J Ind Microbiol Biotechnol (2010) 37:1203–1209
DOI 10.1007/s10295-010-0767-9

ORIGINAL PAPER

Fed-batch production of a bioflocculant from *Corynebacterium glutamicum*

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Received: 24 December 2009 / Accepted: 14 June 2010 / Published online: 30 June 2010

Abstract The constant-rate fed-batch production of the polygalacturonic acid bioflocculant REA-11 was studied. A controlled sucrose-feeding strategy resulted in a slight improvement in biomass and a 7% reduction in flocculating activity compared with the batch process. When fed with a 3 g l^{-1} urea solution, the flocculating activity was enhanced to 720 U ml^{-1} in 36 h. High cell density (2.12 g l^{-1}) and flocculating activity (820 U ml^{-1}) were obtained in a 10-l fermentor by feeding with a sucrose-urea solution, with values of nearly two times and 50% higher than those of the batch process, respectively. Moreover, the residual sucrose declined to 2.4 g l^{-1}, and residual urea decreased to 0.03 g l^{-1}. Even higher flocculating activity of 920 U ml^{-1} and biomass of 3.26 g l^{-1} were obtained by feeding with a sucrose-urea solution in a pilot scale fermentation process, indicating the potential industrial utility of this constant-rate feeding strategy in bioflocculant production by *Corynebacterium glutamicum*.

Keywords Bioflocculant · *Corynebacterium glutamicum* · Polygalacturonic acid · Constant-rate feeding · Flocculation · Fermentation

Abbreviations

μ	Specific cell growth rate
q_s	Specific sucrose consumption rate
q_p	Specific bioflocculant production rate
GLC	Glucose
G6p	Glucose-6-phosphate
G1P	Glucose-1-phosphate
UDPG	UDP-glucose
UDP-GAL	UDP-galactose
UDP-GALA	UDP-galacturonic acid
polyGALA	Polygalacturonic acid
PEP	Phosphoenolpyruvate
PYR	Pyruvic acid
AcCoA	AcetylCoA
ICI	Isocitric acid
OAA	Oxalacetic acid
α-KG	α-ketoglutaric acid
SUC	Succinic acid
MAL	Malic acid
LAC	Lactic acid
AC	Acetic acid

Introduction

The industrial potential of bioflocculants has long been recognized because of their harmlessness, biodegradability and lack of secondary pollution from their degradative intermediates. Considerable scientific attention has been given to this field for nearly 30 years. Although more than 50 microorganisms have been found that produce various types of extracellular bioflocculants [22], none of these have been commercially produced so far. In fact, little

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progress has been made in the study and application of bioflocculants during the last decade. Current research includes screening for microorganisms that are able to produce novel flocculants efficiently, the purification and characterization of bioflocculants, and the search for low-cost substrates [2–4, 13–17, 20, 25, 26, 29].

Low yield and high product cost comprise a bottleneck for the further development of bioflocculants for both scientific research and industrial applications. The yields of some bioflocculants with related information are listed in Table 1. Fifteen grams of purified MMF1 bioflocculant produced by the multiple-microorganism consortium MM1 was recovered from 1.0 l of culture broth, which is the highest bioflocculant productivity currently reported [29]. Researchers are now making great efforts to lower the production cost of bioflocculants. For example, ethanol and fish blood wastewater were used as substitutes for glucose and yeast extract in bioflocculant synthesis [14]. Kurane tried using volatile fatty acids, such as acetic acid and propionic acid, that were byproducts from treating the surplus sludge from wastewater treatment and the waste from food industries in order to cut cultivation costs. Agricultural waste, such as straw, was also developed as a low-cost carbon source for bioflocculant synthesis [21]. Most of these studies, however, are still only at the laboratory level. Using low-cost raw materials has proven to be a direct and efficient way to lower the cost of bioflocculants, while optimization of the cultivation process is a rather powerful approach to improve the production of bioproducts, which have long been neglected in the study of bioflocculants.

Fed-batch processes have been widely applied in the industrial production of many biological products, including antibiotics, vitamins and amino acids. They are particularly well suited to cultivations that exhibit substrate inhibition, catabolite repression, toxic precursors or the glucose effect, thus improving the production of bioproducts [28]. However, relatively few studies have focused on the fed-batch cultivation of *C. glutamicum* [11, 23, 27]. And there have been no such reports on the biosynthesis of flocculants yet.

In our previous studies, a polygalacturonic acid bioflocculant (REA-11) was purified from the culture broth of *Corynebacterium glutamicum* CCTCC M201005 [8]. Its efficiency at both clarification and decolorization of wastewater and its stable physiochemical properties suggest that it has potential applications in industry [5]. To increase the productivity of REA-11 and lower its production cost, two strategies are being carried out: (1) analysis of the metabolic mechanism of bioflocculant and construction of an engineered *Corynebacterium glutamicum* strain with great flocculant synthesis potential [7, 19] and (2) the optimization of cultivation conditions [6, 9]. With a cost-effective medium, preliminary studies in shaking flasks have indicated that the flocculating activity of REA-11 can be greatly enhanced by feeding sucrose and urea during cultivation [6]. Hence, this paper describes the fed-batch production of REA-11 on a pilot scale.

Materials and methods

Microorganism

Corynebacterium glutamicum CCTCC M201005, which was screened by our laboratory and preserved in the China Center for Type Culture Collection (CCTCC, Wuhan, China), was used in this study.

Media and cultivation conditions

The pre-culture medium consisted of (l^{-1}): 10 g glucose, 0.5 g yeast extract, 0.5 g urea, 0.1 g NaCl and 0.2 g $MgSO_4 \cdot 7H_2O$. Unless otherwise specified, the fermentation medium consisted of (l^{-1}): 12 g sucrose, 0.1 g KH_2PO_4, 0.5 ml corn steep liquor (nitrogen content 47 mg ml^{-1}), 0.5 g urea, 0.1 g NaCl and 0.2 g $MgSO_4 \cdot 7H_2O$. The initial pH of all media was adjusted to 7.8.

All feeding solutions were prepared with distilled water, and all media were sterilized at 121°C for 20 min.

Culture conditions

The seed culture from a slant was inoculated into a 250-ml flask containing 50 ml pre-culture medium and incubated for 18 h on a reciprocal shaker at 120 r min^{-1}. Inocula of 5 ml and 300 ml were added to a 250-ml shaking flask containing 100 ml of fermentation medium and a 10-l jar

Table 1 Information on a variety of recently reported bioflocculants

Strain	*Alcaligenes latus*	*Alcaligenes cupidus* KT201	*Paecilomyces*	*Rhodococcus erythropolis*	Multiple-microorganism consortia MM1
Crude bioflocculant/g l^{-1}	2.8	2.438	0.5	0.205	15
Carbon and nitrogen sources in fermentation process	Fructose, urea, yeast extract	Sucrose, $(NH_4)_2SO_4$	Glucose, yeast extract, casamino acid	n-Pentadecane, yeast extract, urea	Brewery water, urea, yeast extract, $(NH_4)_2SO_4$
References	[15]	[26]	[25]	[24]	[29]

fermentor (B. Braun B10STAT ER10L, Germany) containing 6 l of fermentation medium, respectively. A pilot production of the bioflocculant was performed in a 500-l fermentor (Zhenjiang Dongfang GJ-500L, China) containing 300 l of fermentation medium with an inoculum of 1,500 ml.

The agitation speed for shaking flasks was kept at 120 r min^{-1}, and cultures in the jar fermentors were operated at an agitation speed of 150 r min^{-1} with an aeration rate of 1 l^{-1} min^{-1} throughout the whole process. All cultivations were performed at 28°C with pH and dissolved oxygen uncontrolled.

Constant-rate fed-batch production of bioflocculant

The tested feeding strategies were presented in Table 2. All fed-batch cultures were conducted in the 10-l B. Braun fermentor, except that a pilot production of bioflocculant was carried out in the 500-l reactor.

Determination of flocculating activity

Flocculating activity was measured according to the method described by Kurane et al. [14]. Briefly, 1 ml of sample and 2.5 ml of $CaCl_2$ solution (10 g l^{-1}) were mixed with 40 ml kaolin clay solution (10 g l^{-1}), gently shaken and left to stand still for 5 min at room temperature. By measuring the decrease of turbidity in the upper phase, flocculating activity was expressed as flocculating rate (FR) calculated by FR(U mL^{-1}) = (A − B)/A × 100 × D, where *A* and *B* are the optical densities at 550 nm of the control and the sample, respectively. D is the dilution of the culture broth free of cells. Each sample was analyzed in triplicate.

Analyses

Cell growth was measured by dry cell weight (DCW). A total of 5 ml of fermented medium was centrifuged at 10,000 *g* for 15 min, washed twice with distilled water and dried at 105°C until a constant weight was achieved.

Sucrose concentration was measured by the modified Roe spectrometric method [12]. A series of standard sucrose solutions at concentrations of 0, 0.08, 0.16, 0.24, 0.32 and 0.4 mg ml^{-1} were prepared, respectively. Each of the standards was mixed with 0.1 ml of NaOH (2 mol l^{-1}) and heated for 10 min in a boiling water bath. After the solutions were cooled with running water, 1 ml resorcin and 3 ml HCl (10 mol L^{-1}) were added and mixed, then heated at 80°C for 8 min. After cooled off, the optical density of the solutions was measured at 500 nm, and the standard curve was obtained. Then 1 ml of cell-free culture broth was treated in the same way as described above. The sucrose concentration of the culture broth was calculated according to the standard curve.

Urea concentration was measured by paradimethylaminobenzaldehyde (PDAB) method [18]. A series of standard urea solutions at concentrations of 0, 0.04, 0.1, 0.15, 0.2, 0.3 and 0.4 mg ml^{-1} were prepared, respectively; 5.0 ml PDAB solution (0.03 g ml^{-1}) and 3.0 ml HCl (10 mol l^{-1}) were added into each of the standard solutions, mixed and left stand for 15 min. The optical densities of the standard urea solutions were measured at 446 nm, and the standard curve was obtained. Then 1 ml of cell-free culture broth was treated in the same way as described above. The urea concentration of the culture broth was calculated according to the standard curve.

Results and discussion

Batch production of the bioflocculant REA-11 from *C. glutamicum*

Batch production of the REA-11 bioflocculant was used as a control (Fig. 1). The maximum flocculating activity reached 545 U ml^{-1} in 32 h, which was 16 h earlier than in the shaking flask cultures. Cell growth stopped after 18 h of cultivation, and the peak value reached 1.17 g l^{-1}. Sucrose content decreased to nearly 8 g l^{-1} in the first 21 h, but remained almost constant afterwards, suggesting that over 60% of the sucrose remained unutilized

Table 2 Different feeding strategies for the fed-batch production of REA-11 by *C. glutamicum*[a]

Feeding stratergy	Feeding solution	Concentration of feeding solution/g l^{-1}	Feeding time/hour	Feeding rate/ml h^{-1}
1[b]	Sucrose	50	18th	30
2	Urea	3	16th	30
3	Sucrose-urea	20 (sucrose)	16th	30
	Mixture	3 (urea)		(1,500[c])

[a] All the feeding strategies were applicable in a 10-l fermentor unless indicated

[b] The initial sucrose concentration in the fermentation medium was 8 g l^{-1}

[c] Feeding rate in a 500-l fermentor

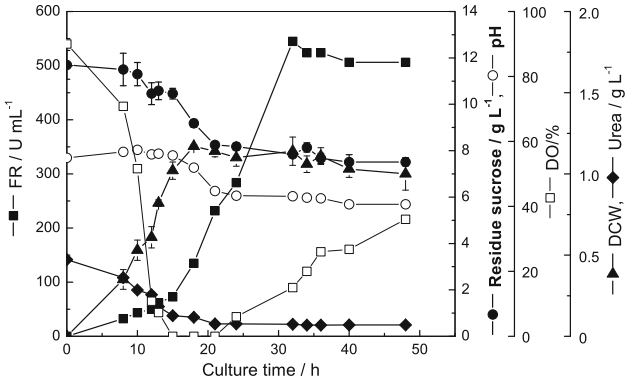

Fig. 1 Batch production of REA-11 by *Corynebacteria glutamicum* CCTCC M201005. FR (*filled square*), residual sucrose (*filled cicle*), DCW (*filled triangle*), urea (*filled diamond*), DO (*open square*), pH (*open circle*)

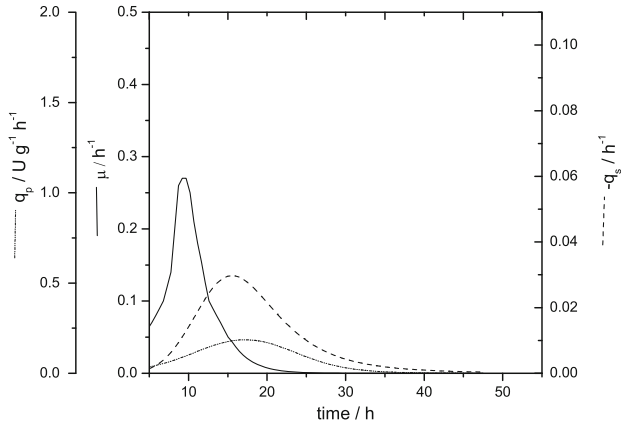

Fig. 2 Kinetic curves in the batch production of REA-11 by *Corynebacteria glutamicum* CCTCC M201005. μ (*continuous line*), q_s (*dashed line*), q_p (*dashed dotted line*)

in the culture. However, 83.8% of the urea was consumed in the first 21 h, with the final residual urea below 0.05 g l^{-1}.

The kinetics of the batch process were analyzed (Fig. 2). The cells grew quickly in the early period of the batch process with a maximum specific growth rate (μ) of 0.27 h^{-1} at the 10th h, but dropped to 0.06 h^{-1} at the 15th h. The specific bioflocculant production rate (q_p) increased to 0.2 U g^{-1} h^{-1} at the 17th h, but fell down to 0.15 U g^{-1} h^{-1} at the 20th h. As for the substrate consumption, the specific sucrose consumption rate (q_s) decreased from 0.03 h^{-1} at the 15th h to 0.018 h^{-1} at the 20th h. The rapid decline of bacteria cells led to poor cell growth and low bioflocculant production, which might be caused by an inappropriate nutrient supply or worsening environmental conditions.

Given that C/N is one of the most important parameters affecting the balance of carbon and nitrogen in fermentation [6], the discovery that there was a high concentration

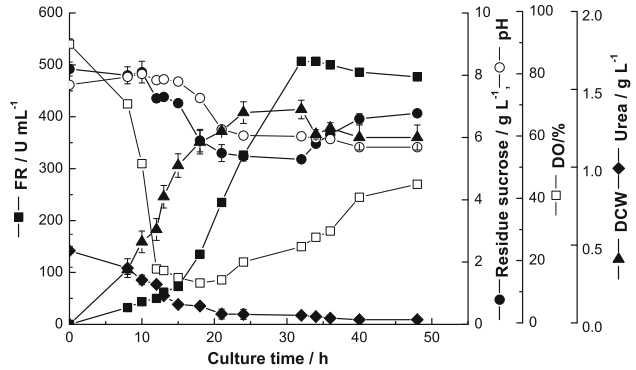

Fig. 3 Sucrose-fed batch production of REA-11 by *Corynebacteria glutamicum* CCTCC M201005. FR (*filled square*), residual sucrose (*filled cicle*), DCW (*filled triangle*), urea (*filled diamond*), DO (*open square*), pH (*open circle*)

of residual sucrose and low sucrose utilization rate during bioflocculant production suggested an unfavorable C/N.

Sucrose-fed batch production of REA-11

The initial sucrose content in the medium was reduced to 8 g l^{-1}. With a sucrose-feeding strategy, the trend of substrate consumption was found to be similar to that in the batch process (Fig. 3). The sucrose concentration decreased from 8 g l^{-1} to 5.5 g l^{-1} in the first 21 h, but tended to increase after 32 h because of the continuous input of sucrose solution. Only 7% of the initial urea was left at the end of cultivation. The fact that the flocculating activity decreased to 507 U ml^{-1} while the maximum biomass increased to 1.36 g l^{-1} in 24 h was due to the excess supply of carbon or the lack of a nitrogen source.

Urea-fed batch production of REA-11

When feeding with a urea solution of 3 g l^{-1}, the final sucrose concentration in the culture decreased to 4.7 g l^{-1} (Fig. 4), suggesting that 60% of the carbon source was utilized during the fermentation process. Compared with the batch process, the consumption efficiency of sucrose was enhanced, and the flocculating activity increased to 720 U ml^{-1} after 36 h. In addition, cell growth was slightly augmented.

Sucrose + urea fed-batch production of REA-11

For a more favorable carbon-to-nitrogen ratio (C/N) in the medium, sucrose and urea were both provided during fed-batch cultivation (Fig. 5). These conditions yielded the highest cell density (2.12 g l^{-1}) and flocculating activity (820 U ml^{-1}), which were nearly two times and 50% over the values obtained in the batch process, respectively. The

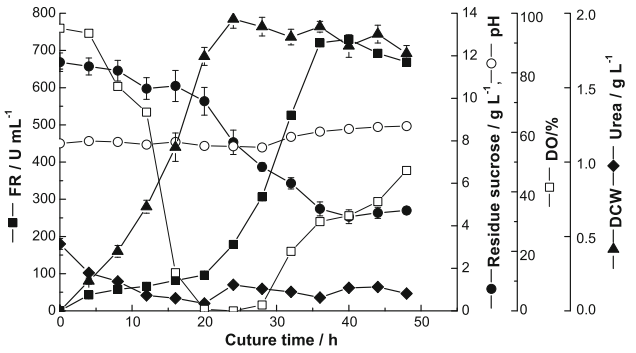

Fig. 4 Urea-fed batch production of REA-11 by *Corynebacteria glutamicum* CCTCC M201005 FR. (*filled square*), residual sucrose (*filled cicle*), DCW (*filled triangle*), urea (*filled diamond*), DO (*open square*), pH (*open circle*)

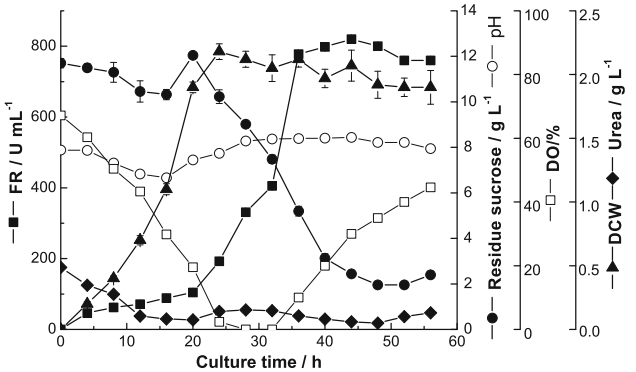

Fig. 5 Sucrose + urea fed-batch production of REA-11 by *Corynebacteria glutamicum* CCTCC M201005 FR. (*filled square*), residual sucrose (*filled cicle*), DCW (*filled triangle*), urea (*filled diamond*), DO (*open square*), pH (*open circle*)

specific cell growth rate stayed at a level of over 0.04 h^{-1} until the 20th h, while the specific sucrose consumption rate reached the maximum (0.1 h^{-1}) at the 35th h (Fig. 6). Although the flocculating activity in the medium continually increased until reaching a maximum at 44 h, the maximum volumetric productivity of the bioflocculant enhanced to 21.6 U ml^{-1} h^{-1} at the 36th h. The high specific productivity of cells lasted until the 35th h. The residual sucrose in the culture was observed to decrease to 2.4 g l^{-1}, and the residual urea was only 0.03 g l^{-1}. The fedbatch of both substrates led to greatly increased substrate utilization and cell productivity; thus, biomass and flocculating activity were both improved. It was noticed, however, that the specific bioflocculant productivity of cells began to decrease to below 0.1 U g^{-1} h^{-1} after 34 h. So we suggest that the production period of bioflocculant should be shortened to 34 h when the flocculating activity of the culture was 780 U ml^{-1}. This is industrially applicable because of the lowered production cost of the bioflocculant.

In our previous studies, a dramatic increase in REA-11 production was detected when the initial C/N of the

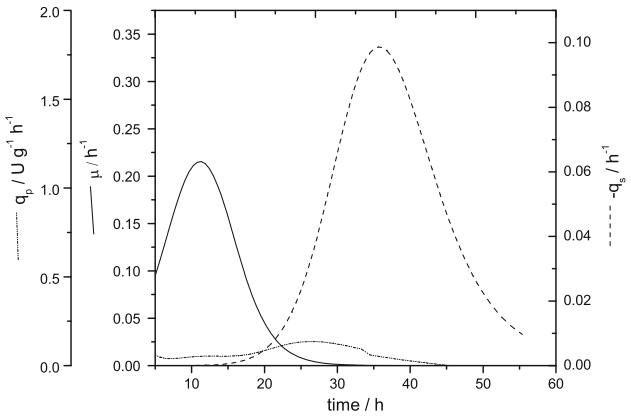

Fig. 6 Kinetic curves in the sucrose + urea fed-batch production of REA-11 by *Corynebacteria glutamicum* CCTCC M201005. μ (*continuous line*), q_s (*dashed line*), q_p (*dashed dotted line*)

medium was increased above 3:1 [6]. The feeding strategy that utilized a sucrose-urea mixture at a constant C/N of 6:1 gave further support to this suggestion: not only the highest levels of bioflocculant and cell weight were obtained, but also the consumption efficiencies of carbon and nitrogen sources were greatly enhanced.

A comparison of key parameters obtained with different cultivation strategies is presented in Table 3.

Pilot scale production of REA-11 with the sucrose + urea feeding strategy

For further verification of this sucrose + urea feeding strategy in industrial application, a pilot scale fermentation of the bioflocculant was conducted in a 500-l jar fermentor (Fig. 7). Results showed that even higher flocculating activity (920 U/ml) and biomass (3.26 g/l) were obtained in this pilot production than in the 10-l reactor. The bacteria grew well on a pilot scale. The specific growth rate of cells kept at a high level (0.16 h^{-1}) until 20 h and the maximum bioflocculant productivity of cells reached 0.22 U g^{-1} h^{-1} at the 24th h. The volumetric production rate of the bioflocculant was 25.3 U ml^{-1} h^{-1} at the 36th h, 17% higher than that in the 10-l one. The pilot scale testing fully proved the industrial potential of this feeding strategy in bioflocculant production.

From the metabolic pathway of bioflocculant REA-11 in *Corynebacterium glutamicum* CCTCC M201005 (Fig. 8), the oversecretion of acetate and lactate is considered to be an essential factor that affects bioflocculant secretion [7]. Inappropriate C/N led to the accumulation of acidic byproducts. In the batch cultivation of *C. glutamicum* CCTCC M201005, high levels of acetate (110 mg l^{-1}) and lactate (140 mg l^{-1}) were detected. When feeding with a sucrose-urea mixture, the final contents of acetate and lactate decreased to 40 and 80 mg l^{-1}, respectively.

Table 3 Comparison of parameters from different cultivation strategies

Cultivation strategies	Maximum DCW, g l^{-1}	Maximum FR, U ml^{-1}	Time needed to maximum FR, h	Maximum bioflocculant productivity[a], U ml^{-1} h^{-1}	Maximum cell productivity[b], g l^{-1} h^{-1}
Batch cultivation	1.17	545	32	17.0	0.068
Constant-rate fed cultivation[c]					
Strategy 1	1.38	507	32	15.8	0.068
Strategy 2	1.97	730	40	20.0	0.086
Strategy 3	2.12	820	44	21.6	0.095

[a] Flocculating activity per milliliter fermentation medium per hour

[b] Dry cell weight per liter fermentation medium per hour

[c] Detailed information on the strategies is presented in Table 2

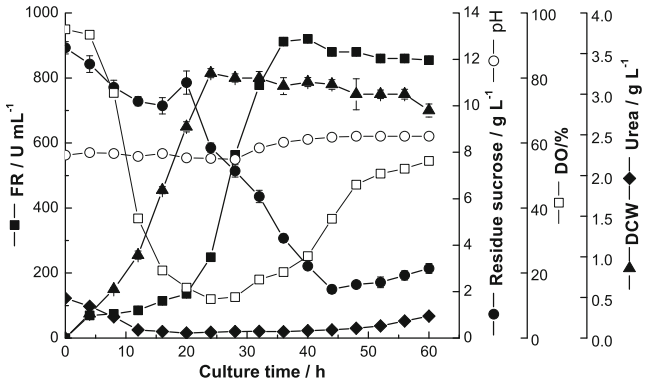

Fig. 7 Sucrose + urea fed-batch production of REA-11 by *Corynebacteria glutamicum* CCTCC M201005 in a 500-l fermentor FR. (*filled square*), residual sucrose (*filled cicle*), DCW (*filled triangle*), urea (*filled diamond*), DO (*open square*), pH (*open circle*)

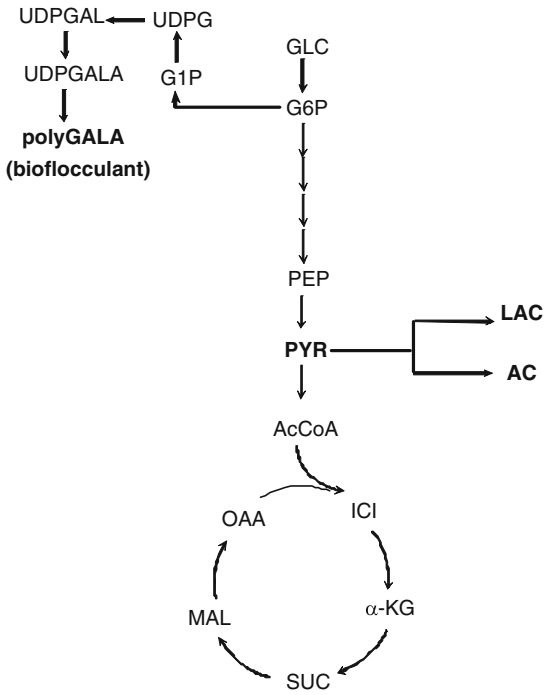

Fig. 8 Metabolic pathway of REA-11 in *Corynebacteria glutamicum* CCTCC

The fact that higher productions of biomass and bioflocculant were obtained in the 500-l fermentor than in the 10-l fermentor is due to a superior oxygen supply in a pilot scale fermentation process. It was also determined that the concentration of acidic byproducts in the 500-l fermentor was nearly 12% lower than that in the 10-l fermentor. The metabolic flux analysis of the metabolic pathway of bioflocculant in our previous studies gave further proof to the results. The metabolic flux distribution at the pyruvic acid node in *C. glutamicum* differs with the oxygen supply [7]. About 40% of the metabolic flux flew to acetate and lactate with 70% dissolved oxygen, while 55% flux was directed to acidic byproducts when dissolved oxygen was decreased to 10%.

Recently, a stepwise oxygen supply strategy during REA-11 production has been developed in our laboratory. Preliminary results have shown that this strategy can efficiently stimulate both the bacterial growth and bioflocculant synthesis by restricting the metabolic flux to acetate and lactate (unpublished). One important scale-up criterion for a bioprocess is the ratio of oxygen uptake to sugar uptake [1, 10]. Thus, combining substrate feeding with DO control is expected to be a more powerful way for *C. glutamicum* to produce an even higher yield of biomass and bioflocculant. Like many bioproducts already manufactured in industries, bioflocculants have practical applications, and their large-scale production is imminent.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (no. 30700020), the Xiamen Science and Technology Committee (no. 3502Z20093006), and Fujian Provincial Department of Science and Technology, China (key project no. 2008Y0060).

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