**Note**

Optimization of L-lactic Acid Production from Banana Peel by Multiple Parallel Fermentation with *Bacillus licheniformis* and *Aspergillus awamori*

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This study investigated the optimization of L-lactic acid production from banana peel as an unutilized biomass, by multiple parallel fermentation (MPF) with *Bacillus licheniformis* and *Aspergillus awamori*. To optimize L-lactic acid production, the factors studied consisted of banana peel, potassium dihydrogen phosphate, Tween 80, magnesium sulfate heptahydrate, sodium chloride, yeast extract, and ammonium sulfate. Optimization of these component factors was performed using the Taguchi method with an L8 orthogonal array. The optimal concentration for MPF using biomass substrate was as follows: L-lactic acid production was 28.01 g/L in the medium containing 10% banana peel, 0.5% potassium dihydrogen phosphate, 0.05% Tween 80, 0.1% magnesium sulfate heptahydrate, 0.1% sodium chloride, 1.5% yeast extract, and 0.2% ammonium sulfate. The result indicates that MPF with *B. licheniformis* and *A. awamori* could constitute part of a potential industrial application for banana peel as a currently unutilized biomass for L-lactic acid production.

Keywords: L-lactic acid, banana peel, multiple parallel fermentation, *Bacillus licheniformis*, *Aspergillus awamori*

**Introduction**

Lactic acid, also known as milk acid, is a chemical compound that plays a role in several biochemical processes (Richard and Domasius, 2010). It has been widely applied to food, pharmaceutical, and chemical industries (Gerngross and Slater, 2000). The efficient fermentation of L-lactic acid from unutilized biomass has been investigated (Hofvendahl and Hagerdal, 2000). The efficient fermentation of L-lactic acid from unutilized biomass has been investigated (Hofvendahl and Hagerdal, 2000).

Agricultural residue such as by-products of banana, corn, potato, rice, wheat and others containing substantial amounts of starch has been utilized as raw materials for lactic acid production. Bananas are a popular fruit consumed worldwide, with a yearly production of hundreds of million tons in 2011, and originate from the tropical region of South Asia (Anhwange et al., 2009). The organic waste arising from banana peel approaches 30 – 40% of the gross weight and indicates that banana peel is not effectively used after the fruits are consumed. Cellulose, hemicellulose, starch, and pectin are contained as polysaccharides in banana peel (Essien et al., 2005), and can be used as a supplementary source of the production of industrial enzymes (Oberoi et al., 2011). Banana peel is also an agricultural residue that provides renewable energy resources (Rehman et al., 2014; Jeevan et al., 2011).

Lactic acid production is not typically a highly complex procedure. However, for unutilized biomass to be used as a carbon source, it must be saccharified and/or pretreated because most lactic acid bacteria cannot utilize it directly (Luo et al., 1997; Ghowdaman and Ponnusami, 2015). Thus, the types of biomass and hydrolytic enzymes as well as lactic acid producing microorganisms can affect the efficiency of lactic acid production (Sasaki et al., 2012).
consideration when selecting the L-lactic acid producing microorganism. The use of thermotolerant Bacillus licheniformis has been reported for L-lactic acid production from kitchen waste under open condition (Sakai and Yamanami, 2006) and B. licheniformis fermented glucose for lactic acid production in a mineral salts medium (Wang et al., 2011). The use of thermotolerant microorganisms such as L-Lactic acid fermentation by thermotolerant microorganisms such as B. licheniformis for L-lactic acid fermentation can reduce the cooling water requirement of the fermentation process, particularly in tropical countries like Indonesia, where the average temperature is usually high throughout the year. We have focused on banana peel as an unutilized biomass substrate for L-lactic acid fermentation. There have been no reports on L-lactic acid production from banana peel using B. licheniformis. Prior to this study, we isolated eight strains of B. licheniformis on medium with xylan as the sole carbon source at 50°C. The utility of these isolates as L-lactic acid producers was examined and one strain (YN15) was selected for multiple parallel fermentation (MPF).

The present study investigated the optimization of L-lactic acid production from banana peel as an unutilized biomass substrate by MPF with B. licheniformis YN15 and Aspergillus awamori NBRC 4388, a koji mold used in Japanese spirit brewing.

Materials and Methods

Microorganisms The lactic acid producing bacteria used in this study were B. licheniformis strains (YN5, YN8, YN9, YN10, YN11-1, YN13-1, YN13-3, and YN15) isolated from soil samples collected in Kusatsu, Shiga in 2013, Lactococcus lactis subsp. lactis NK2 isolated from pickled turnip in 2015, and Lactobacillus plantarum ZZU299 isolated from cabbage in 2015. For degradation of biomass substrates in MPF, A. awamori NBRC 4388 purchased from NITE Biological Resource Center was used.

Cultivation of fungi The growth medium was composed of 1% banana peel, 0.25% potassium dihydrogen phosphate, 0.1% yeast extract, 0.035% magnesium sulfate heptahydrate, 0.035% urea, 0.1% Tween 80, 0.005% ferrous sulfate heptahydrate, 0.001% manganese (II) sulfate, and 0.1% sodium chloride. The initial pH was adjusted to 5.6. A total of 5 mL of inoculum and 3% of manganese (II) sulfate, and 0.1% sodium chloride. The initial pH was adjusted to 5.6. A total of 5 mL of inoculum and 3% of calcium carbonate was transferred to a 100-mL Erlenmeyer flask containing 100 mL of each medium. Cultivation was carried out in flask cultures at 30 – 60°C with a rotary shaker at 100 rpm.

Pretreatment of fermentation substrates Banana peel (Musa acuminata balbisiana Colla) was collected from household waste. Before the banana peel could be utilized, it was cut into small 5- to 8-cm pieces using a stainless steel knife. The banana peel was then soaked in 1% sodium thiosulfate solution for 5 h at 30°C to inhibit the oxidation processes, thus preventing browning and obtaining a better banana peel texture for use in lactic acid production (unpublished data), followed by a drying process in a hot air oven. The small dried pieces of banana peel were collected by sieving with a < 1 mm mesh net. In a preliminary experiment, the effect of pretreatment of biomass substrates on lactic acid production was examined. Lactic acid production using banana peel with pretreatment was higher than that using banana peel without pretreatment (unpublished data). The result indicated that pretreatment of banana peel in multiple parallel fermentation (MPF) is strongly recommended.

Multiple parallel fermentation MPF involves a combination of degradation of the biomass substrate; (banana peel) by A. awamori with L-lactic acid fermentation by B. licheniformis YN15. The basic liquid medium for MPF included 3% banana peel, 0.25% potassium dihydrogen phosphate, 0.5% yeast extract, 0.05% magnesium sulfate heptahydrate, 0.05% urea, 0.1% Tween 80, 0.005% ferrous sulfate heptahydrate, 0.001% manganese(II) sulfate, 0.2% ammonium sulfate and 0.1% sodium chloride. The initial pH was adjusted to 5.6. A total of 10 mL of inoculum (A. awamori culture) and 1% of calcium carbonate was transferred to a 300-mL Erlenmeyer flask containing 200 mL of medium at 30°C in an incubator with shaking at 120 rpm. MPF was performed at 37°C in an incubator with gentle shaking at 100 rpm after inoculation (10 mL) of B. licheniformis YN15 to allow for homogeneous mixing of the medium and inoculum.

Analytical methods Reducing sugar was measured by the Somogyi-Nelson method (Somogyi, 1952). The protein concentration was determined using Lowry’s method with bovine serum albumin as the standard (Lowry et al., 1951) or by monitoring the optical density at 280 nm. The activities of xylanase, pectinase, amyrase, β-glucosidase, and endoglucanases in the culture medium containing banana peel as the biomass substrate were determined as below.

Crude enzyme preparation (100 µL) was incubated in the presence of substrate (1% w/v) (beechwood xylan for xylanase, citrus pectin for pectinase, starch for amyrase, salicin for β-glucosidase, carboxy methyl cellulose for endoglucanase) in
Lactic Acid Production from Banana Peel by MPF

The amount of reducing sugars was determined by the Somogyi-Nelson method against standard curves of xylose, galacturonic acid, and glucose. The amount of enzyme that releases one µmole of reducing sugar per min under standard assay conditions was defined as one international unit (U) of enzyme.

**L-Lactic acid** was determined according to the Boehringer Mannheim / R-Biopharm method with minor modification. The reaction solution consisted of 222 mM glycylglycine buffer (buffer pH 10.0), 100 mM L-glutamic acid, 52.8 mM NAD’, 13.66 U of glutamate-pyruvate transaminase, and 43.48 U of L-lactate dehydrogenase. The absorbance increase of NADH at 340 nm was determined. Measurement of D-lactic acid was also carried out using 6.79 U of D-lactate dehydrogenase instead of L-lactate dehydrogenase. The absorbance increase of NADH at 340 nm was determined. Measurement of D-lactic acid was also carried out using 6.79 U of D-lactate dehydrogenase instead of L-lactate dehydrogenase.

**Taguchi experimental design** The optimal MPF conditions for production of L-lactic acid were described using the Taguchi method (Ravella et al., 2008; Taguchi, 1987), in which variables or factors were arranged in an orthogonal array (OA). All calculations and analyses were performed using Qualitek-4 software for automatic design and analysis of Taguchi experiments. The L8 OA layout and factor levels are presented in Tables 1 and 2, respectively. The signal-to-noise ratio (S/N Ratio) shows the extent of all factor effects. This approach introduces the S/N ratio to examine the influence of the noise factor on variation. For the S/N ratio, characteristic types defined by LTB (Larger The Better) means the highest value is the best of quality (Ravella et al., 2008).

**Reagents** Beechwood xylan, salicin, and carboxy methyl cellulose were purchased from Nacalai Tesque (Kyoto, Japan). Glutamate-pyruvate transaminase, L-lactate dehydrogenase and D-lactate dehydrogenase were from Wako Pure Chemical Ind. (Osaka, Japan). Other reagents were chemically pure grade commercial products.

**Results and Discussion**

**Screening of microorganisms** The liquid fermentation abilities of the microorganisms used in this study were assessed for the bacteria that produced the highest amounts of L-lactic acid. Among the B. licheniformis strains examined, B. licheniformis YN15 produced the highest amounts of L-lactic acid by simple fermentation using MBL medium for 48 h at 37°C (unpublished data). Production of L-lactic acid by B. licheniformis YN15 was compared with those by typical L-lactic acid producing bacteria, L. lactis and L. plantarum, production was lower compared to L. lactis, but somewhat higher compared to L. plantarum (unpublished data). To determine the temperature that is most suitable for production of L-lactic acid, B. licheniformis YN15 was cultured under various temperature and time cultivation condition using simple fermentation. B. licheniformis YN15 produced the highest L-lactic acid for 48 h at 37°C (unpublished data). This temperature and time cultivation condition was adopted for MPF, since L-lactic acid production by B. licheniformis YN15 for 72 – 96 h at 40 – 50°C was almost the same as that for 48 h at 37°C. Saccharification of biomass substrates by the fungi Aspergillus niger NBRC 4414, A. awamori NBRC 4388, Rhizopus oryzae NBRC 4706, R. microsporus NBRC 3200, A. oryzae NBRC 100959, and A. sojae NBRC 33802 was evaluated in a preliminary experiment (unpublished data).

**A. awamori** NBRC 4388 was used as the fungus for effective hydrolysis of banana peel as the biomass substrate, in L-lactic acid production by MPF. A marginal increase in the pH of the media during the hydrolytic process of banana peel by A. awamori NBRC 4388 could change the MPF condition to pH 6.3 for optimal growth of B. licheniformis (pH optimum 6.5 – 7.0).

On the 2nd day of banana peel hydrolysis, the highest reducing...
sugar level was obtained in using 3% banana peel (unpublished data). After a 36-h incubation of *A. awamori* with banana peel, thin layer chromatography (TLC) revealed that glucose and xylose were detectable as visible spots (data not shown). However, as the method used crude products, the spots detected by TLC analysis were unclear and also appeared as other scattered spots. Based on the results, MPF was conducted by inoculating *B. licheniformis* YN15 to the culture medium of *A. awamori* after 36-h hydrolysis of banana peel.

**L-Lactic acid production from banana peel by MPF** The carbon source noticeably affected L-lactic acid production in MPF. The effect, variance, and contribution of each selected factor were assessed by analysis of variance (ANOVA) (Table 3). The calculated F-ratio suggested that all parameters were significant within 95% confidence intervals. The concentration of banana peel was paramount for MPF and optimal L-lactic acid production. The contribution of banana peel to L-lactic acid production was 55.84%, and was supported by other factors such as yeast extract (12.34%), Tween 80 (11.41%), ammonium sulfate (11.37%), magnesium sulfate heptahydrate (8.80%), potassium dihydrogen phosphate (0.17%), and sodium chloride (0.03%). The result indicates that the carbon source concentration is the most important factor in determining optimal L-lactic acid production.

Thus, utilization of banana peel as a carbon source has great potential for L-lactic acid production. Notably, each factor contributes differently to L-lactic acid production; for example, sodium chloride showed minor contribution to production.

The optimization process revealed that L-lactic acid production (28.01 g/L) from banana peel as biomass substrate by MPF for 48 h at 37°C was most efficient with 10% banana peel, 0.5% potassium dihydrogen phosphate, 0.05% Tween 80, 0.1% magnesium sulfate heptahydrate, 0.1% sodium chloride, 1.5% yeast extract, and 0.2% ammonium sulfate after saccharification of biomass substrates by *A. awamori* for 36 h (Table 4). D-Lactic acid was mostly undetected (below 0.01% of L-lactic acid); the optical purity of L-lactic acid in MPF using banana peel was calculated to be 99.99%.

**The activities of main polysaccharide-lytic enzymes in MPF using banana peel substrate** Figure 1 shows the time course of polysaccharide-lytic enzyme activities and L-lactic acid production in MPF. The enzymes produced are thought to be involved in the degradation of constituent polysaccharides of banana peel. In MPF, the carbon sources utilized by *B. licheniformis* for L-lactic acid fermentation are made available from banana peel by these enzymes, which are produced by *A. awamori*. Thus, this enzymatic treatment is thought to affect the production of L-lactic acid by *B. licheniformis*. During hydrolytic processing with banana peel as

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**Table 3. Analysis of variance (ANOVA)**

<table>
<thead>
<tr>
<th>Factor</th>
<th>DOF (f)</th>
<th>Sum of sqrs. (S)</th>
<th>Variance (V)</th>
<th>F-Ratio (F)</th>
<th>Pure Sum (S’)</th>
<th>Percent P (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banana peel</td>
<td>1</td>
<td>546.699</td>
<td>546.699</td>
<td>26584.34</td>
<td>546.678</td>
<td>55.839</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1</td>
<td>1.646</td>
<td>1.646</td>
<td>80.048</td>
<td>1.625</td>
<td>0.166</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>1</td>
<td>0.333</td>
<td>0.333</td>
<td>16.238</td>
<td>0.313</td>
<td>0.032</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1</td>
<td>111.706</td>
<td>111.706</td>
<td>5431.948</td>
<td>111.685</td>
<td>11.408</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1</td>
<td>120.799</td>
<td>120.799</td>
<td>5874.097</td>
<td>120.778</td>
<td>12.336</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>1</td>
<td>86.154</td>
<td>86.154</td>
<td>4189.408</td>
<td>86.133</td>
<td>8.797</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>1</td>
<td>111.344</td>
<td>111.344</td>
<td>5414.338</td>
<td>111.323</td>
<td>11.371</td>
</tr>
<tr>
<td>Other/Error</td>
<td>16</td>
<td>0.328</td>
<td>0.02</td>
<td>0.051</td>
<td>0.051</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>979.012</td>
<td></td>
<td></td>
<td>979.012</td>
<td>100%</td>
</tr>
</tbody>
</table>

**Table 4. Optimal condition and performance, in validation of L-lactic acid production using banana peel as carbon source**

<table>
<thead>
<tr>
<th>Factor (%)</th>
<th>Level description</th>
<th>Level</th>
<th>Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banana peel</td>
<td>10</td>
<td>2</td>
<td>4.772</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>0.5</td>
<td>1</td>
<td>0.261</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.1</td>
<td>2</td>
<td>0.117</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.05</td>
<td>1</td>
<td>2.157</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5</td>
<td>2</td>
<td>2.243</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>0.1</td>
<td>2</td>
<td>1.894</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>0.2</td>
<td>1</td>
<td>2.153</td>
</tr>
<tr>
<td>Total contribution from all factors</td>
<td></td>
<td></td>
<td>13.597</td>
</tr>
<tr>
<td>Current grand average of performance</td>
<td></td>
<td></td>
<td>13.758</td>
</tr>
<tr>
<td>Expected result at optimum condition</td>
<td></td>
<td></td>
<td>27.355</td>
</tr>
</tbody>
</table>

Validation result: 28.011 (g/L)

All calculations and analysis were performed using Qualitek-4 software for automatic design.
the carbon source, β-glucosidase showed the highest enzyme activity at 36 h, followed by amylase, xylanase, endoglucanase, and then pectinase. β-Glucosidase, endoglucanase, and amylase activity decreased on the first day after inoculation with B. licheniformis, whereas xylanase activity increased and then decreased on the following day. B. licheniformis YN15 produced the highest L-lactic acid for 2 d (Fig. 1). At 3 d, α-amylase activity re-increased. In this case, it is proposed that α-amylase is produced by B. licheniformis. It has been reported that B. licheniformis can produce high levels of α-amylase activity using some of the substrate by submerged fermentation (Chandran et al., 2011).

It is known that B. licheniformis produces polysaccharide-lytic enzymes such as those described above (Seo et al., 2014). In a preliminary experiment, L-lactic acid production only using B. licheniformis YN15 was investigated. However minimal L-lactic acid was produced without saccharification of banana peel by A. awamori. (unpublished data). Therefore, we concluded that MPF with both B. licheniformis and A. awamori was necessary for the effective production of L-lactic acid. Banana peel is a potential carbon source for the production of β-glucosidase, amylase and xylanase from A. awamori NBRC 4388.

Table 5 shows examples of L-lactic acid production from unutilized biomass using L-lactic acid producing microorganisms. L-Lactic acid production from banana peel by MPF with B. licheniformis and A. awamori was compared with those of Acromonium cellulose and Rhizopus sp. using corn cob in an SSF process, and Lactobacillus rhamnosus CECT-288 using apple pomace in a batch fermentation process. Even though L-lactic acid production from banana peel by MPF show room for improvement, our study indicated that banana peel biomass has high potential for L-lactic acid production using B. licheniformis and A. awamori in combination.

As described in Table 5, sugar cane bagasse hemicellulose, hydrolyzed by dilute sulfuric acid was used for high lactic acid production (Patel et al., 2004). Acid pretreatment of biomass is not expensive because acids such as sulfuric acid or hydrochloric acid employed are low cost. However, the process is carried out at high temperatures and requires high-energy input, which is costly. Because acid pretreatment at high temperatures can easily result in corrosive conditions, the process requires specialized reaction vessels that are corrosion resistant. In addition, acid treatment generates inhibitors that must be removed, and as a result, downstream processing is costly (Chaturvedi and Verma, 2013). In our L-lactic acid fermentation experiment, a simple pretreatment of banana peel was used without the need for strong acids. Limiting the use of strong acids is one way to reduce their corrosive effects, while the recycling of acids could lower the cost of pretreatment. To the best of our knowledge, no studies on L-lactic acid production using banana peel with simple pretreatment by MPF (using A. awamori and B. licheniformis) has been reported to date. We believe this eco-friendly work has valuable application for the utilization of such biomass substrates for L-lactic acid production.

L-Lactic acid production by B. licheniformis YN15 at 40 or 50°C was almost the same level as that at 37°C (unpublished data), indicating that L-lactic acid production using B. licheniformis YN15 could be possible under non-controlled temperatures below 50°C. In this study, L-lactic acid production using banana peel by MPF was performed at 37°C. L-Lactic acid fermentation from unutilized biomass, such as banana peel in tropical countries, by MPF using B. licheniformis is attractive from an economic
viewpoint.

Accurate calculation of the costs of L-lactic acid production by MPF using banana peel is difficult, because of fluctuations in various factors such as the countries and regions in which the production is implemented, labor costs, and so on. However, it can be presumed that the cost of L-lactic acid production by MPF using banana peel would not fundamentally differ from L-lactic acid production using other raw materials such as corn cob and wood hydrolysate, aside from the costs for procurement of banana peel and its pretreatment. On the other hand, sufficient access to banana peel as a raw material could be achieved by collaboration with banana manufacturing companies, which consume banana as a raw material for producing banana chips, baked goods etc. For example, Indonesia is the sixth largest banana producer in the world and is home to many confectionary companies. Therefore, countries and regions producing banana products, such as Indonesia, would be appropriate for establishing a L-lactic acid production system using banana peel.

### Conclusion

The Taguchi method using an L8 orthogonal array enabled us to analyze the influence of several factors and their interactions on L-lactic acid production from banana peel biomass substrate by MPF with *B. licheniformis* and *A. awamori*. Our data revealed that optimal L-lactic acid production by MPF from banana peel (28.01 g/L, optical purity 99.99%) was achieved using 10% banana peel, 0.5% potassium dihydrogen phosphate, 0.05% Tween 80, 0.1% magnesium sulfate heptahydrate, 0.1% sodium chloride, 1.5% yeast extract, and 0.2% ammonium sulfate. This result indicated that application of banana peel as an unutilized biomass substrate to MPF has high potential for L-lactic acid production.

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### Table 5. Comparison of L-lactic acid production from biomass by lactic acid producing bacteria

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Organisms</th>
<th>Fermentation process</th>
<th>L-Lactic acid (g/L)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corncob molasses</td>
<td><em>Bacillus sp.</em></td>
<td>Fed batch</td>
<td>74.7</td>
<td>Wang L et al., 2000</td>
</tr>
<tr>
<td>Corncob</td>
<td><em>Acremonium cellulose and Rhizopus sp.</em></td>
<td>SSF</td>
<td>24.0</td>
<td>Miura et al., 2004</td>
</tr>
<tr>
<td>Sugarcane bagasse</td>
<td><em>Bacillus sp.</em></td>
<td>Fed batch</td>
<td>55.5</td>
<td>Patel et al., 2004</td>
</tr>
<tr>
<td>Wood hydrolyzate</td>
<td><em>Enterococcus faecalis</em> RKY1</td>
<td>Batch</td>
<td>93.0</td>
<td>Wee et al., 2004</td>
</tr>
<tr>
<td>Apple pomace</td>
<td><em>Lactobacillus rhamnosus</em> CECT-288</td>
<td>Batch</td>
<td>32.5</td>
<td>Gullon et al., 2008</td>
</tr>
<tr>
<td>Banana peel</td>
<td><em>Aspergillus awamori</em> and <em>Bacillus licheniformis</em></td>
<td>MPF</td>
<td>28.0</td>
<td>Present study</td>
</tr>
</tbody>
</table>

SSF : Simultaneous saccharification and fermentation
MPF : Multiple parallel fermentation

### References


URL Cited

i) http://www.nutek-us.com/wp-q4w.html (Nov. 4, 2014)