. However, the feeding rate was firstly set to 1 $g.L^{-1}.h^{-1}$ for the first 4 h of feeding phase, and then the rate was increased for 2 and 3 $g.L^{-1}.h^{-1}$ for the following 8 hours (4 h each). The obtained results (Fig. 3) clearly proved that the increased rate feeding significantly led to a potential improvement in the kinetics of cell growth and α-amylase production. From the results, it can be seen that the cultivation before feeding phase proceeded similarly to that in the previous experiments in terms of maximal cell growth, cell growth rate, production rate and maximal enzyme concentration. However, the feeding phase was characterized by a great improvement in cultivation parameters. In response to the increased feeding rate, the cell growth rate was doubled (from 0.175 to 0.363 $g.L^{-1}.h^{-1}$ before and during the feeding phase, respectively). This doubling in the cell growth rate resulted also in 120% increase in maximal cell growth obtained (from 3.62 to 7.97 $g.L^{-1}$ before and during feeding, respectively). Furthermore, the α-amylase production rate doubled from 174.8 to 354.6 $\mu Kat.L^{-1}.h^{-1}$ before and during feeding phases, respectively. As a result of such doubling, the maximal volumetric productivity of α-amylase reached 8160 $\mu Kat.L^{-1}$ by the end of the feeding phase. Additionally, it can be observed that during the increased-rate feeding, the DO% reached a minimum of 25.2% by the end of the feeding. Recently, Ju *et al.*[22] evaluated different feeding strategies for the improvement of acetyl esterase production in the fed-batch fermentation of *Pseudomonas* sp. ECU1011. They found that conducting the fed-batch cultivation under variable feeding rates depending on different process parameters (DO and pH feedback), significantly increased the maximal cell growth and enzyme activities in comparison to the fed-batch cultivation performed under constant feeding rates. Moreover, the total volumetric productivity of 2-deoxyribose-5-phosphate aldolase by *Escherichia coli* was about 5-folds higher than that obtained in the traditional batch cultivation upon applying an exponential feeding strategy[14].

Conclusion

Table (1) summarized the obtained kinetic parameters for the batch cultivations in shake flask and bioreactor levels, as well as the fed-batch cultivations under different feeding strategies. Generally, it can be seen that the production of α-amylase in 3-L stirred tank bioreactor was greatly enhanced than in shake flask levels.

Table 1—Kinetic parameters of cell growth and α-amylase production by *Bacillus amyloliquefaciens* as affected by different cultivation strategies

Parameter	Batch cultivation		Fed-Batch cultivation			
	Shake flask	Bioreactor	Constant feeding rate		Increased feeding rate	
			Before feeding	During feeding	Before feeding	During feeding
$X_{max\text{-}conc.}$ [$g.L^{-1}$]	2.645*	3.57*	3.74*	5.54	3.62*	7.97
dX/dt [$g.L^{-1}.h^{-1}$]	0.127*	0.174*	0.182*	0.151	0.175*	0.363
$P_{max\text{-}conc.}$ [$\mu Kat.L^{-1}$]	2245		6910		8160	
Q_p [$\mu Kat.L^{-1}.h^{-1}$]	105.5*	162.19*	169.9*	253.5*	174.8*	354.6
$Y_{p/x}$ [$\mu Kat.g^{-1}$ cells]	879.3	1181.9	1238.8		1079.4	
Overall cultivation time [h]	32	32	40		40	

* Data taken at the end of the exponential growth phase.

Abbreviations: $X_{max\text{-}conc.}$, maximal cell dry weight; dx/dt, cell growth rate; $P_{max\text{-}conc.}$, maximal α-amylase production; Q_p, α-amylase production rate; $Y_{p/x}$, μKat of α-amylase produced per g biomass.

Acknowledgement

This work was financially supported by the Deanship of Scientific Research at king Saud University (Research Group Project RG-1435-047).

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