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Highly efficient production of D-lactate by *Sporolactobacillus* sp. CASD with simultaneous enzymatic hydrolysis of peanut meal

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Abstract Highly efficient D-lactate production by *Sporolactobacillus* sp. strain CASD was demonstrated in this study. Peanut meal was found to be a better nutrient than yeast extract, soybean meal, soybean peptone, corn steep, liquor beef extract, and ammonium sulfate in the production of D-lactate. To improve the utilization of peanut meal, the material was enzymatically hydrolyzed and simultaneously utilized as the nitrogen source in D-lactate fermentation. Very high D-lactate production (207 g/L) was obtained using 40 g/L of peanut meal in 30-L fed-batch fermentation, with the average productivity of 3.8 g/(L·h) and optical purity of 99.3%. The production of such a high concentration of optically pure D-lactate by strain CASD, with the simultaneous enzymatic hydrolysis of peanut meal and fermentation, represents a new cost-efficient and integrated method for D-lactate production using agricultural by-products.

Keywords D-Lactate · *Sporolactobacillus* sp. · Peanut meal · Simultaneous enzymatic hydrolysis

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Introduction

Lactic acid (2-hydroxypropionic acid) and its derivatives are widely used in food, cosmetic, pharmaceutical, and leather industries. Recently, new applications of lactic acid have been found as a building block for biodegradable plastic, poly (lactic acid) (PLA). PLA is considered to be one of the most promising biodegradable polymers that can be applied to drug delivery systems, orthopedic screws, textiles, packaging materials, and agricultural films. PLA, which is usually produced from optically pure L-lactic acid, has some desirable properties such as good processability, biocompatibility, and biodegradability. However, its application is limited by its melting temperature which is lower than that of other petroleum-based polymers (Fukushima et al. 2007). The development of a stereocomplex of poly (L-lactic acid) and poly (D-lactic acid) is considered an advance in PLA modification, because the melting point of this stereocomplex (230 °C) exceeds that of poly (L-lactic acid) (180 °C; Ikada et al. 1987). Recently, it was reported that stereocomplexation could be used to improve the mechanical performance, thermal resistance, and hydrolysis resistance of PLA-based materials (Fukushima et al. 2007). These discoveries have provided a new and simple approach for PLA modification and have opened a potential market for D-lactic acid.

Although the biotechnological production of L-lactic acid has been extensively investigated, there are relatively few studies on D-lactic acid fermentation. The titer of D-lactic acid production is generally much lower than that of L-lactic acid production. Several strains have been reported to be homofermentative D-lactic acid producers, such as *Lactobacillus delbrueckii* (Demirci and Pometto 1992), *Bacillus laevolacticus* (Boer et al. 1990), *Lactobacillus coryniformis* (Bustos et al. 2004), *Corynebacterium*

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glutamicum (Okino et al. 2008), *Lactobacillus bulgaricus* (Benthin and Villadsen 1995), metabolically engineered *Saccharomyces cerevisiae* (Ishida et al. 2006), and *Escherichia coli* (Zhou et al. 2005). Although *Sporolactobacillus inulinus* mainly produces the D-isomer of lactic acid (Fukushima et al. 2004), there have been few studies on its potential ability to produce optically pure D-lactic acid (Zhao et al. 2010).

A major concern in lactic acid fermentation is to improve the productivity and reduce the cost of raw materials. The nitrogen source and yeast extract account for 38% of the total fermentation cost during lactic acid fermentation (Altaf et al. 2007). To reduce production costs, the use of various low-cost raw materials has been extensively investigated, such as rice starch (Fukushima et al. 2004), defatted rice bran (Tanaka et al. 2006), tryptic soy (Nancib et al. 2005), casamino acids, soybean hydrolyzate, ram horn protein hydrolyzate, and corn steep liquor (Kwon et al. 2000; Wee et al. 2008). However, lactic acid production is limited when such low-cost, poor nutrient quality are used. Oil crops, such as peanut, soybean, and rapeseed are abundant in China. Oil mill wastes, such as peanut meal, are relatively inexpensive and abundant by-products of oil production from oil crops (Sharma et al. 2002). Proteins and other ingredients in peanut meal can be used as nutrients for lactic acid production. However, to our knowledge, there are few studies on the use of peanut meal as the nitrogen source for D-lactic acid production.

In this paper, we report a highly efficient D-lactate production method using *Sporolactobacillus* sp. CASD as the fermentative microorganism and peanut meal as the nitrogen source. Conventionally, raw nitrogen sources are hydrolyzed at high temperatures and under acidic conditions. The high temperatures would increase the energy requirement and acidic conditions would have deleterious effects on the produced amino acids (Gao et al. 2008). In this study, peanut meal was hydrolyzed by a protease and simultaneously utilized by strain CASD to convert glucose into D-lactate. This new strategy of combining protein hydrolysis and utilization was considered to be more advantageous than the traditionally separated sequential processes of pretreatment and fermentation. The results might provide a reference for the efficient D-lactate production on an industrial scale.

Materials and methods

Chemicals

Peanut meal was purchased from Comwin Pharm-culture Co., Ltd. (Beijing, China). It is a dried powder that contains 7.6% (*w*/*w*) total nitrogen. Yeast extract, beef extract, and soybean peptone were purchased from Aoboxing Biotech Company Ltd. (Beijing, China). Soybean meal and corn steep liquor were purchased from Comwin Pharm-culture Co., Ltd. (Beijing, China). Ammonium sulfate was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Commercially available neutral protease (Neutrase EC 3.4.24.28), with an activity of 5×10^4 U/g according to the manufacturer's data, was purchased from Novozymes (Denmark). All other chemicals were of analytical grade and are commercially available.

Microorganism and culture conditions

Sporolactobacillus sp. strain CASD used in this study is a homofermentative D-lactic acid producer (Xu et al. 2007; Zhao et al. 2010). The strain has been deposited in the China General Microbiological Culture Collection Center (№ 2185). The strain was maintained on De Man, Rogosa, and Sharpe (MRS) agar slants. The pH was adjusted to 6.2–6.5. The slant was cultivated at 42 °C for 48 h and stored at 4 °C. The stock culture was transferred to fresh MRS agar slants every 3–4 weeks.

The medium for the seed culture had the following composition (in g/L): glucose, 40; and yeast extract, 10. Calcium carbonate (30 g/L) was added to neutralize D-lactic acid during the fermentation. The seed culture was prepared as follows: a loop of cells from the fully grown slant was inoculated into 20 ml of the above sterile medium in 50-ml conical flasks and incubated for 48 h at 42 °C without agitation. The seed culture was then inoculated into 300-ml Erlenmeyer flasks or fermentors for D-lactate production. The inoculum volume was 10% (*v*/*v*).

Effects of different nitrogen sources on D-lactate fermentation

The effects of different nitrogen sources on D-lactate fermentation were determined in a medium containing 90 g/L glucose and 80 g/L $CaCO_3$. According to the product instructions, the total nitrogen concentration (*w*/*w*) in yeast extract, beef extract, soybean peptone, peanut meal, soybean meal, corn steep liquor, and ammonium sulfate is 11.3%, 13.1%, 8.5%, 7.6%, 8.1%, 7.4%, and 22.7%, respectively. Quantities of the above nitrogen sources used for D-lactate production, including yeast extract, beef extract, soybean peptone, peanut meal, soybean meal, corn steep liquor, and ammonium sulfate, were 30, 26, 40, 46, 42, 46, and 15 g/L, respectively, which corresponded to a nitrogen concentration of 3.4 g/L. Fermentations were carried out at 42 °C without agitation in 300-ml Erlenmeyer flasks, each containing 100 ml of medium. Samples were

taken for a 48-h period and the D-lactate concentration was determined.

Comparison of different peanut meal hydrolysis strategies

The enzymatic and chemical hydrolysis strategies were tested using the following medium (in g/L): peanut meal, 40; glucose, 135; and $CaCO_3$, 80. Two enzyme feeding strategies were used. The first one involved the fermentation with peanut meal hydrolyzate. Neutrase (0.5 g/L) was added to the peanut meal solution (30%, *w*/*v*) and treated for 6 h at 45 °C. The reaction was stopped by heating in boiling water for 15 min. After cooling the solution to room temperature, the hydrolyzed peanut meal was used as the nitrogen source for D-lactate fermentation. The second strategy was simultaneous enzymatic hydrolysis and fermentation. In this method, 0.5 g/L Neutrase was added to the medium after filtration through a 0.22-μm filter (Carrigtwohill, Co. Cork, Ireland) and the peanut meal was simultaneously hydrolyzed and utilized by strain CASD to produce D-lactate.

For chemical hydrolysis, the peanut meal was mixed with deionized water at a ratio of 3:10 (*w*/*w*). The initial pH of the mixture was set to 1.0 by adding 3 M H_2SO_4 and the resulting mixture was hydrolyzed at 121 °C for 20 min. After cooling the solution to room temperature, the pH was adjusted to 7.0 with 1 M NaOH and the hydrolyzate was stored at −20 °C.

Samples were taken every 6 h and the D-lactate concentration was assayed.

Optimization of the fermentation conditions

To study the optimal peanut meal concentration, medium of the following composition was used (in g/L): glucose, 135; Neutrase, 0.5; and $CaCO_3$, 80. The initial peanut meal concentration was 10, 20, 30, 40, 50, or 60 g/L. Samples were taken for a 36-h period and the concentrations of residual glucose and D-lactate were determined.

The medium used to optimize the protease concentration had the following composition (in g/L): peanut meal, 40; glucose, 160; and $CaCO_3$, 100. Different concentrations of Neutrase (0, 0.05, 0.1, 0.2, 0.3, 0.4, or 0.5 g/L) were added after filtration. Samples were taken for a 72-h period and the concentrations of residual glucose and D-lactate were determined.

The optimal fermentation temperature of strain CASD was determined using the medium of the following composition (in g/L): glucose, 80; peanut meal, 40; Neutrase, 0.3; and $CaCO_3$, 50. Fermentations were carried out at 37 °C, 42 °C or 47 °C. Samples were taken for a 36-h period and the concentrations of residual glucose and D-lactate were determined.

To study the substrate tolerance of strain CASD, medium of the following composition was used (in g/L): peanut meal, 40; Neutrase, 0.3; and $CaCO_3$, 130. The initial glucose concentrations were 0, 52, 80, 113, 150, or 180 g/L. Samples were taken after 0, 12, 18, and 24-h periods and the concentrations of residual glucose and D-lactate were determined.

The above-described fermentations were carried out at 42 °C without agitation in 300-ml Erlenmeyer flasks, each containing 100 ml of medium.

Fed-batch fermentations

Fed-batch fermentations were carried out in a 5-L bioreactor (Biostat B., B. Braun Biotech International GmbH, Melsungen, Germany) containing 4 L medium and in a 30-L bioreactor (Biotech-30BS, Shanghai Baoxing Inc. China) with a working volume of 24 L. Two feeding strategies were used with the 5-L bioreactor. In the first strategy, when the residual glucose concentration was below 10 g/L, glucose was added to the bioreactor to maintain the residual glucose concentration at about 50 g/L. In the second strategy, glucose and $CaCO_3$ were supplied after 30 h of fermentation. In the case of the 30-L bioreactor, only the second strategy was used.

Samples were taken every 6 h and the temperature was controlled at 42 °C in all fed-batch fermentations. The culture pH was automatically maintained at 5.5–6.0 using $CaCO_3$ present in the fermentation medium. The pre-culture for the fed-batch fermentation was prepared in the same manner as that used for flask experiments and the inoculum volume was 10% (*v*/*v*).

Analytical methods

Samples were heated at 100 °C for 5 min and then centrifuged at 6,000 rpm for 5 min. The supernatants were diluted to the desired extent with deionized water before analysis. The D-lactate concentration was assayed using a high-performance liquid chromatography system (Agilent 1100 series, Hewlett-Packard, USA) equipped with a chiral column (MCI GEL CRS10W, Japan) and a tunable UV detector at 254 nm. The mobile phase was 2 mM $CuSO_4$, which was pumped at a flow rate of 0.5 ml/min (25 °C). The optical purity of D-lactate was calculated as follows:

$$\text{D-lactate optical purity} = \frac{\text{D-lactate}}{\text{D-lactate} + \text{L-lactate}} \times 100\%$$

The concentration of residual glucose was measured by a SBA-40 C biosensor analyzer (Institute of Biology, Shandong Academy of Sciences, China).

Results

Effects of different nitrogen sources on D-lactate production

Seven nitrogen sources were used to evaluate the effects of different nitrogen sources on D-lactate production by strain CASD. The pH values of the fermentation broth were monitored and they were maintained at 5.5–6.0 by adding $CaCO_3$ during the fermentation (data not shown). As shown in Fig. 1, yeast extract, beef extract, soybean peptone, peanut meal, soybean meal, corn steep liquor, and ammonium sulfate could be used as the nitrogen source by strain CASD to produce D-lactate. In comparison with the other nitrogen sources, peanut meal and soybean meal resulted in more D-lactate production. The D-lactate concentration reached 72 g/L after 48 h of cultivation in the medium containing peanut meal. The D-lactate concentration obtained with yeast extract was lower than the concentrations obtained with peanut meal and soybean meal. Use of ammonium sulfate resulted in low D-lactate production and only 10 g/L of D-lactate was obtained.

Comparison of different peanut meal hydrolysis strategies

Fermentations with peanut meal were studied using three strategies: simultaneous enzymatic hydrolysis and fermentation, fermentation with peanut meal enzymatic hydrolyzate, and fermentation with peanut meal acidic hydrolyzate. When the fermentation was carried out with enzyme or acid-treated peanut meal, the time courses of D-lactate production were similar. The D-lactate concentration dramatically increased after 6 h and reached the maximum value at 36 h (Fig. 2). The simultaneous enzymatic hydrolysis and fermentation strategy was better than the other approaches and the D-lactate concentration reached a maximum value of 98 g/L at 48 h. Considering that the simultaneous enzymatic hydrolysis and fermentation strategy could reduce fermentation time, it was used in further investigations.

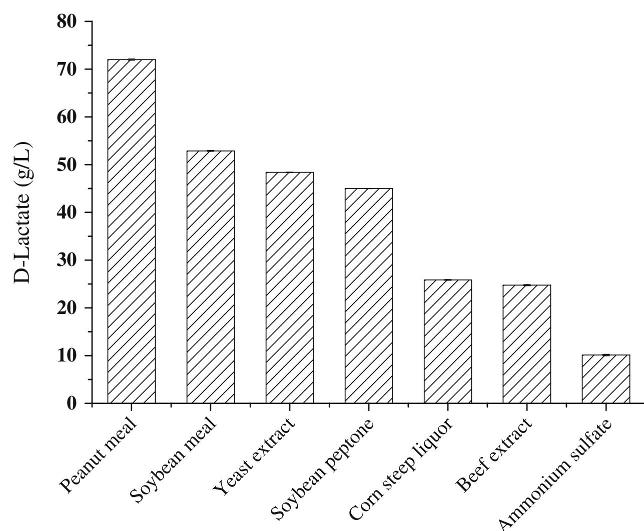

Fig. 1 Comparison of the various nitrogen sources used for D-lactate production

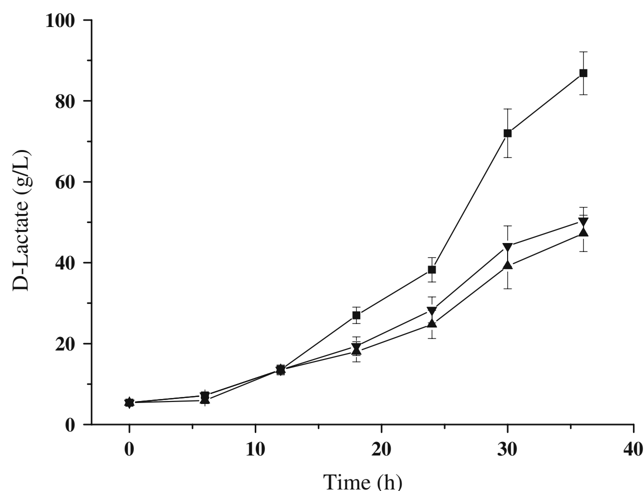

Fig. 2 Comparisons of different peanut meal hydrolysis methods used for the production of D-lactate by strain CASD. Symbols: enzymatic hydrolysis of peanut meal and simultaneous fermentation (*filled square*), peanut meal enzymatic hydrolyzate (*filled triangle*) and peanut meal acidic hydrolyzate (*filled inverted triangle*)

Effects of the concentrations of peanut meal and protease on D-lactate production

Strain CASD was cultivated using peanut meal of different initial concentrations in the range of 10–60 g/L to study the nutrient requirement (Fig. 3). The D-lactate concentration dramatically increased with increase in the amount of added

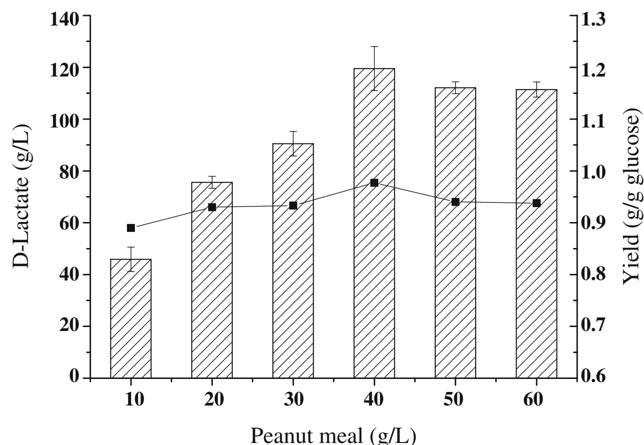

Fig. 3 Effect of peanut meal concentrations on D-lactate production by strain CASD. Symbols: D-lactate concentration (hatched bar); yield (*filled square*)

peanut meal and then gradually decreased when peanut meal concentration was higher than 40 g/L. Maximum D-lactate production (120 g/L) was obtained with the peanut meal concentration of 40 g/L, which resulted in a yield of 0.98 g/g glucose.

To determine the optimum concentration of neutrase required, different concentrations (0–0.5 g/L) of protease were used for D-lactate production. As shown in Fig. 4, approximately 90 g/L D-lactate accumulated during the fermentation without protease supplementation. A higher level of D-lactate was produced by adding protease. The highest D-lactate concentration (149 g/L) was obtained with 0.3 g/L protease. Addition of more protease resulted in a lower D-lactate production. The yield was also influenced by the protease concentration. It increased slightly with protease supplementation from 0.05 to 0.3 g/L. However, excess protease had a negative effect on the production of D-lactate. The yield decreased from the maximum value of 0.92–0.81 g/g glucose when the protease concentration increased from 0.3 to 0.5 g/L.

Effects of temperature and initial glucose concentration on D-lactate production

The obtained D-lactate concentrations at 37 °C and 47 °C were 83 and 99 g/L, respectively. The highest D-lactate concentration of 113 g/L was obtained at 42 °C; therefore, this temperature was selected for further investigations.

To examine the substrate tolerance of strain CASD, different initial glucose concentrations were used for D-lactate production (Fig. 5). In the previous study, we found that peanut meal contains 22.8% (*w*/*w*) of starch (data not shown). The experiment without glucose addition was performed to see if the fermentable carbohydrates

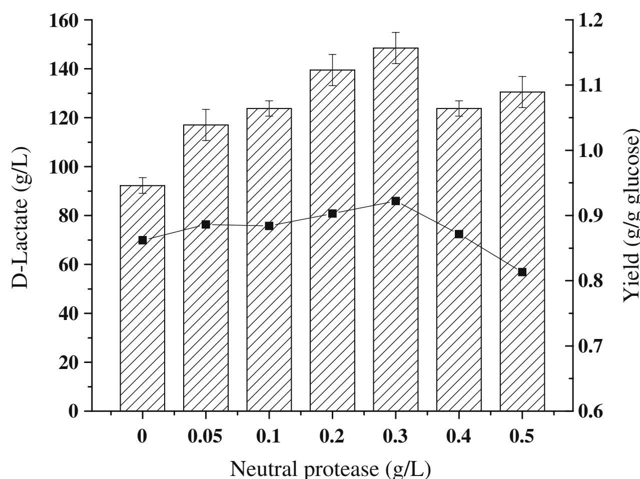

Fig. 4 Effect of protease concentrations on D-lactate production by strain CASD. Symbols: D-lactate concentration (hatched bar); yield (*field square*)

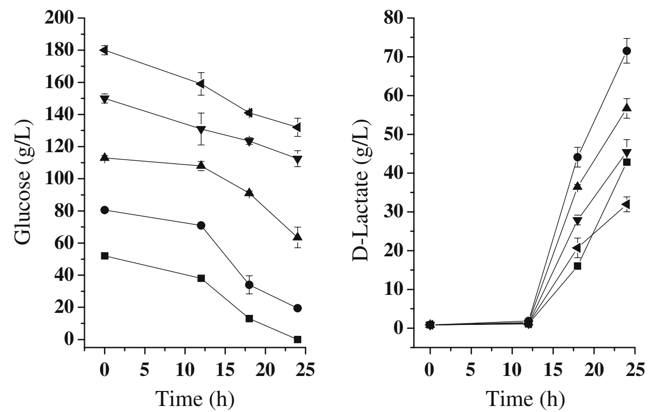

Fig. 5 Effect of the initial glucose concentrations on D-lactate production by strain CASD. Symbols: the initial glucose concentrations were 52 (*filled square*), 80 (*filled circle*), 113 (*filled triangle*), 150 (*filled inverted triangle*), and 180 g/L (*filled left-pointing pointer*). **a** Glucose consumption; **b** D-lactate production

present in peanut meal could be used to produce D-lactate by strain CASD. Study showed that no D-lactate was produced in the medium without glucose addition.

When glucose concentration was below 80 g/L, the D-lactate concentration increased with an increase in the initial glucose concentration. The highest D-lactate concentration (72 g/L) was obtained after 24 h with an initial glucose concentration of 80 g/L (Fig. 5b). Higher glucose concentration resulted in low D-lactate concentration. Glucose was hardly consumed when the initial glucose concentration was higher than 150 g/L (Fig. 5a) and D-lactate production was inhibited (Fig. 5b).

Fed-batch fermentations

Fed-batch fermentations were first tested in a 5-L bioreactor by using two-pulse feeding strategies to avoid inhibition caused by high concentration of initial glucose. The first one was the pulse fed-batch strategy and the second one was the multipulse fed-batch strategy. All fermentations were completed within 54 h and more than 200 g/L D-lactate, with the optical purity of 99.2%, was accumulated (Fig. 6a, b). Up to 226 g/L D-lactate was obtained in the multipulse fed-batch strategy, which also led to a high productivity of 4.4 g/(L·h). These values were higher than those obtained in the pulse fed-batch strategy (206 g/L and 3.8 g/(L·h), respectively). Although the multipulse fed-batch strategy seems to be superior to the pulse fed-batch strategy in terms of end-product concentration and productivity, its yield of 0.84 g/g glucose was much lower than that obtained by pulse fed-batch method (0.92 g/g glucose; Table 1). Therefore, the fed-batch fermentation strategy was used for the 30-L fermentation.

For the 30-L fed-batch fermentation, feeding was started at 30 h when the residual glucose concentration was less

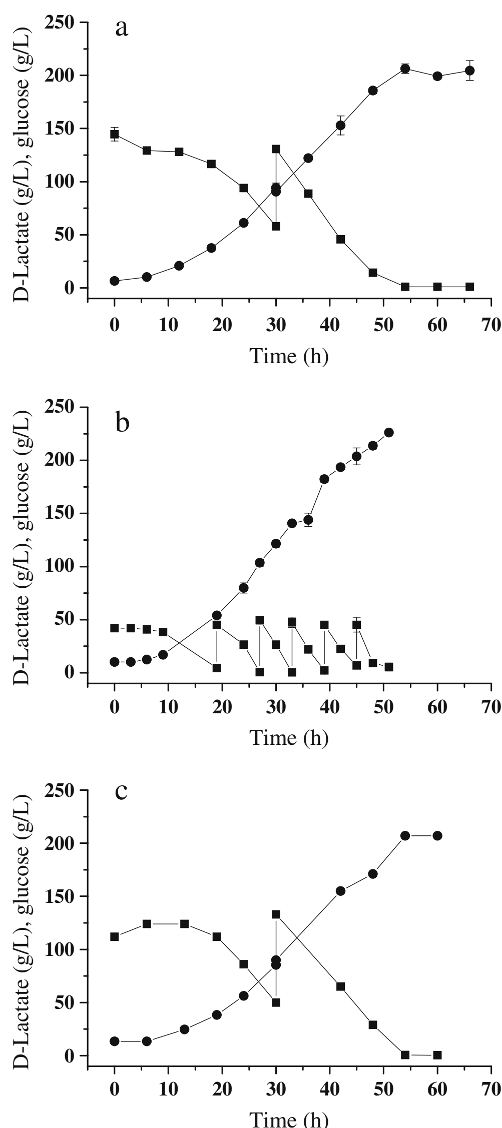

Fig. 6 Fed-batch fermentations for D-lactate production by strain CASD using the optimized medium in 5-L and 30-L bioreactor. **a** 5-L pulse fed-batch strategy; **b** 5-L multipulse fed-batch strategy; **c**, 30-L pulse fed-batch strategy. Symbols: glucose (*filled square*); D-lactate (*filled circle*)

Table 1 Fed-batch fermentation in 5- and 30-L reactors

Strategy	Time (h)	D-Lactate (g/L)	Productivity[b] [g/(L·h)]	Yield[a] (g/g glucose)
5-L pulse fed-batch	54	206	3.8	0.92
5-L multipulse fed-batch	51	226	4.4	0.84
30-L pulse fed-batch	54	207	3.8	0.93

[a] Yield (g/g glucose) was calculated as the ratio of D-lactate produced (g) to total glucose consumed (g)

[b] Volumetric productivity [g/(L·h)] was calculated as the ratio of D-lactate concentration (g/L) to the fermentation time (h)

than 50 g/L (Fig. 6c). Based on the D-lactate formation curve, the fermentation could be divided into three phases. During the first 19 h, 38 g/L D-lactate was produced and the average productivity was only 2.02 g/(L·h). From 19 to 48 h, the D-lactate concentration was accumulated at a higher rate to 171 g/L and the average productivity reached 4.43 g/(L·h). After 48 h, D-lactate formation slowed down and the average productivity was 3.0 g/(L·h). At the end of the fed-batch fermentation, final D-lactate concentration was 207 g/L and the glucose was exhausted. The optical purity of D-lactate was 99.3%.

Discussion

The limitations in the study of D-lactic acid production are the low productivity and high costs. Efforts have been made to find an excellent D-lactic acid-producing strain and to reduce the costs by using agricultural by-products. Cellulose (Yáńez et al. 2003), corn steep liquor (Bustos et al. 2004), rice saccharificate (Fukushima et al. 2004), rice bran (Tanaka et al. 2006), and sugarcane juice (Calabia and Tokiwa 2007) have been used for D-lactic acid production by *Lactobacillus* species. However, the concentration achieved in the fermentation is comparatively low and *Lactobacillus* species are generally fastidious organisms that require an easily available nitrogen source for lactic acid production. The nitrogen source has a strong positive effect on cell growth. It can stimulate strains to grow faster and reach higher cell densities (Adolf et al. 2002). Lactic acid production depends strictly on cell growth, as fermentation is associated with growth (Wee and Ryu 2009). Therefore, nitrogen source has a significant impact on D-lactate production. Yeast extract, which contains various vitamins, is widely reported to be an efficient nutrient for lactic acid production. However, in this study, yeast extract was poorly utilized by strain CASD. Peanut meal was the most suitable nitrogen source for strain CASD to produce D-lactate under the same conditions. Soybean meal is generally considered to be less effective than yeast extract. Study showed that yeast extract was more efficient for D-lactate production than soybean meal and vitamins supplementation was needed to enhance lactate production (Sunhoon et al. 2000). In this study, using strain CASD as the fermentative microorganism and soybean meal as the nitrogen source, 52.9 g/L D-lactate was obtained. This value is higher than that obtained with yeast extract (48.4 g/L).

A previous study has shown that some lactic acid bacteria have a complex proteolytic system that could utilize milk casein for cell growth and acid production. The proteolytic system is composed of proteinases and peptidases that cleave proteins into peptides and further hydrolyze these into smaller peptides and amino acids

Table 2 D-Lactate producing microorganisms and their performances

Microorganisms	Substrates	D-Lactate concentration (g/L)	Yield[a] (g/g glucose)	Productivity[b] [g/(L·h)]	Optical purity (%)	Reference
Bacillus laevolacticus	YE[c], glucose	–	–	13[d]	–	Boer et al. 1990
Lactobacillus delbrueckii	YE, glucose	117	0.76	6.5[e]	–	Demirci and Pometto 1992
Lactobacillus bulgaricus	YE, lactose	40	0.72	8.1[e]	99	Benthin and Villadsen 1995
Lactobacillus coryniformis	Cellulose	25	0.89	0.5	100	Yáńez et al. 2003
Lactobacillus coryniformis	CSL[f], YE, peptone, glucose	59	–	0.6	–	Bustos et al. 2004
Lactobacillus delbrueckii	Rice saccharificate	63	0.89	–	98.4	Fukushima et al. 2004
Escherichia coli SZ132	YE, glucose	90	0.95	6.8[e]	–	Zhou et al. 2005
Escherichia coli SZ132	YE, sucrose	96	0.93	2.6[e]	–	Zhou et al. 2005
Escherichia coli SZ132	Mineral salts, glucose	63	0.92	1[e]	–	Zhou et al. 2005
Escherichia coli SZ132	Mineral salts, sucrose	60	0.88	0.8[e]	–	Zhou et al. 2005
Lactobacillus delbrueckii	Rice bran	28	0.78	0.8	95	Tanaka et al. 2006
Saccharomyces cerevisiae	YE, glucose	61.5	0.61	0.9	99.9	Ishida et al. 2006
Lactobacillus delbrueckii	Sugarcane juice	118	0.95	1.7	96.7	Calabia and Tokiwa 2007
Corynebacterium glutamicum	Mineral salts, glucose	120	0.87	4	99.9	Okino et al. 2008
Sporolactobacillus sp. CASD	Peanut meal, glucose	207	0.93	3.8	99.3	This study

[a] Yield (g/g glucose) was calculated as the ratio of D-lactate produced (g) to total glucose consumed (g)

[b] Productivity [g/(L·h)] was calculated as the ratio of D-lactate concentration (g/L) to the fermentation time (h)

[c] Yeast extract

[d] Continuous culture

[e] Maximum volumetric productivity

[f] Corn steep liquor

(Jean and Alfred 1997). In our study, 72 g/L D-lactate, with a yield of 0.99 g/g glucose, was produced by using 46 g/L peanut meal without protease treatment. The results compared favorably with those reported elsewhere. Chienyan et al. (1999) reported that unhydrolyzed soy protein could not be significantly utilized by *Lactobacillus amylovorus*. Although *L. bulgaricus* could use unhydrolyzed whey protein as a nutrient, the lactate production was low (25 g/L) (Mel and Marvin 1989). The ability of strain CASD to directly utilize peanut meal may be due to the proteolytic system which is capable of cleaving proteins into smaller peptides and amino acids and these peptides and amino acids could be used as nitrogen source for D-lactate production. Strain CASD's potential to utilize raw protein as sole nutrient allowed expensive nutrients such as yeast extract to be replaced by less costly agricultural by-products. The possibility of directly using raw protein without hydrolysis would further contribute to the development of low-cost biological process for lactic acid production.

Conventionally, protein hydrolysis and fermentation are separated. Protein is generally hydrolyzed by enzymes or acid prior to its use as the nitrogen source in fermentation. This process requires additional treatment and separation steps, which might damage the nutrients. In simultaneous saccharification and fermentation (SSF), cellulose hydrolysis and glucose assimilation were combined into a single fermentation process (Milind et al. 2006). Equipment and labor could be reduced by using SSF. Similarly, protein hydrolysis by enzyme and protein hydrolyzate utilization might be integrated into a single process. Figure 2 showed that neither the acidic nor the enzymatic hydrolyzate of peanut meal gave better results than the simultaneous enzymatic hydrolysis and fermentation process. The simultaneous enzymatic hydrolysis and fermentation strategy for D-lactate production not only eliminated the pretreatment process, but also circumvented the inhibitory effects of peanut meal hydrolyzate on strain CASD.

Although the peanut meal without protease treatment could directly be used by strain CASD, protease addition improved the utilization efficiency. However, further protease addition resulted in a decrease in D-lactate production. The negative effects of a protease concentration higher than 0.3 g/L might be due to the inhibitory effect of the overhydrolyzed proteins in peanut meal on cell growth. The results were consistent with those reported elsewhere.

Hsieh et al. (1999) reported that the hydrolyzed soy protein significantly increased lactate production, while the over-hydrolyzed protein (molecular weight <700 Da) had an inhibitory effect on the lactate production rate. Leh and Charles (1989) reported that whey protein hydrolyzate with an average molecular weight of 700 Da resulted in the highest average volumetric productivity of lactate.

To date, D-lactic acid has attracted increasing attention because of its applications in the modification of PLA. However, in contrast to L-lactic acid production, which has been studied extensively in terms of strains, substrates, and culture processes, the biological production of D-lactic acid has been less well investigated. Only a few microorganisms are reported to be D-lactic acid producers and the concentration obtained is comparatively low (Table 2). To our knowledge, the maximum D-lactate concentration obtained was 120 g/L (Okino et al. 2008). In this study, the average D-lactate concentration in the fed-batch and multipulse fed-batch fermentation was 206 and 226 g/L, respectively. These values were comparable to those obtained in L-lactate production. *Lactobacillus lactis*, the representative L-lactic acid producer, produced an average concentration of 210 g/L L-lactate (Bai et al. 2003). Multipulse fed-batch fermentation allowed the substrate concentration to be controlled within a narrower range and resulted in a better fermentation performance. Considering that the multipulse fed-batch strategy would add to the complexity of fermentation, the simpler pulse fed-batch strategy was scaled up to 30 L and similar results were obtained. A high D-lactate concentration (207 g/L) was achieved and the conversion ratio from glucose to D-lactate was nearly 93% of the theoretical value, which is among the best reported for D-lactate production.

In summary, an efficient and simplified strategy for the industrial production of polymer-grade D-lactate was established by combining the strain's excellent production traits with the simultaneous enzymatic hydrolysis of peanut meal and fermentation.

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