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Efficient and Cost-Reduced Glucoamylase Fed-Batch Production with Alternative Carbon Sources

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Glucoamylase is an important industrial enzyme. Glucoamylase production by industrial *Aspergillus niger* strain featured with two major problems: (i) empirical substrate feeding methods deteriorating the fermentation performance; and (ii) the high raw materials cost limiting the economics of the glucoamylase product with delegated specification. In this study, we first proposed a novel three-stage varied-rate substrate feeding strategy for efficient glucoamylase production in a 5 L bioreactor using the standard feeding medium, by comparing the changing patterns of the important physiological parameters such as DO, OUR, RQ, etc., when using different substrate feeding strategies. With this strategy, the glucoamylase activity and productivity reached higher levels of 11,000 U/ml and 84.6 U/ml/h, respectively. The performance enhancement in this case was beneficial from the following results: DO and OUR could be controlled at the higher levels (30%, 43.83 mmol/l/h), while RQ was maintained at a stable/lower level of 0.60 simultaneously throughout the fed-batch phase. Based on this three-stage varied-rate substrate feeding strategy, we further evaluated the economics of using alternative carbon sources, attempting to reduce the raw materials cost. The results revealed that cornstarch hydrolysate could be considered as the best carbon source to replace the standard and expensive feeding medium. In this case, the production cost of the glucoamylase with delegated specification (5,000 U/ml) could be saved by more than 61% while the product quality be ensured simultaneously. The proposed strategy showed application potential in improving the economics of industrial glucoamylase production.

Keywords: Glucoamylase, *Aspergillus niger*, fed-batch fermentation, feeding strategy, raw materials cost

Introduction

Glucoamylase (E.C. 3.2.1.3), the widely used enzyme in foods, pharmaceuticals, and fermentation industries, including the productions of biofuels, amino acids, *etc*., is a biocatalyst capable of hydrolyzing α-1,4-glycosidic linkages from the nonreducing ends of starch and oligosaccharides to release β-D-glucose. Glucoamylase has become the second largest market within the industrial enzymes sectors [9]. Unlike α-amylase, most of the glucoamylase could also hydrolyze the α-1,6-glycosidic linkages at the branching points of starch, but with a lower hydrolysis ability than that for α-1,4-linkages [16, 17]. Many relevant reviews and academic investigations on glucoamylase production and its applications have been published in recent years [9, 20]. Glucoamylase and α-amylase are the two most important enzymes to degrade the starch substances to produce glucose, which is used as the basic carbon substrate unit for many fermentation processes.

Industrially, glucoamylase is mostly produced by a filamentous fungus, *Aspergillus niger*, which has an excellent ability to excrete large amounts of glucoamylase over 20 g/l [6, 19]. Glucoamylase could be produced by a couple of operation modes, including submerged fermentation (SmF), solid-state fermentation (SSF), and semi-solid state fermentation [1, 25]. Among them, SmF remains the major

production mode because of the high production yield on substrates [19]. The performance of batch, continuous, and fed-batch SmFs was comprehensively compared, and the reports suggested that the maximum glucoamylase production could be obtained by fed-batch fermentation [14, 18]. Glucoamylase fermentation is an extremely high oxygen consuming process, and a large amount of oxygen is required as an electron acceptor for energy (ATP) generation [3, 24]. The requirements of dissolved oxygen (DO) concentrations on cell growth, fungal morphology, and enzyme biosynthesis are different [24, 27]. Wongwicharn *et al.* [27] reported that oxygen enriched environments (30%–50%) were favorable for glucoamylase production, whereas oxygen limitation deteriorated the enzyme biosynthesis. Therefore, controlling the DO at appropriately high levels through a suitable substrate feeding strategy is very important.

In industrial glucoamylase production, maltose syrup and α-lactose are used as the major substrates or raw materials [2, 18, 23]. In these cases, the raw materials cost almost occupies more than 60% of the total production cost, which has largely limited the future development and economics of the glucoamylase industry, by considering the inexpensive features of glucoamylase. Furthermore, the prices of those raw materials has continued to rise during the past years, leading to a very low profit margin in many glucoamylase production companies. As a result, it would be of great commercial interest to produce glucoamylase products by using low value-added raw materials. Recently, utilization of low value-added raw materials such as food waste, wheat bran, tea waste, and rice flake manufacturing waste products for glucoamylase production was investigated by many academic groups [1, 5, 22, 25]. However, owing to the lower nutrients (especially carbon source) contents in those raw materials, the glucoamylase activities stayed at the range of 100–300 U/ml, which was much lower than the standard activity level required by the glucoamylase industry (about 5,000–10,000 U/ml). Reports on glucoamylase production process using low value-added raw materials and industrial production strain are very limited [19], and its industrial potential should be extensively explored and investigated.

In this study, focusing on using low value-added raw materials and an industrial production strain to produce glucoamylase in efficient and economical ways, we proposed a metabolic-activity-based substrates feeding strategy using different raw materials. The effectiveness and performance of the proposed approach were evaluated and compared in terms of product quality (glucoamylase activity) and cost reduction, and the best combination (optimal substrate feeding strategy and type of raw materials) was selected as the candidate for effective glucoamylase production in the future.

Materials and Methods

Strain and Media

Aspergillus niger JJS-01, an industrial glucoamylase-producing strain, which was kindly provided by CSPC Hebei Zhongrun Pharmaceutical Co., Ltd (Shijiazhuang, Hebei Province, China) and kept in the laboratory, was used throughout this study. The medium composition for slant agar culture contained (g/l) $NaNO_3$ 3, K_2HPO_4 1, KCl 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, $FeSO_4{\cdot}7H_2O$ 0.01, sucrose 30, and agar 17, at pH 6.0. The seed medium contained (g/l) sucrose 70, bean cake powder 80, corn steep liquor 10, $CaCl_2$ 0.2, $MgSO_4{\cdot}7H_2O$ 0.9, and 0.2 ml of α-amylase (20,000 U/ml), pH 5.5. The medium for glucoamylase production in 5 L bioreactor contained (g/l) starch 30, bean cake powder 10, corn steep liquor 20, $CaCl_2$ 0.2, $(NH_4)_2SO_4$ 5, Na_2HPO_4 1.3, KH_2PO_4 3.5, $MgSO_4{\cdot}7H_2O$ 3.5, trisodium citrate 1.25, and 0.15 ml of α-amylase, pH 5.0. $(NH_4)_2SO_4$ solution was sterilized separately. Feeding medium #1 (Batches #A–D) contained (g/l) maltose 600, α-lactose 100, and corn steep liquor 25. All media were sterilized at 121°C for 30 min.

Fermentation Conditions

The seed culture was cultivated on a rotary shaking incubator at 31°C and 180 rpm for 42 h. The fed-batch glucoamylase fermentation was implemented in a 5 L stirred bioreactor equipped with the standard DO/pH on-line measurement probes and control system (BIOTECH-5BG, Baoxing Co., Ltd., China), with the working volume of 3 L. The temperature was controlled at 31°C and the pH was maintained at 5.0 by adding either H_2SO_4 solution (20% (v/v)) or ammonia water (36% (w/w)). The aeration rate was controlled at 2 vvm throughout the fermentation. The inoculation size was 10% (v/v). The agitation rate started at 150 rpm, and it was manually increased by an increment of 20 rpm when the DO dropped down to a low control limit (15%–20% of saturation). Fermentation was divided into two phases: batch phase (about 0–20 h) and fed-batch phase (about 20–130 h). In the first phase, cells grew exponentially until the carbon/nitrogen sources were completely consumed at about 20 h, which could be reflected by the "DO sudden rise up" (to a high level of about 40%). Then, feeding medium #1 was fed into the bioreactor based on the following feeding strategies until the end of fermentations. The usage amounts of the feeding medium were determined by an electronic balance (JA1102; Haikang Instrument Co., Ltd., China), which was connected with an industrial computer *via* a multi-channels A/D–D/A converter (PCL-812PG; Advantech Co., Ltd., Taiwan), by measuring the weight loss of the feeding medium reservoir. A gas analyzer (LKM2000A; Lokas Automation Co., Ltd., Korea) was used to on-line measure the O_2 and CO_2 partial pressures

in the exhaust gas, and based on the pressures, the oxygen uptake rate (OUR), CO_2 evolution rate (CER), and respiratory quotient (RQ) were determined by the standard calculation formula.

Substrate Feeding Strategies

In this study, we used the medium #1 mentioned above as the standard feeding medium to investigate the effects of different feeding strategies on glucoamylase production performance.

Strategy I: DO-stat-based feeding method (Batch #A). When the carbon source was consumed at the end of the batch phase (about 20 h) and DO rose up to an upper limit (40% in this case), DO-stat-based substrate feeding was initiated by switching on a programmable peristaltic pump *via* the multi-channel A/D–D/A converter, with the aid of the self-developed control programs (Visual Basic ver. 6.0) embedded in the industrial computer. This automatic feeding strategy was successfully applied in cephalosporin C production by another fungus (*A. chrysogenum* HC-3) in our previous study [12].

Strategy II: Constant-rate feeding (Batch #B). In the fed-batch phase (20–132 h in this case), the feeding rate was maintained at a constant value of 5 g-sugar/l/h by observing the electronic balance readings for the feeding medium reservoir and regulating the programmable peristaltic pump. Here, the sugar was referred to as the mixture of maltose and α-lactose with a weight ratio of 6:1 (medium #1).

Strategy III: Two-stage varied-rate feeding (Batch #C). The feeding rates during the fed-batch phase was kept at 4.5 g-sugar/l/h during 20–70 h (Stage I), and then shifted to 5.5 g-sugar/l/h after 70 h (Stage II).

Strategy IV: Three-stage varied-rate feeding (Batches #D, #E, #F, and #G). During the fed-batch phase (20–130 h), feeding rates were set at three different levels: 4.0 g-sugar/l/h in 20–50 h (Stage I), 5.5 g-sugar/l/h in 50–100 h (Stage II), and 4.5 g-sugar/l/h in 100–130 h (Stage III).

Pretreatment of Cassava and Cornstarch

The cassava meal (starch content 70% (w/w)) was provided by Henan Tianguan Fuel Ethanol Co., Ltd., China. Cornstarch was purchased in a local supermarket. The cassava powders were sieved up to a mesh size of 40. Then, the modified dual-enzymatic hydrolysis method was utilized to hydrolyze the cornstarch and cassava powders [10]. More specifically, a minuscule amount of α-amylase (8 U/g-cornstarch and 6 U/g-cassava, treated at 95°C for 45 min) and then glucoamylase (120 U/g-cornstarch and 90 U/g-cassava, treated at 62°C for 60 min) were added into the cassava- and cornstarch-based media. Subsequently, the viscosity-reduced media containing the corn steep liquor was sterilized at 121°C for 25 min.

Analytical Methods

At a specified time, 10 ml of broth was centrifuged at 10,000 ×*g* and 4°C for 10 min. The supernatant and pellets were separated aseptically. Pellets were used for biomass determination. The supernatant was used for the determination of residual concentrations of total reducing sugar and glucose, as well as the glucoamylase activity. All experiments were performed in triplicate, and the data are presented as the mean ± standard deviation (SD).

The biomass concentration was determined by dry cell weight (DCW) analysis. The cell pellets were washed twice with deionized water, and dried at 105°C until constant weight was reached. Residual reducing sugar concentration was determined by the modified 3,5-dinitrosalicylic acid (DNS) reagent method, using glucose as the standard [15]. The glucose concentration was measured by a SBA-40C glucose analyzer (Shandong Academy of Sciences, China).

One milliliter of supernatant was quantitatively diluted with 0.05 mol/l sodium acetate buffer solution (pH 4.6). One milliliter of the diluted enzyme solution was mixed with 5 ml of soluble starch solution (20 g/l) and 1 ml of sodium acetate buffer solution (pH 4.6), and then placed at 40°C for reaction for 20 min. The reaction was ended by adding 0.1 ml of 200 g/l NaOH solution. One glucoamylase activity unit (1 U) was defined as one μmol glucose released from the soluble starch mixture per minute.

Results and Discussion

Fed-Batch Glucoamylase Fermentation Performance with Different Feeding Strategies

In this study, we first investigated the submerged fed-batch glucoamylase fermentation performance (glucoamylase activity and productivity, biomass concentration, *etc*.) with different feeding strategies, using feeding medium #1 as the standard feeding medium. In these cases, the same conditions (aeration, agitation, pH, initial nutrients concentrations, *etc*.) were applied for all fermentations during the batch phase (0–20 h).

DO-Stat Substrate Feeding Strategy

In fed-batch glucoamylase fermentation, the substrate (reducing sugar, maltose + α-lactose in this case) was continuously fed into the bioreactor. Then, the substrate was degraded into glucose by the glucoamylase released to supply the basic carbon source unit for further and enhanced glucoamylase production. DO-stat and its derived feeding methods are widely applied in protein/enzyme production to prevent the formation of inhibitory/undesirable by-products and substrate overfeeding/glucose accumulation [8, 12]. A modified DO-stat method featured with a specified delay time (3 min) was adopted for feeding in this work: when the DO rose up over its upper limit (40%), the programmable peristaltic pump was switched on and then continued to run during the specified feeding delay period even though the DO had dropped down below its upper

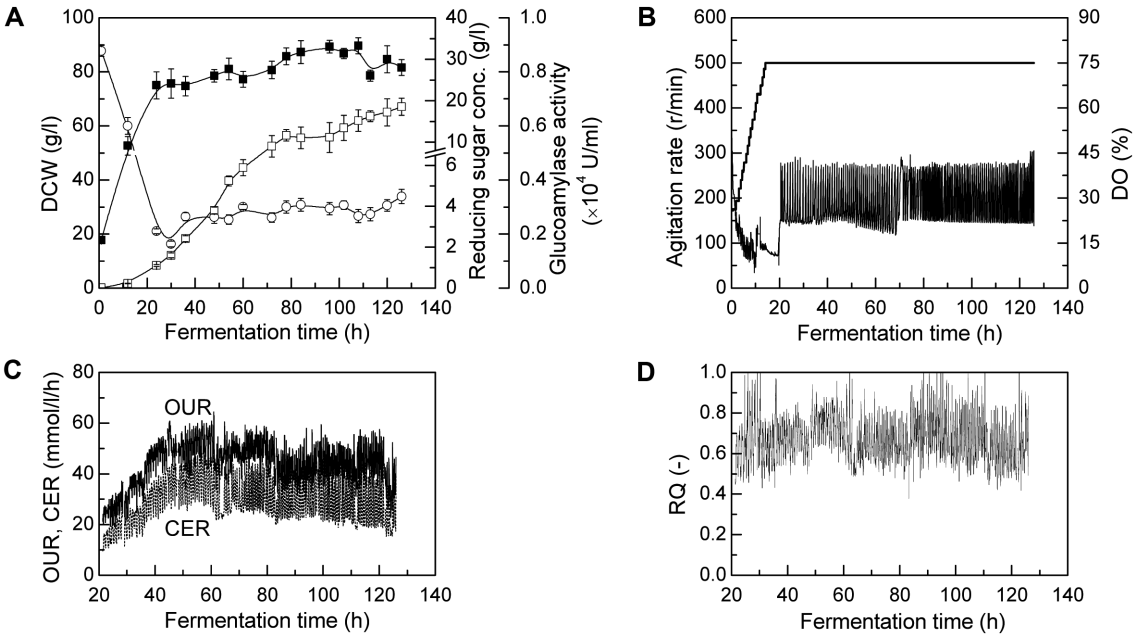

Fig. 1. Glucoamylase fermentation curves with DO-stat feeding strategy (I, Batch #A).
(**A**) DCW (■), glucoamylase activity (□), and reducing sugar concentration (○); (**B**) Agitation rate (bold line), DO (thin line); (**C**) The changing patterns of OUR and CER; (**D**) The changing patterns of RQ.

limit. When the delay time had run out and the DO declined below this upper limit, the peristaltic pump was switched off to stop the feeding. The above substrate feeding operation was repeatedly continued until the end of the fermentation. The fermentation curves in this case are depicted in Fig. 1 (Batch #A). The DO oscillated continuously in a range of 20%–40% and the agitation rate was kept at 500 rpm throughout the fed-batch phase (20–126 h). During the batch phase, cells grew rapidly and its concentration reached a stable level of 70–80 g-DCW/l, while glucoamylase activity stayed at a low level of 600 U/ml at 20 h before initiating the substrate feeding (Fig. 1A). After entering the fed-batch stage, glucoamylase activity significantly and quickly increased from 600 to 5,000 U/ml during the period of 20–70 h. These results indicated that the glucoamylase production is a non-growth-associated process, which is in accordance with the conclusion reported by other research groups [18, 19].

The OUR, CER, and RQ are very important fermentation parameters, which could reflect the physiological status and cellular metabolic activity of the fermentation processes [21, 29]. For example, a higher OUR could be considered as a direct indicator for an efficient targeted metabolites biosynthesis by fungal microorganism [12, 29]. Figs. 1C–1D show the changing patterns of OUR, CER, and RQ during the fed-batch phase. During the early stage of the fed-batch phase (20–60 h), the OUR and CER increased rapidly from the lower levels of 20 mmol/l/h and 10 mmol/l/h (average) to the higher levels of 50 mmol/l/h and 30 mmol/l/h (average), respectively. However, the average OUR and CER levels tended to decline slowly and gradually after 60 h. The severe oscillations in OUR and CER (also RQ, RQ=CER/OUR) were in accordance with the continuous DO oscillations, which were caused by the DO-stat substrate feeding strategy itself. With this strategy, the fermentation process shifted in between glucose transient excess and starvation frequently, leading to a deteriorated cellular metabolic activity. As a result, the effective glucoamylase production period could not be extended or prolonged, and its activity was only enhanced about 1,000 U/ml during the late fed-batch stage of 70–126 h. The average RQ varied at about 0.7 throughout the fed-batch phase.

Constant-Rate Substrate Feeding Strategy

The frequent shift in between glucose transient excess and starvation of the fermentation process led to a declined OUR and a shorter glucoamylase production phase, and glucoamylase activity could not be further increased. To solve this problem, the strategy of feeding the substrate at a constant rate was adopted, where the substrate feeding and agitation rates were set at 5.0 g-sugar/l/h and 525 rpm constantly throughout the fed-batch stage (20–132 h). Fig. 2

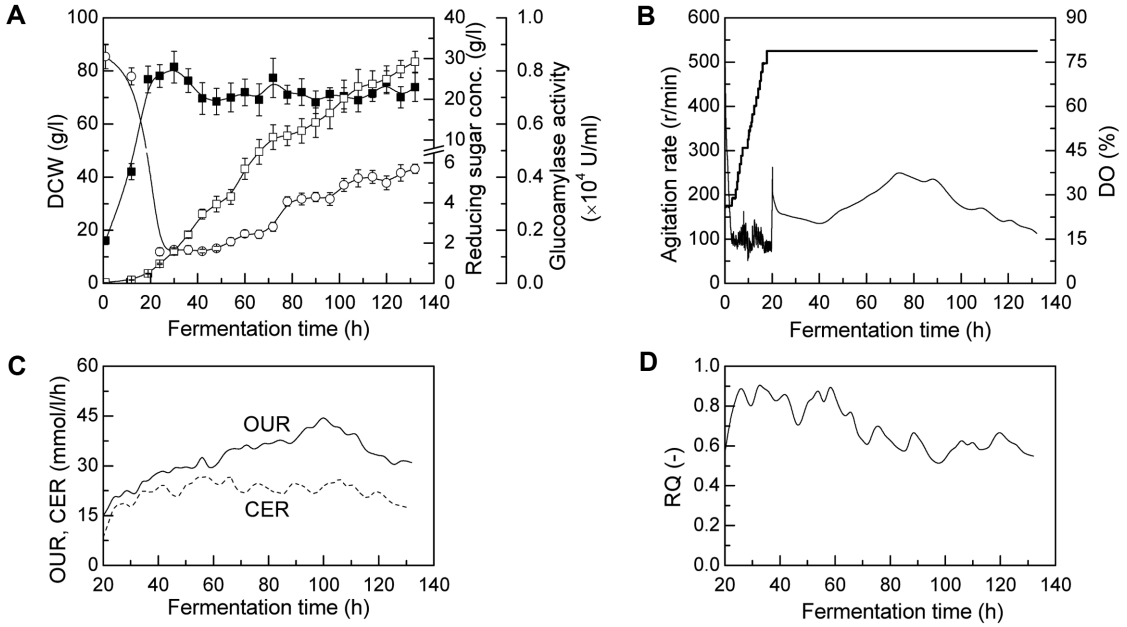

Fig. 2. Glucoamylase fermentation curves with constant-rate feeding strategy (II, Batch #B).
(**A**) DCW (■), glucoamylase activity (□), and reducing sugar concentration (○); (**B**) Agitation rate (bold line), DO (thin line); (**C**) The changing patterns of OUR and CER; (**D**) The changing patterns of RQ.

shows the fermentation curves in this case (Batch #B). The glucoamylase activity increasing patterns during the early feeding stage (20–70 h) were similar to those of Batch #A. However, the effective glucoamylase production period was prolonged to a certain extent, and glucoamylase activity gradually and slowly increased even after 70 h and eventually reached a level of 8,300 U/ml at 132 h, which was 1.24-fold of that obtained with strategy I (Batch #A). In this case, the DO varied in the range of 20%–35% during the early fed-batch stage of 40–110 h, but declined to 15%–20% in the late stage of 110–132 h. RQ stayed at a higher level of 0.8 during 20–70 h and then declined to a lower level of 0.6 after 70 h. In addition, the total reducing sugar accumulated to a level of about 6 g/l.

Dissolved oxygen concentration control is very important in glucoamylase production by fungal aerobic microorganisms. Bhargava *et al.* [3] reported that glucoamylase activity increased rapidly if the DO was controlled above a high level of 40%, whereas it remained unchanged or even reduced if the DO was below a lower level of 30%. Therefore, the low DO level (15%–20%) during the late fed-batch stage (110–132 h) was not favorable for glucoamylase production. On the other hand, RQ basically reflects the carbon sources utilization efficiency for the targeted metabolite biosynthesis. Fig. 3 shows a simplified metabolic map for glucoamylase production by *A. niger* JJS-01, using

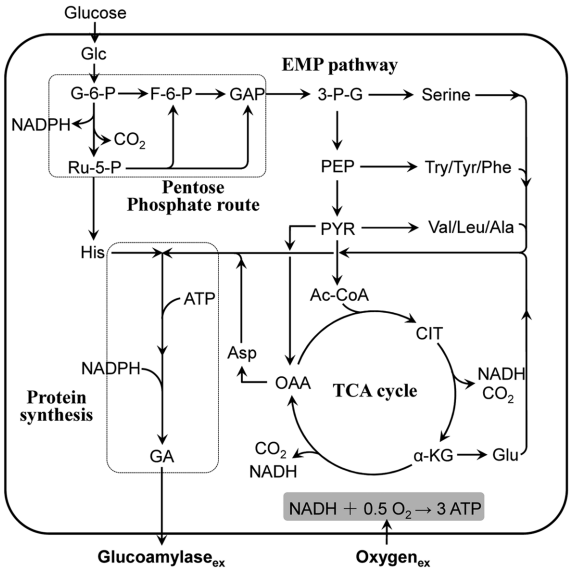

Fig. 3. Simplified metabolic map of glucoamylase synthesis by *A. niger* JJS-01.
Glc: glucose; G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; GAP: glyceraldehyde-3-phosphate; 3-P-G: 3-phosphoglycerate; Ru-5-P: ribulose-5-phosphate; PEP: phosphoenolpyruvate; PYR: pyruvate; Ac-CoA: acetyl-CoA; CIT: citrate; α-KG: α-ketoglutarate; OAA: oxaloacetic acid; His: histidine; Try: tryptophane; Tyr: tyrosine; Phe: phenylalanine; Val: valine; Leu: leucine; Ala: alanine; Glu: glutamate; Asp: aspartate; ATP: adenosine triphosphate; NADH: reduced diphosphopyridine nucleotide; NADPH: reduced nicotinamide adenine dinucleotide phosphate.

several reports as the references [4, 11, 13]. The metabolic map contained the Embden-Meyerhof-Parnas (EMP) pathway, tricarboxylic acid (TCA) cycle, pentose phosphate (PP) route, the synthesis pathways of various amino acids, and the glucoamylase biosynthesis pathway. In addition, the energy metabolism (ATP, NADH, and NADPH) and O_2 consumption (oxidative phosphorylation reaction) route were also considered. As shown in Fig. 3, CO_2 is produced by the PP route and TCA cycle [4], and CO_2 release is partially associated with the protein synthesis route. Theoretically, RQ = 1.0 means that the TCA cycle is fully activated and the carbon source (*e.g.*, glucose) is completely converted to H_2O and CO_2 with the TCA cycle and oxidative phosphorylation reaction [28]; in this case, 100% glucose is consumed for energy regeneration and cellular growth/maintenance without targeted metabolite formation. If the energy substance (ATP, *etc.*) created in the TCA cycle and related route is sufficient enough in supporting the targeted metabolite synthesis, then more carbon source should be directed into the metabolite synthesis pathway, indicating a relatively lower RQ would be desirable. With the constant-rate feeding strategy (strategy II), RQ stayed at a higher level of 0.8 (versus 0.7 when using strategy I) during the early stage of 20–70 h. This result implied that the substrate feeding rate might be too high and carbon metabolism efficiency too low during this stage, and thus the following two-stage varied-rate substrate feeding strategy was proposed.

Two-Stage Varied-Rate Substrate Feeding Strategy

This strategy aimed at achieving the following two targets: (i) to stably control the DO above a higher level of 20% and RQ at a lower level of 0.6 throughout the fed-batch stage; (ii) to obtain a stable OUR as higher as possible

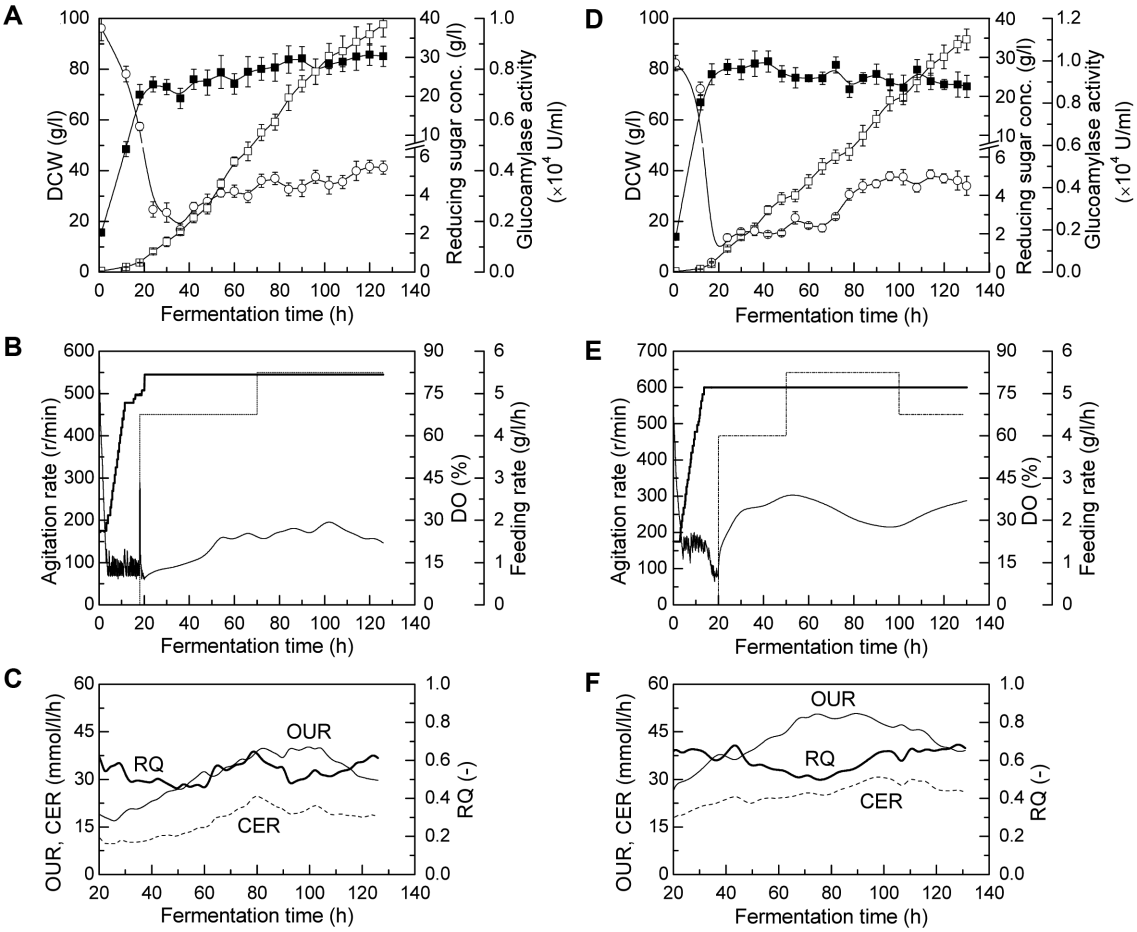

Fig. 4. Glucoamylase fermentation curves with multi-stage varied-rate feeding strategies (III and IV, Batches #C and #D).
(**A**–**C**): two-stage varied-rate feeding strategy (III, Batch #C); (**D**–**F**): three-stage varied-rate feeding strategy (IV, Batch #D). (**A**, **D**) DCW (■), glucoamylase activity (□), and reducing sugar concentration (○); (**B**, **E**) Agitation rate (bold line), DO (thin line), and sugar feeding rate (dotted line).

during the late fed-batch stage. To do this, (i) the agitation rate was raised to 550 rpm at the beginning of the substrate feeding until the end of fermentation; and (ii) the substrate feeding was reduced to 4.5 g-sugar/l/h during 20–70 h and then raised to 5.5 g-sugar/l/h in 70–126 h, while maintaining the total substrate feeding amount approximately equivalent with that of strategy II.

Figs. 4A–4C show the fermentative curves using this feeding strategy (Batch #C). With this strategy, the DO was stabilized over 20% after 70 h, and RQ varied in the range of 0.5–0.6 throughout the fed-batch stage. As a result, the glucoamylase activity increased continuously almost during the entire fed-batch phase, and a higher glucoamylase activity level of 9,800 U/ml was obtained at 126 h. This enzymatic activity level was 1.46-fold of that using DO-stat-based strategy (I) and 1.18-fold of that adopting constant-rate strategy (II). However, two other problems appeared by observing the fermentation curves: (i) OUR was much lower than those when adopting strategies I and II, particularly during the early fed-batch stage of 20–70 h. We speculated that the long-term and lower reducing sugar feeding rate (4.5 g-sugar/l/h) led to a very low glucose concentration, which reduced the OUR and limited glucoamylase production in turn (Table 1); and (ii) the reducing sugar at the late fed-batch stage (70–126 h) accumulated to a level around 5.0–6.0 g/l, due to the higher reducing sugar feeding rate (5.5 g-sugar/l/h), and the higher reducing sugar concentration not only consumed glucoamylase accumulated (to produce glucose) but also inhibited glucoamylase production itself [4]. Even though the overall glucoamylase production performance was largely enhanced in this case, it seemed that there is still a room or space for further performance improvement by properly eliminating the two above-mentioned problems. As a result, the following "three-stage varied-rate substrate feeding strategy (IV)" was proposed and subsequently testified.

Three-Stage Varied-Rate Substrate Feeding Strategy

In this case, the agitation rate was kept at 600 rpm throughout the feeding phase. The basic idea of this strategy was as follows: (i) during the early stage (20–50 h) of the fed-batch phase, to feed the reducing sugar at a lower rate of 4.0 g-sugar/l/h during a shorter period to bring the DO up to 30% quickly *via* creating a glucose relative limitation environment; (ii) during the middle stage (50–100 h) and when DO continued to rise, then to feed at a higher rate of 5.5 g-sugar/l/h to ensure the relative enriched glucose environment to increase the OUR and cellular metabolic activity; and (iii) during the late stage (100–130 h) and when DO continued to go down, decreasing the feeding rate to 4.5 g-sugar/l/h, to maintain a DO over 30% and avoid the excessive reducing sugar accumulation to keep the glucoamylase production rate at a constant level. Figs. 4D–4F show the fermentation curves in this case (Batch #D). With this strategy, the DO was controlled to over 30% most of the time during the entire feeding phase, RQ was stabilized around 0.6 throughout the fed-batch stage, a higher OUR ranging 40–50 mmol/l/h was obtained, and the residual reducing sugar concentration

Table 1. Glucoamylase fermentation performance with different feeding strategies and standard feeding medium[a].

Fermentation batches	Batch #A	Batch #B	Batch #C	Batch #D
Feeding strategy	Strategy I	Strategy II	Strategy III	Strategy IV
Fermentation time (h)	126	132	126	130
Ave. sugar feeding rate (g/l/h)	4.25	5.00	5.03	4.82
Final reducing sugar conc. (g/l)	4.5	6.0	5.5	4.4
Ave. OUR (mmol/l/h)	42.32	33.23	31.62	43.83
Ave. RQ (-)	0.69	0.67	0.56	0.60
Ave. residual glucose conc. (g/l)	0.57 (20–126 h)	0.74 (20–132 h)	0.45 (20–70 h)	0.40 (20–50 h)
			1.10 (70–126 h)	1.05 (50–100 h)
				0.76 (100–130 h)
Biomass (g-DCW/l)	81.6 ± 3.0	73.8 ± 5.4	85.1 ± 4.1	73.2 ± 4.3
Glucoamylase activity (U/ml)	6,700 ± 300	8,300 ± 450	9,800 ± 500	11,000 ± 490
Productivity (U/ml/h)	53.2 ± 2.4	62.9 ± 3.4	77.8 ± 4.0	84.6 ± 3.8
Yield on biomass (U/g-DCW)	82.1 ± 3.7	112.5 ± 8.3	115.2 ± 5.9	150.3 ± 6.7

[a]The standard feeding medium refers to feeding medium #1, containing 600 g/l maltose, 100 g/l α-lactose, and 25 g/l corn steep liquid.

stayed at a lower level of 4.0 g/l, simultaneously. The glucoamylase production was performed almost at a constant rate without any decline until the fermentation end, and the final glucoamylase activity reached the highest level of 11,000 U/ml (1.12-fold of that when adopting strategy III) among the four feeding control cases.

Glucoamylase Fermentation Performance Comparison with Different Feeding Strategies

Table 1 summarizes the comparison results of glucoamylase fermentation performance using the different feeding strategies. The glucoamylase fermentation performance, including glucoamylase activity, productivity, and yield on biomass, was the best when adopting the three-stage varied-rate substrate feeding strategy (IV, Batch #D). Using the DO-stat-based substrate feeding strategy as the comparison base, with feeding strategy IV, the glucoamylase activity increased 64% from 6,700 U/ml to 11,000 U/ml; glucoamylase productivity enhanced from 53.2 U/ml/h to 84.6 U/ml/h with an increment of 59%; and glucoamylase yield on biomass changed from 82.1 U/g-DCW to 150.3 U/g-DCW with an enhancement of 83%.

In Table 1, the average values of the important fermentation parameters, such as reducing sugar feeding rate (r_{sugar}), OUR, and RQ during the fed-batch phase, were also listed for physiological interpretation. The averaged values were calculated by Eqs. (1)–(4). With the "three-stage varied-rate substrate feeding strategy (IV)", the highest average OUR (43.83 mmol/l/h) and stable/lower RQ (0.60) were achieved simultaneously, and these largely contributed to the enhancement of glucoamylase production performance in this case.

$$\mathrm{Ave.r_{sugar}} = \frac{\int_{t_0}^{t_f} r_{sugar}(t)dt}{t_f - t_0} \quad (1)$$

$$\mathrm{Ave.OUR} = \frac{\int_{t_0}^{t_f} \mathrm{OUR}(t)dt}{t_f - t_0} \quad (2)$$

$$\mathrm{Ave.CER} = \frac{\int_{t_0}^{t_f} \mathrm{CER}(t)dt}{t_f - t_0} \quad (3)$$

$$\mathrm{Ave.RQ} = \frac{\mathrm{Ave.CER}}{\mathrm{Ave.OUR}} = \frac{\int_{t_0}^{t_f} \mathrm{CER}(t)dt}{\int_{t_0}^{t_f} \mathrm{OUR}(t)dt} \quad (4)$$

Here, t_f and t_0 represent the ending and starting instants of the substrate feeding, respectively.

Effective Glucoamylase Production Using Cost-Reduced Raw Materials and the Three-Stage Varied-Rate Feeding Strategy

Since the glucoamylase fermentation performance was the best when using the three-stage varied-rate substrate feeding strategy (IV, Batch #D) and the standard feeding medium (feeding medium #1), the optimal feeding strategy was then applied for the cases of glucoamylase production with cost-reduced raw materials to decrease production costs and increase profits. Several research groups have investigated the performance and economics of glucoamylase production using other renewable feedstock and low value-added materials [7, 25, 26], or the combination of traditional feedstock and low-valued nutrients/substances fermentation [1, 18]. However, the glucoamylase activities in those cases stayed at the levels of 100–300 U/ml or U/g-dried substrate, which were much lower than our data when using different feeding strategies and the standard/traditional feeding medium (Batches #A–#D, 6,700–11,000 U/ml). In addition, the fermentations, such as substrate feeding strategies, were not properly optimized or controlled, so that the potentials of the low value-added materials were not fully utilized.

Here, in our case, we tried to replace the standard feeding medium (maltose + α-lactose, carbon source; corn steep liquor, nitrogen source) with the low valued-added substances, as the potential substrate candidates. The standard carbon-source combinations for glucoamylase production are expensive: maltose syrup, \$550/ton; α-lactose, \$1,700/ton. Pedersen *et al.* [18] reported that almost no difference on glucoamylase productivity by *A. niger* BO-1 with fed-batch fermentation mode was observed, when the expensive maltose was replaced by the cheaper glucose (glucose, industrial grade, \$400/ton). On the other hand, enzymatic hydrolysis of renewable starch substances to prepare high glucose concentration hydrolysate for glucoamylase fermentation is also attractive. Cassava and cornstarch are the two available starch substances. Cassava is particularly attractive for its features of low cost (\$220/ton), high starch content (70% (w/w)), and being abundantly producible even in in-arable land. Cornstarch also has the price advantage (\$450/ton) over the traditional maltose/α-lactose carbon-source combinations. Table 2 summarizes the composition details of the feeding medium prepared by various alternative raw materials, including glucose (medium #2), cassava hydrolysate (medium #3), and cornstarch hydrolysate (medium #4). It should be noted that, in this study, we kept the carbon-source/corn steep liquor ratio in different feeding media at a constant value.

Table 2. Compositions of the feeding medium prepared by various alternative raw materials and the unit prices of the raw materials.

Feeding media	Fermentation batches	Carbon-sources conc.	Nitrogen-source conc.
Medium #1	#A–D	Maltose (600 g/l) α-Lactose (100 g/l)	Corn steep liquor (25 g/l)
Medium #2	#E	Glucose (700 g/l)	Corn steep liquor (25 g/l)
Medium #3	#F	Cassava hydrolysate (400 g/l)	Corn steep liquor (10 g/l)
Medium #4	#G	Cornstarch hydrolysate (700 g/l)	Corn steep liquor (25 g/l)

Raw materials	Maltose syrup	α-Lactose	Glucose	Cassava	Cornstarch
Unit price ($/ton)	550	1,700	400	220	450

Thus, the corn steep liquor concentration in feeding medium #3 was set at 10 g/l.

Fig. 5 shows the glucoamylase production curves when adopting the three-stage varied-rate feeding strategy and different feeding media prepared by the low-cost carbon materials. Among the fermentations with the four feeding media, the cassava hydrolysate-based medium produced the lowest glucoamylase activity (Batch #F, 5,400 U/ml). We speculated that the impurity in cassava was harmful to *A. niger* cells and thus glucoamylase biosynthesis was restricted. On the other hand, in Batches #E and G, during the middle fed-batch phase of 50–100 h, glucoamylase production rates were comparably higher than that of the control (standard medium, Batch #D). Furthermore, during the late fed-batch phase of 100–130 h, glucoamylase production still remained at a high rate when using the cornstarch hydrolysate-based feeding medium (Batch #G), and in this case, the highest glucoamylase activity of 14,500 U/ml and productivity of 111.54 U/ml/h were obtained, which was about a 32% increase over the control. The glucose-based feeding medium also yielded an equivalently high glucoamylase production (10,800 U/ml) as compared with that of the control. The cornstarch hydrolysate-based feeding medium seemed to be the best candidate for glucoamylase production in terms of both productivity and economics.

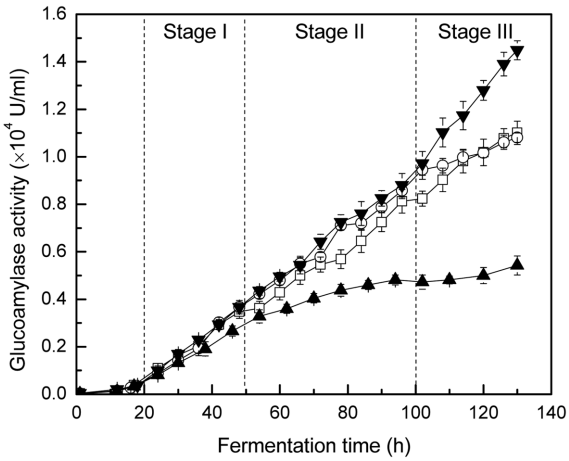

Fig. 5. Glucoamylase production curves with the optimal three-stage varied-rate feeding strategy and different low-cost feeding media.

Batch #D (standard maltose/α-lactose combination, □), Batch #E (glucose, ○), Batch #F (cassava hydrolysate, ▲), and Batch #G (cornstarch hydrolysate, ▼).

Economic Evaluation of Glucoamylase Production Using Different Raw Materials

We also carried out the economic evaluation of glucoamylase production using different raw materials, under the conditions of (i) using the proposed three-stage

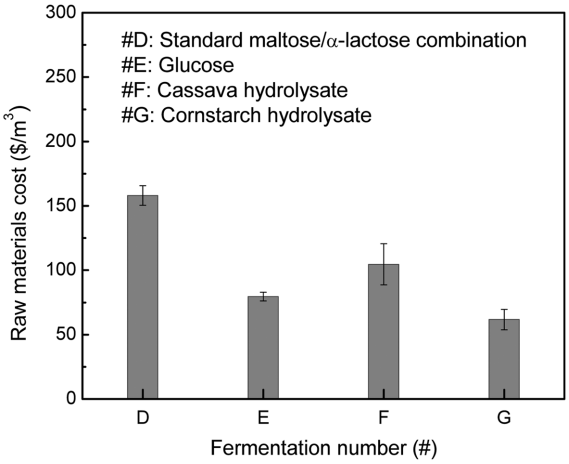

Fig. 6. Economic evaluation of using different raw materials for production of glucoamylase with delegated specification.

varied-rate substrate feeding strategy; and (ii) the final supernatant volumes of fermentations in 5 L bioreactor finished at 3 L, as well as the assumption that the glucoamylase commercial specification (enzymatic activity) is 5,000 U/ml (a slightly lower level of the minimum activity obtained using cassava hydrolysate-based medium). Based on the above experimental results and assumption, Fig. 6 depicts the prices of raw carbon-source materials to produce one ton (or 1 m^3) of glucoamylase with the specified enzymatic activity of 5,000 U/ml (Batches #D, #E, #F, and #G). As shown in Fig. 6, $158.3 of the traditional substrate (maltose/α-lactose combinations) is required to produce one ton of specified glucoamylase product (Batch #D). On the other hand, only $61.7 and $75.0 are required to produce one ton of specified glucoamylase product (5,000 U/ml) when using cornstarch hydrolysate and glucose as the carbon-source alternatives. Furthermore, both of them could produce glucoamylase with much higher enzymatic activities than that of using cassava hydrolysate-based medium (10,800–14,500 U/ml versus 5,400 U/ml; Batches #E and #G versus Batch #F), so that their consumed amounts to produce the specified product could be largely reduced. In contrast, the economic aspect of using cassava hydrolysate-based medium for the specified glucoamylase production is not desirable, because the very low enzymatic activity (5,400 U/ml, Batch #F) led to a large amount of raw materials utilization even though the unit price of the cassava is the lowest (Table 2). In summary, the economics evaluation results indicated that cornstarch hydrolysate (Batch #G) is the best candidate for the production of glucoamylase with delegated specification, as it could reduce the raw materials cost by more than 61% while satisfying the product quality as compared with that using the standard medium (maltose/α-lactose combination).

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