Optically Pure L-Lactic Acid Production Directly from Leftover Bits and Pieces of Potato Starch Using an Amylolytic Pellet-Form Complex

Rhizopus Oryzae ASC081

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Abstract

Optically pure L-(+)-lactic acid fermentation directly from leftover bits and pieces of potato starch (LBPPS) using an amylolytic pellet-form complex Rhizopus oryzae ASC081 was studied. The complex ASC081, which have the capability for the simultaneously saccharification and production of pure L-(+)-lactic acid, was mutagenized and screened from two parent Rhizopus oryzaes As1 and As2. In the ASC081 fermentation, the variation in temperature, inoculum size, and pH obviously affected the amylolytic capacity, the L-(+)-lactic acid production and the fungal biomass formation. The addition of phytic acid and bitter salt (an alternate name: magnesium sulfate) significantly stimulated the L-(+)-lactic acid fermentation process, and enabled complete bioconversion within 90 h. A condition, LBPPS concentration 140 g/L, pH 5.2, temperature 32 °C, inoculum size 15%, phytic acid addition 0.05 g/L and bitter salt addition 0.5 g/L, was favourable for the optically pure L-(+)-lactic acid production, resulting in the optically pure L-(+)-lactic acid yield of 0.12 g / 1.0 g LBPPS.

Key Words: Amylolytic, Rhizopus Oryzae ASC081, Leftover Bits and Pieces of Potato Starch, Optically Pure L-Lactic Acid

1. Introduction

Lactic acid, or named as milk acid, is a chemical compound playing roles in several chemical and biochemical processes. Lactic acid has a chiral carbon atom and occurs naturally in two optical isomers. One is known as L-(+)-lactic acid or (S)-lactic acid and the other, its mirror image, is D-(−)-lactic acid or (R)-lactic acid. L-(+)-lactic acid is the biologically important isomer. Since elevated levels of the D-(−)-lactic acid are harmful to human beings, optically pure L-(+)-lactic acid is the preferred isomer in pharmaceutical and food industries [1–7]. The chemical structure of L-(+)-lactic acid and D-(−)-lactic acid are shown in Figure 1.

Figure 1. Chemical structures of L-(+)-lactic acid and D-(−)-lactic acid.
microorganism, substrate, metabolism control and growth conditions used.

Leftover bits and pieces of potato starch is an abundant byproduct in the potato processing industry. The main processes to produce potato starch are following: 1) the potatoes are washed and rasped into fine particles, 2) fruit juice and solid matter are separated into two streams, 3) fresh water is added to the stream of solid matter and pulp is separated from the starch by centrifugation 4) starch is refined in hydro cyclones and vacuum filters, 5) the concentrated slurry is dried in a warm air stream. The byproducts (waste products) mainly include potato juice and potato pulp. The potato pulp is a side product of washing the starch from the mash. The byproducts contain (approximate composition) 0.5\%/c45 1% of potato, 25\%/c45 35% of starch (not extracted because of economical reasons), 50\%/c45 60% protein, 2\%/c45 3% minerals, 1\%/c45 2% fat, 5\%/c45 7% nitrogen and other. In this study, leftover bits and pieces of potato starch (LBPPS) were used as an inexpensive fermentation medium. To achieve the production of optically pure L-(+)-lactic acid directly from LBPPS simultaneously without D-(-)-lactic acid produced, a complex \textit{Rhizopus oryzae} ASC081 (\textit{R. ASC081}) was breeded and screened by induced mutagenesis method. Direct fermentation process using the amylolytic pellet-form complex \textit{R. ASC081} was developed. The \textit{R. ASC081} was found to be an excellent fungus for the direct degradation of LBPPS. The culture pH, temperature, and an addition of phytic acid and minerals of medium were the main influential factors on the productivity of optically pure L-(+)-lactic acid.

2. Material and Methods

2.1 Bacterial Strain, Media and Instruments

\textit{R. ASC081}, a highly pure L-(+)-lactic acid producing strain bred by using \textit{Rhizopus oryzae} As1 and \textit{Rhizopus oryzae} As2 as parents, was used in the experiments. The strain was maintained on potato dextrose agar slants at 4 °C. For fermentation study, agar slant containing sporulated fungus was washed by sterile water to obtain spore suspension. 5 mL of spore suspension was transferred to a new growth medium (100 mL) in a 250 mL vial every 12 h. A 25 mL of this culture was then transferred to a 500 mL vial that contained 400 mL sterile growth medium. The inoculum was incubated at 32 °C for 8 h on an orbital shaker at 200 rpm before inoculation at 15 % (v/v) to the fermenter.

2.2 Inoculum Preparation

Spores were grown on a potato dextrose agar slants at 32 °C for 60 h. They were collected with an inoculating loop and suspended in distilled sterilized water. 5 mL of spore suspension was transferred to a new growth medium (100 mL) in a 250 mL vial every 12 h. A 25 mL of this culture was then transferred to a 500 mL vial that contained 400 mL sterile growth medium. The inoculum was incubated at 32 °C for 8 h on an orbital shaker at 200 rpm before inoculation at 15 % (v/v) to the fermenter.

2.3 Fermentation Conditions

Fermentations were performed in BIOF-2005 5-liter automatic fermenter with 3.5 liter working volume. The experiments were carried out at 32 °C, agitation speed 600 rpm, aeration rate 30 L/h, and the pH was maintained at 5.7 by automatic addition of neutralization agent. The samples were withdrawn at intervals for analysis.

2.4 Analytical Methods

The culture broth was first centrifuged, and then the resulting supernatant was used for analysis. The measurements of glucose, lactic acid, malate, fumarate, acetic and citric acid were carried out by high-performance liquid chromatography (HPLC) equipped with a PDA/UV detector at 210 nm. A Thermo Hypersil Gold C18 column (150 * 2.1 mm, 3 μm) was used with methanol and 0.1% phosphoric acid as a mobile phase at a flow rate of
0.2 ml/min, while the column temperature was maintained at 25 °C. Dry cell weight was determined by harvesting the culture samples, filtering and then washing the mycelia three times with distilled water, and drying at 95 °C until constant weight was achieved.

L-(+)-lactic acid was determined using an enzyme-UV method. In the presence of L-lactate dehydrogenase (L-LDH), L-(+)-lactic acid is oxidized to pyruvate by nicotinamide-adenine dinucleotide (NAD), L-Lactate + NADH = pyruvate + NADH. The equilibrium of this reaction lies on the side of lactate. The amount of NADH formed in the above reaction is stoichiometric to the amount of L-(+)-lactic acid. The increase in NADH is quantified by measuring its light absorbance at 340 nm.

3. Results and Discussion

3.1 Chemical Composition of LBPPS Samples

In order to determine the probability of producing L-(+)-lactic acid from LBPPS, the variation in chemical composition of LBPPS collected were first characterized. The compositions of samples are presented in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Starch (%)</th>
<th>Moisture (%)</th>
<th>Crude protein (%)</th>
<th>Crude fat (%)</th>
<th>Crude fibre (%)</th>
<th>Ash (%)</th>
<th>Other components (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>23.6</td>
<td>74.4</td>
<td>1.1</td>
<td>0.06</td>
<td>0.1</td>
<td>0.54</td>
<td>0.2</td>
</tr>
<tr>
<td>Sample 2</td>
<td>15.8</td>
<td>81.3</td>
<td>1.7</td>
<td>0.02</td>
<td>0.1</td>
<td>0.92</td>
<td>0.16</td>
</tr>
<tr>
<td>Sample 3</td>
<td>80.2</td>
<td>13.0</td>
<td>3.1</td>
<td>0.23</td>
<td>0.3</td>
<td>2.01</td>
<td>1.16</td>
</tr>
<tr>
<td>Sample 4</td>
<td>73.5</td>
<td>13.0</td>
<td>6.3</td>
<td>0.14</td>
<td>0.5</td>
<td>4.06</td>
<td>2.50</td>
</tr>
</tbody>
</table>

3.2 Effect of pH Control on Fermentation

In order to determine the impact of pH on the fermentation of L-(+)-lactic acid by R.ASC081, the pH was controlled at 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0. Without pH control, 2.7 g lactic acid was produced from 55 g starch after 120 h fermentation, and culture pH finally dropped to 3.0. With pH control at 4.0 to 7.0, production of lactic acid varied correspondingly (See Table 2). When the pH lower than 4.5, the productivity of L-(+)-lactic acid was restrained. When the pH higher than 6.0, the productivity of total lactic acid was raised; however, D-(-)-lactic acid was detected in the medium, and the yield of D-(-)-lactic acid increased with increasing pH up to pH at 7.5. Therefore, the pH for optically pure L-(+)-lactic acid production ranged from 4.5 to 6.0 was recommended. The results of L-(+)-lactic acid production and biomass increase under different pH value are summarized in Table 2. The fermentation conditions of the experiments are as follows: the fermentation medium had the following components (g/L) : LBPPS 320, (NH4)2SO4 6, KH2PO4 0.7, MgSO4 0.3, ZnSO4 0.2, phytic acid 0.06. The pH of medium lies between 5.7-5.8 after sterilizing. A sterilization time of 30 min at 121 °C is recommended. Inoculum size: 15 % (v/v). The experiments were carried out at 32 °C, agitation speed 600 rpm, aeration rate 30 L/h, and the pH was maintained at 4.8, 5.0, 5.2, 5.4, 5.6, 6.0 6.5 and 7.0, respectively, by automatic addition of neutralization agent.

The results also indicated that R.ASC081 had a high enzymatic capability for saccharification of LBPPS in the pH range of 4.8-7.0. The starch hydrolysis and lactic acid accumulation were influenced obviously by growth pH. When the pH was controlled at 5.2, the highest production of L-(+)-lactic acid was achieved after 120 h fermentation, and at the same time, no D-(-)-lactic acid was found in the medium. If the pH was controlled higher than 6.0, D-(-)-lactic acid was found in the medium after 80 h fermentation. Because in the culture controlled at pH ≥
6.0, the heterofermentation occurred, resulting in the accumulations of D-(-)-lactic acid and acetic acid. However, if pH was controlled lower than 4.5, higher Ca\(^{2+}\) was injected, and the higher concentration of Ca\(^{2+}\) was inhibitory to the organism, leading to lower production of L-(+)-lactic acid, consequently, a growth pH higher than 6.0 or lower than 4.5 was unfavourable for the production of L-(+)-lactic acid with high optical purity.

### 3.3 Effect of Inoculum Size on L-(+)-lactic Acid Fermentation

The influences of inoculated spore concentration on the L-(+)-lactic acid production and biomass increase were investigated. The results are shown in Figure 2. In the fermentation on 5 L fermenter, an inoculum size 15% (spore concentration of 1×10\(^7\) spores per micro liter) yield the highest L-(+)-lactic acid production (32.3 g) after 100 h fermentation. When inoculum size was lower than 10% (spore concentration < 1×10\(^4\) spores per micro liter), L-(+)-lactic acid yield decreased, due to inadequacy of enzymatic efficiency. When inoculum size was more than 20% (spore concentration more than 1×10\(^8\) spores per micro liter), irregularly shaped masses or pieces of spores were formed in production medium, while the L-(+)-lactic acid production was lower than that of inoculum size 10% after 60 h fermentation (see Figure 2A). The productivity of L-(+)-lactic acid was weakened perhaps due to the nutrient and dissolved oxygen limitation in the irregularly shaped masses or pieces. However, the biomass concentration increased correspondingly with the inoculum size (see Figure 2B). Therefore, with inoculum level of 5×10\(^7\) spores per micro liter was the optimum for L-(+)-lactic acid production and biomass formation.

### 3.4 Effect of Phytic Acid on L-(+)-lactic Acid Fermentation

The influence of adding phytic acid on L-(+)-lactic acid production and mycelial morphology by \textit{R. ASC081} was investigated. The results are shown in Table 3. In the fermentation culture, an addition of phytic acid (0.05 g/L), made for forming a great deal of mycelial pellets in fermentation medium during 40-80 h, yields the highest L-(+)-lactic acid production (31.0 g) after 90-h culture. In the L-(+)-lactic acid fermentation by \textit{R. ASC081}, the growth of mycelium, formation of mycelial pellet and accumulation of irregularly shaped masses or pieces were observed during the growth of \textit{R. ASC081}. The formation of mycelial pellet was crucial for optically pure L-(+)-lac-
tic acid production. Phytic acid can accelerate the formation of mycelial pellet of R.ASC081, and then stimulate the L-(+)-lactic acid production. The results are listed in Table 3. The fermentation conditions of the experiments are as follows: the fermentation medium had the following components (g/L): LBPPS 310, (NH4)2SO4 5, KH2PO4 0.6, MgSO4 0.6, ZnSO4 0.3. The pH of medium lies between 5.7-5.8 after sterilizing. A sterilization time of 30 min at 121°C is recommended. Inoculum size: 15% (v/v). The experiments were carried out at 32°C, agitation speed 600 rpm, aeration rate 30 L/h, and the pH was maintained at 5.2 by automatic addition of neutralization agent.

When adding phytic acid (0.05 g/L) at the beginning of fermentation, L-(+)-lactic acid production was increased by 1.4 times higher than without adding in 90 h fermentation. Nevertheless, that can be taken effect after 40 h. For the growth of R.ASC081, addition of phytic acid also promoted the increase of biomass, but the promotion (1.1 times higher than that without adding phytic acid) was less than that for L-(+)-lactic acid production. The promoting effect of phytic acid for the growth of R.ASC081 was also delayed. After 30 h, the promotion took effect obviously, and the fungal biomass formation reached 8.28 g after 90 h. If no phytic acid was added, lots of mycelia formation led to a long passing phase, and in a short duration, the mycelia formed irregularly shaped masses or pieces of spores. Therefore, addition of phytic acid was favorable not only for lactic acid production, but for fungal biomass formation, due to the enhanced amylolytic capacity and decreased limitation of nutrients and dissolved oxygen in the inner parts of spores. The formation of mycelial pellets during 40-50 h by adding 0.05-gram phytic acid per micro-liter substrate was recommended condition for L-(+)-lactic acid production.

3.5 Effect of Mg2+

By comparing the fermentation with or without Mg2+ in the medium, it was found that Mg2+ would not directly increase the L-(+)-lactic acid production. However, it is a valuable factor to shorten the fermentation period of R.ASC081. The L-(+)-lactic acid production rate after 60 h was decreased to 0.10 g/L·h in medium without Mg2+ addition. But in Mg2+ supplemented medium, the initial production rate can reach to 0.18 g/L·h. By addition of Mg2+ in a range of 0.1–5.0 g/L, the L-(+)-lactic acid fermentation period was shortened. It suggested the ability of Mg2+ to act as an inducer of Mg-amylolysis activity agent. The above analysis of LBPPS also indicated that Mg2+ was a deficient element. But Mg2+ was an essential fermentation factor for R.ASC081. Therefore, when Mg2+

Table 3. Influence of adding phytic acid on L-(+)-lactic acid production and fungal biomass formation

<table>
<thead>
<tr>
<th>Fermentation time (h)</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>80</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L-Lactic acid production (g)</strong></td>
<td>Adding phytic acid</td>
<td>0.60</td>
<td>1.80</td>
<td>3.20</td>
<td>4.10</td>
<td>8.60</td>
<td>18.80</td>
<td>30.0</td>
</tr>
<tr>
<td><strong>Without phytic acid</strong></td>
<td>0.68</td>
<td>1.78</td>
<td>2.98</td>
<td>4.0</td>
<td>6.60</td>
<td>10.66</td>
<td>16.50</td>
<td>16.50</td>
</tr>
<tr>
<td><strong>Fungal biomass formation (g)</strong></td>
<td>Adding phytic acid</td>
<td>0.08</td>
<td>0.12</td>
<td>0.66</td>
<td>1.65</td>
<td>3.21</td>
<td>4.05</td>
<td>7.55</td>
</tr>
<tr>
<td><strong>Without phytic acid</strong></td>
<td>0.09</td>
<td>0.11</td>
<td>0.68</td>
<td>1.56</td>
<td>2.88</td>
<td>3.15</td>
<td>6.55</td>
<td>7.02</td>
</tr>
</tbody>
</table>

![Figure 2. Effect of inoculum size on L(+)-lactic acid fermentation and fungal biomass formation.](image-url)
was added to fermentation medium at 0.5 g/L, L-(+)-lactic acid fermentation was completed within 90 h cultivation. Therefore, the addition of 0.5 gram per liter was the recommended in view of whole fermentation time.

3.6 Effect of Temperature

The impact of the cultivation temperature on the L-(+)-lactic acid fermentation was investigated. When the temperature was below 28 °C or above 35 °C, the L-(+)-lactic acid production and biomass growth were obviously varied by the temperature. The fermentation performed by R.ASC081 at 28 °C and 35 °C appeared to be relatively less sensitive than at 30–33 °C. The peak L-(+)-lactic acid production was found at 32 °C. Consequently, 32 °C was regarded as optimal condition in view of L-(+)-lactic acid production.

4. Conclusions

Optically pure L-(+)-lactic acid has been successfully produced directly from LBPPS using R.ASC081 fermentation. The formation of mycelial pellets and the effective expression of amylase become the key factors for direct production of optically pure L-(+)-lactic acid. For simultaneous saccharification and selective bioconversion of LBPPS to optically pure L-(+)-lactic acid, the optimum pH was controlled at 5.2. The magnesium would not directly increase the L-(+)-lactic acid production, however, it is a valuable factor to shorten the fermentation period of R.ASC081. Phytic acid, as a new growth factor for Rhizopus oryzae, can also stimulated L-(+)-lactic acid production and biomass growth.

References


