L-LACTIC ACID PRODUCTION FROM *LACTOBACILLUS CASEI* BY SOLID STATE FERMENTATION USING RICE STRAW

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In order to make full use of rice straw (RS) produced at large quantity in China and to reduce the production cost of L-lactic acid, attempts were made to utilize the hydrolysate of RS as sole carbon source and the lignocellulose as inert support for producing L-lactic acid using solid state fermentation (SSF). The pretreated rice straw was enzymatically hydrolyzed by cellulase, and the hydrolyzate, containing reducing sugars supplemented with a minimum of $(NH_4)_2SO_4$, $MnSO_4$, and yeast extract, was used as moistening agent to impregnate 5g of RS, which was used as the inert support for SSF. Maximum L-lactic acid production of 3.467g per 5g of support was obtained at 37 ^oC, using Lactobacillus casei as inoculum, after 5 days of fermentation with optimized process parameters such as 72% moisture content, 4g per 5g support of reducing sugars, 2.5ml per 5g support of inoculum size, 3g per 5g support of $CaCO_3$, and pH 6.5.

Keywords: L-lactic acid, Solid state fermentation, Rice straw, Hydrolysate, Lactobacillus casei

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INTRODUCTION

Lactic acid is a versatile chemical, widely used in the food, pharmaceutical, textile, cosmetic, and chemical industries (Yun et al. 2004). In recent years there has been increasing interest in the manufacture of poly(lactic acid), a biocompatible and biodegradable plastic. Selective production of stereospecific L(+) lactic acid has been extensively studied by microbial fermentation because chemical synthesis results in racemic mixture of lactic acid (Garlotta 2001; Naveena et al. 2005a). However, only a few lactic acid bacteria, such as *Lactobacillus casei*, produce a predominately optically pure L(+) form of Lactic acid (Senthuran et al. 1997).

The economics of L(+)-lactic acid production is still a problem, and media composition contributes significantly to the overall cost of L(+)-lactic acid fermentative production. In order to bring down the production cost of L(+)-lactic acid, research efforts have been undertaken to replace the expensive carbon and nitrogen sources with cheap raw materials in the media (Hofvendahl and Hahn-Hagerdal 2000). Utilization of renewable, cheap, and abundant agro-industrial waste for the production of L(+)-lactic acid is an increasing trend in recent years. Two bioprocess schemes, termed simultaneous saccharification and fermentation, and separate hydrolysis and fermentation, have been developed for lactic acid production using lignocellulosics (Yun et al. 2004; Bustos et al. 2005; Shen and Xia 2006).

Solid state fermentation (SSF) processes involve the growth of microorganism on a solid material in the absence or near-absence of free-flowing water (Krishna 2005). It usually uses agro-industrial waste as support and/or carbon source for

production of various value-added products, such as single-cell protein, industrial enzymes, secondary metabolites, and fine chemicals. The subject has been widely studied (Robinson et al. 2001; Soccol and Vandenberghe 2003; Couto and Sanroman 2005; Couto and Sanroman 2006). Compared to submerged fermentation, the SSF process is a simple process with improved product characteristics, higher product yields, reduced energy requirements and initial capital cost, lower water output, and easier product recovery (Ooijkaas et al. 2000; Adinarayana et al. 2003).

There have been quite a few studies published in these years concerning lactic acid production from lignocellulosic materials under submerged liquid fermentation (John et al. 2007; Zhang et al. 2007). However, to the best of our knowledge, there have been no reports regarding the production of L(+)-lactic acid by *Lactobacillus casei* under SSF using rice straw (RS) as an inert support and hydrolysate of pretreated RS hydrolyzed by cellulase as sole carbon source.

EXPERIMENTAL

Microorganism and Inoculum Preparation

Trichoderma viride YQ-02, isolated by our laboratory, was used for cellulase production. The stock culture was stored on potato-glucose-agar (PDA) at 4℃ and subcultured every two months. *Lactobacillus casei* GIM 1.159 (originally from ATCC 334), a homofermentative lactic acid producer, was used for L(+)-lactic acid production. The strain was maintained on deMan, Rogosa and Sharpe (MRS) slant (DeMan et al. 1960), and subcultured every month. A loop of culture was inoculated into 250ml conical flasks containing 45ml MRS medium and incubated at 37℃ for 20 h. The 20h old bacterial culture was used as the inoculum for SSF.

Lignocellulosic Materials and Pretreatment

Raw RS was obtained locally in Hefei, Anhui province, China. It was ground to 80-120mesh with an electric grinder and was used in the experiments. The smashed RS was stored at room temperature until its use. The initial composition of the RS was determined to be 33.3% cellulose, 23.32% hemicellulose and 17.5% lignin. A two-step chemical pretreatment: dilute acid pretreatment and alkaline peroxide pretreatment, was carried out as described by Curreli et al. (2002). The cellulose, hemicellulose, and lignin contents of pretreated RS were 68.8%, 9.4%, and 8.9%, respectively. The pretreated RS was used as substrate for enzymatic saccharification.

Cellulase Production and Extraction

Cellulase was produced by SSF. A two-stage technique was employed. In the first stage, the fungus was grown on PDA slants for 5 days at 30℃. In the second, 10ml deionized sterile water was added to a PDA slant and a spore suspension containing 10^7 spores/ml was used to inoculate the solid state medium (2.5cm thick) in 500ml Erlenmeyer flasks containing RS and wheat bran (dry weight ratio is 3:2) moistened with the following mineral medium (g/l): $(NH_4)_2SO_4$ 10; KH_2PO_4 3; $MgSO_4 \cdot 7H_2O$ 0.5, and $CaCl_2$ 0.5 (Jecu 2000). The initial pH value of the medium was adjusted to 5.0 after sterilization at 121℃ for 30 min, and the water content of the substrate was 75%. The flasks were incubated at 30℃ for 96 h under static conditions.

Cellulase was extracted by suspending the solid state medium in 10-fold 0.1 M

citrate buffer of pH 4.8 for one hour and centrifuged to remove spores of the organism. The supernatants obtained were further concentrated by ammonium sulfate precipitation. The enzyme salt solutions were dialyzed for removing ammonium salt and were further concentrated with poly(ethylene glycol) (PEG) 20,000. Concentrated cellulase solutions with a filter paper activity (FPU) of 5.028IU/ml and a cellobiase activity (CBU) of 3.017IU/ml were used in the hydrolysis experiments.

Enzymatic Hydrolysis of Pretreated RS

Enzymatic saccharification was carried out at 50℃ for 48 h with the pH adjustted to 4.8 by addition of 0.1M citrate buffer solution. The enzyme loading was 50 FPU/g substrate, and the substrate concentration was 50g/l. The hydrolysate were centrifuged, and clear supernatants containing reducing sugars were used as sole carbon source for SSF.

Solid State Fermentation (SSF) for L-Lactic Acid Production

Experiments were conducted in 250 ml conical flasks containing 5g inert support (powered RS). The concentrated hydrolyzate, supplemented with $(NH_4)_2SO_4$ (0.5g/5 g support) and $MnSO_4 \cdot H_2O$ (0.1g/5 g support) and a growth promoter, yeast extract (0.5g/5 g support), was used as moistening agent to impregnate the support. 2.5g/5 g support of $CaCO_3$ was also added into it as a buffering agent to neutralize the lactic acid produced by microbial fermentation. The flasks were autoclaved at 121℃ for 15min. After cooling, the sterilized solid medium was inoculated with 2 ml cell suspension (20h old), mixed thoroughly, and incubated at 37℃ for 5 days.

Optimization of Process Parameters

Optimum physico-chemical and nutrient parameters required for maximum L(+)-lactic acid production by *Lactobacillus casei* under SSF were determined for initial moisture content of the support (45-90% w/w), initial reducing sugar concentration (2-4.5g/5g support), initial pH of the medium (pH 5 to 7), Tween 80 concentration (0-0.2g/5g support), incubation temperature (25-42℃), inoculum size (1.5-3.5ml), yeast extract concentration (0-2g/5g support), $MnSO_4 \cdot H_2O$ concentration (0-0.15g/5g support), and $CaCO_3$ concentration (1.5-3.5g/5g support). The protocol used for optimization of various process parameters was to evaluate the effect of an individual parameter and to incorporate it at the optimized level into the experiment before optimizing the next parameter. After optimizing various parameters, a time course experiment was conducted, incorporating all the optimized parameters.

Analytical Methods

Filter paper activity (FPU) and cellobiase activity (CBU) were assayed according to a standard cellulase activities analytical procedure recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose 1987). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1μmol of reducing sugars as glucose per minute per milliliter of crude enzyme extract under assay conditions. Cellulose, hemicellulose, and lignin were determined according to the procedures described elsewhere (Goering and VanSoest 1970). The total of reducing sugars was determined by the Dinitrosalicylic acid method (Miller 1959). L(+) lactic acid was extracted with 1M H_2SO_4 and was estimated by the colorimetric method of Kimberley and Taylor (1996). The moisture content of the RS was estimated according

to the method described by Adinarayana et al. (2003).

Three parallel samples were used in the analytic determination, and data are presented as the mean of three replicates. Relative standard deviations in all the assays were below 5%.

RESULTS AND DISCUSSION

For SSF, a certain moisture content of the substrate is a critical factor, because this parameter has influence on the growth and biosynthesis and secretion of different metabolites (Krishna and Chandrasekaran 1996). In the case of some fungi a wide moisture level, ranging from 20 to 70%, supports superior growth and metabolic activity, but in the case of bacteria, only a higher moisture content of the support can yield superior performance (Sabu et al. 2006). The effect of moisture level of the support on lactic acid production was investigated by moistening the support with a minimum amount of concentrated rice straw hydrolyzate-based medium in such a way that the initial reducing sugars were kept constant at 3g/5g support. The results are presented in Fig. 1.

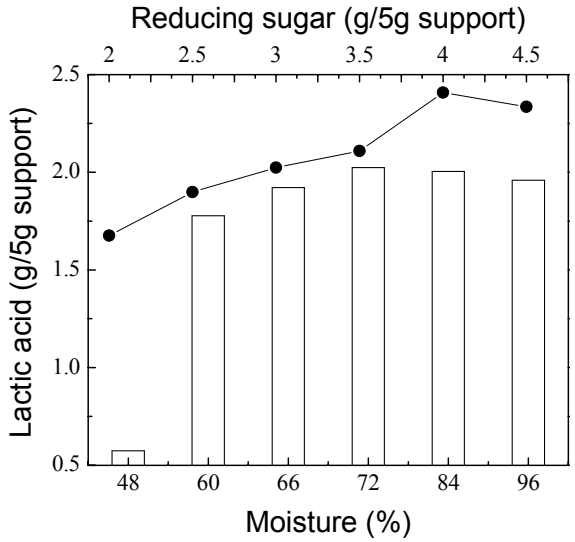

Fig. 1. Effects of moisture content of the support (□) and reducing sugar concentration (●) on lactic acid production using *Lactobacillus casei* under SSF.

As shown in Fig. 1, maximum lactic acid production was observed at an initial 72% moisture content of the support. A decrease in lactic acid production was observed when the moisture level was higher or lower than the optimum. This result was in good agreement with that of Rojan et al. (2005) and John et al. (2006), who also reported that an initial moisture content 72% was best for lactic acid production using *Lactobacillus casei* and *Lactobacillus delbrueckii*, respectively. Higher initial moisture content in SSF leads to suboptimal product formation due to reduced mass and oxygen transfer process and a decrease in porosity of the solid matrix. On the other hand, lower moisture content causes a reduction in solubility of nutrients of the substrate, a low

degree of swelling and a high water tension (Lonsane et al. 1985; Babitha et al. 2006). Initial reducing sugar is also a major factor that can significantly influence the lactic acid production. To optimize the concentrations of reducing sugars from the hydrolysate of pretreated RS, 5g of inert support was moistened with different volumes of concentrated hydrolyzate, varying in concentrations of reducing sugars; however, the moisture content of the support was maintained at 72%. As shown in Fig. 1, 4g initial reducing sugars per 5g of support was found to be optimum for conversion of sugars to lactic acid.

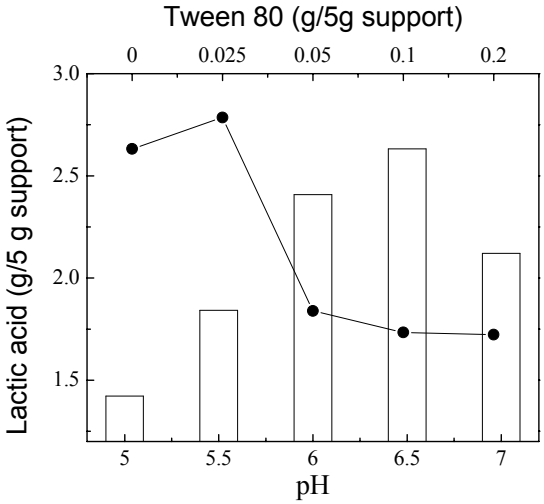

Fig. 2. Effects of pH of medium (□) and Tween 80 concentration (●) on lactic acid production using *Lactobacillus casei* under SSF.

The effect of initial pH of the medium on L(+) lactic acid production was tested using a pH range of 5 to 7 with a constant $CaCO_3$ addition of 2.5g per 5g of support. As shown in Fig. 2, the lactic acid yield increased with an increase in initial pH of the medium up to pH 6.5 (2.63g/5g support), and thereafter followed a marked decrease. In SSF, an optimum pH value 6.5 for *Lactobacillus casei* obtained in this investigation did not agree with those of Rojan et al. (2005) who found pH 5.5 to be the best for producing lactic acid. L(+) lactic acid production increased to 2.79g/5g support with addition of 0.025g/5g support of Tween 80 (Fig. 2). Tween 80, a non-ionic surfactant, has been reported to enhance the microbial ability to produce some enzymes (Reese and Manguire 1969; Goes and Sheppard 1999; Feng et al. 2006; Zeng et al. 2006). Tween 80 has also been proved beneficial in the cultivation and fermentation process of some *lactobacillus* (Duggan et al. 1959), in the case of most *Lactobacillus delbrueckii* strain, unsaturated fatty acids such as Tween 80 are essential growth factors (Partanen et al. 2001). Oh et al. (1995) reported that the growth of *Lactobacillus casei* was strongly affected by Tween 80. In the production of L(+) lactic acid from wheat bran by *Lactobacillus amylophilus* in SSF, both Naveena et al. (2005b) and Nagarjun et al. (2005) pointed out that Tween 80 was found to influence the lactic acid productivity. When more than 0.025g/5g support of Tween 80 was used, Tween 80 had an adverse effect on lactic acid production (Fig. 2). An explanation to this may be that Tween 80 as a surfactant could dissolve the lipid in the cell membrane, destroy the membrane

structure, and then cause the death of the cell.

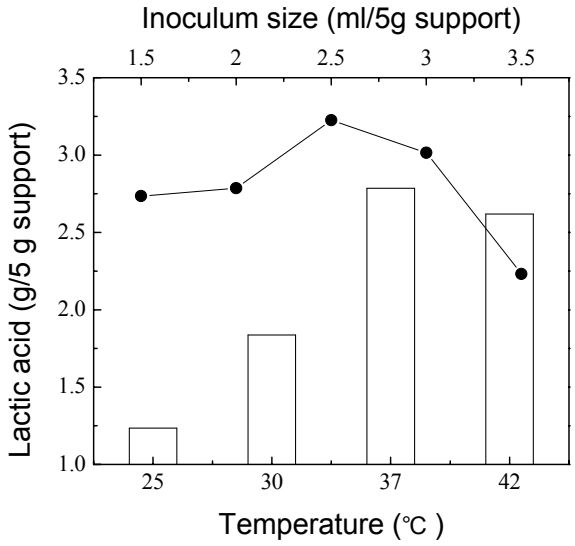

Fig. 3. Effect of temperature (□) and inoculum size (●) on lactic acid production using *Lactobacillus casei* under SSF.

Temperature influences the bioactivity of proteins, including enzymatic activity. Thus is can be expected that temperature could promote or inhibit production of a particular metabolite. Figure 3 shows that the maximum production of L(+) lactic acid was obtained at 37℃. Higher or lower temperatures than the optimum resulted in reduced lactic acid production, although *Lactobacillus casei* could produce lactic acid within the temperature range of 30℃ to 44℃ (Linko and Javanainen 1996). The size of the inoculum plays a significant role in the production of metabolites under SSF. In the present study, bacterial cells were used as inoculum, and different inoculum sizes were tested in order to enhance the lactic acid production. As shown in Fig. 3, an inoculum size of 2.5ml was found to be best for production of lactic acid (3.23g/5g support). Lower inoculum sizes resulted in insufficient biomass for product production, which causes decreased lactic acid production, whereas higher inoculum size produced too much biomass and caused the depletion of nutrients necessary for lactic acid production.

Yeast extract, a costly source of nitrogen, has been commonly used in laboratory scale lactic acid fermentation, because no other nitrogen sources were competitive to yeast extract in the production of lactic acid (Wee et al. 2006). In an economic analysis for lactic acid production, the cost of yeast extract accounted for over 30% total production cost (Mulligan et al. 1991). Therefore, it is necessary to minimize the yeast extract addition while still maintaining a high lactic acid production. Figure 4 shows that addition of 0.5g/5g support of yeast extract gave the maximum lactic acid production.

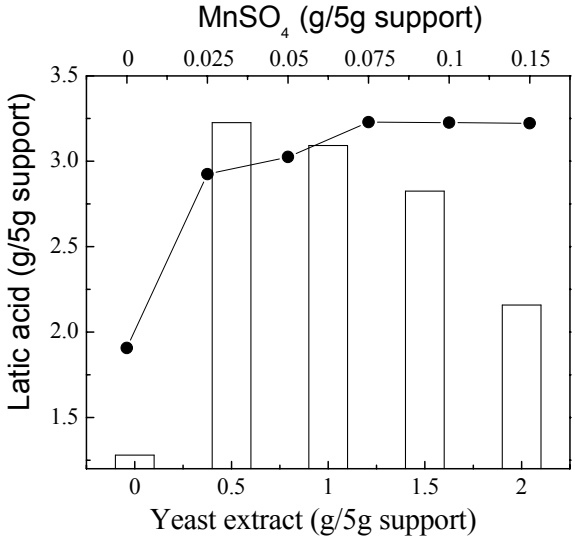

Fig. 4. Effects of yeast extract (□) and Mn^{2+} (●) on lactic acid production using *Lactobacillus casei* under SSF.

The effect of Mn^{2+} on lactic acid production was studied by supplementing the SSF medium with different amounts of $MnSO_4$. It was found that *Lactobacillus casei* produced the maximum amount of lactic acid with addition of 0.075g/5g support of Mn^{2+}. Archibald and Fridovich (1981b) reported that some lactobacilli strains required high concentrations of manganese for growth. It has been known that lactobacilli strains were deficient in catalase activity, and the presence of manganese could act as a scavenger of toxic oxygen species such as superoxide anion (O_2^-) or hydrogen peroxide in the microaerophilic condition (Archibald and Fridovich 1981a). In the case of *Lactobacillus casei*, it also had been reported that manganese could serve as a constituent of lactate dehydrogenase responsible for lactic acid production (Krischke et al. 1991).

Table 1. Effect of $CaCO_3$ Concentration on Lactic Ccid Production using *Lactobacillus casei* under SSF.

$CaCO_3$ (g/5 g support)	1.5	2	2.5	3	3.5
Lactic acid (g/5 g support)	2.495	2.880	3.225	3.468	2.901

$CaCO_3$ was used as a neutralizer of lactic acid produced by bacterial fermentation to prevent pH decrease of the medium. Table 1 reveals that $CaCO_3$ had a significant effect on the production of L(+)-lactic acid. Addition of 3g/5g support of $CaCO_3$ resulted in a 14% increase in L(+)-lactic acid production compared to addition of 2.5g/5g support of $CaCO_3$. Altaf et al. (2006) also reported that lactic acid production was significantly effected by $CaCO_3$ in single step fermentation of starch to L(+)-lactic acid by *Lactobacillus amylophilus* GV6 under SSF.

Finally, after optimizing various process parameters, a time course study was conducted to see the cumulative effect of various parameters. The experiment was carried out, incorporating all the optimized parameters, and samples were taken every 24h. Figure 5 shows that it took just 120h to get maximum production of L(+)-lactic

acid of 3.467g/5g support, and thereafter, lactic acid production leveled off.

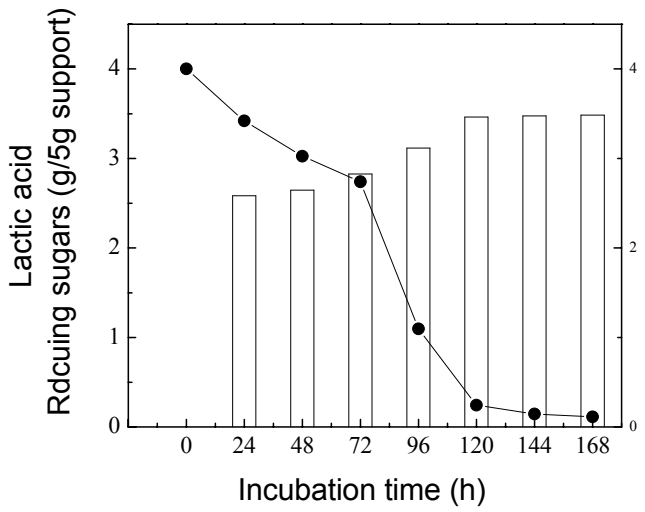

Fig. 5. Time course study of production of lactic acid (□) and consumption of reducing sugars (●).

CONCLUSIONS

SSF for the production of L-lactic acid by *Lactobacillus casei* were conducted using RS as the inert support. It was found that *Lactobacillus casei* was able to grow on RS and effectively utilize the sugar prepared from enzymatic hydrolysis of pretreated RS. Optimization results showed that the strain produced 3.467g/5g support of L-lactic acid after 5 days of fermentation. This process provides a cost-effective and eco-friendly technology to produce L-lactic acid. Our continuing research will be focused on replacing yeast extract with an inexpensive nitrogen source to further reduce the production cost of L-lactic acid.

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