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Statistical optimization of simultaneous saccharification and L(+)-lactic acid fermentation from cassava bagasse using mixed culture of lactobacilli by response surface methodology

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Abstract

Optimization of five process parameters (concentrations of cassava bagasse, enzyme, yeast extract, NH_4Cl and inoculum) was attempted using a Box-Behnken design for the optimal production of L(+)-lactic acid by a mixed culture of *Lactobacillus casei* and *Lactobacillus delbrueckii* by simultaneous saccharification and fermentation. Maximum lactic acid yield of 81 g/L was obtained when 15% (w/v) cassava bagasse treated with 12.5 mL/L enzyme mixture was supplemented with 7.5 g/L yeast extract and 3 g/L NH_4Cl and inoculated with 3×10^{10} CFU/L of lactobacilli and incubated for 60 h at 37 °C as static culture.

Keywords: Cassava bagasse; L(+)-Lactic acid; Lactobacillus; Response surface optimization; Saccharification; Fermentation

1. Introduction

Lactic acid is a versatile organic acid used as acidulant, flavor, pickling agent and preservative in food, pharmaceutical, leather and textile industries, for the production of base chemicals like acetaldehyde, acrylic acid, propionic acid, etc., and for polymerization to biodegradable polylactic acid (PLA). Lactic acid exists as two optical isomers, D- and L-lactic acid. Both isomeric forms of lactic acid can be polymerized and polymers with different properties can be produced depending on the composition. Of the 80,000 tonnes of lactic acid produced worldwide every year, about 90% are made by fermentation employing lactic acid bacteria and the rest is produced synthetically by the hydrolysis of lactonitrile [1]. Fermentative production has the advantage that an optically pure product can be obtained by choosing a strain of lactic acid bacteria producing only one of the isomers, whereas synthetic production always results in a racemic mixture of lactic acid. It is also possible to use renewable resources as substrates, such as starch and cellulose in fermentative production [2,3]. Cellulose, hemicellulose and starch are the most abundant compounds in the world, and when hydrolyzed to fermentable sugars they can be utilized by a number of microorganisms. Generally, lactic acid is produced using refined sugars as carbon source. The main obstacle in the production of lactic acid in large scale is the cost of raw material. The use of starchy material in the place of refined sugars reduces the cost of production. Cassava (*Manihot esculenta* Crantz) ranks the fourth among the staple food crops in the world and is consumed by more than 800 million people [4]. The waste, cassava bagasse obtained from the cassava tubers in the starch industries can be used as the carbon source because of its high starch content (more than 50%). Fermentative lactic acid production from renewable resources comprises the following steps: pretreatment of substrate including hydrolysis to sugars, fermentation of sugars to lactic acid or simultaneous saccharification and fermentation, separation of bacteria and solid particles from the broth, and purification of lactic acid. The negative impact of large concentration of sugar can be avoided by simultaneous saccharification and fermentation where the sugar formed is utilized as soon as it is formed [5,6].

Mixed culture or co-culture systems have been recognized to be effective for certain fermentations. Mixed cultures of lactic acid bacteria are currently used in the dairy industry

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for manufacturing cheeses and fermented milks [7,8]. The existence of symbiotic relationship among various bacteria has been clearly demonstrated. The differences between single *Lactobacillus* cultures and mixed-type lactobacilli cultures, by comparing their fermentative abilities, were investigated using industrial media (glucose and corn steep liquor) [9] and molasses [10]. In the current study, optimization of process parameters for the production of lactic acid by a mixed culture was carried out using a response surface Box-Behnken design.

2. Materials and methods

2.1. Microorganisms and inoculum preparation

L-Lactic acid producing cultures namely *Lactobacillus casei* NCIMB 3254, National Collection of Industrial and Marine Bacteria Ltd. Aberdeen, Scotland and *Lactobacillus delbrueckii* NCIM 2025, National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India were used in the present study. The cultures were maintained in separate stabs in MRS agar medium and were subcultured every 2 weeks. Inoculum having a cell density of 10^9 CFU/mL was obtained by growing the culture overnight in MRS broth at 37 °C. For mixed culture study, both the strains were mixed at 1:1 ratio. The ratio of the strains was same in all the experiments and it was maintained on the basis of the optical density (at 620 nm) of each inoculum used.

2.2. Enzymes

Commercial amylases, α-amylase (5000 IU/mL) and glucoamylase (2000 IU/mL) (Rashesh and Co., Mumbai, India) were used for the hydrolysis of cassava bagasse starch.

2.3. Fermentation medium

Cassava bagasse was collected from Varalakshmi Starch Industries, Salem, Tamil Nadu. Unless otherwise mentioned, the medium consisted of cassava bagasse in distilled water enriched with yeast extract and NH_4Cl. $CaCO_3$ (60%, w/w of starch) was added for buffering. The medium was autoclaved at 121 °C for 15 min and the enzymes (α- and glucoamylase) were added to the medium along with the inoculum (18-h-old). The inoculated flasks were incubated at 37 °C for 60 h.

2.4. Optimization of simultaneous saccharification and fermentation

Effect of cassava bagasse concentration, enzyme concentration, yeast extract concentration, NH_4Cl concentration and inoculum size was studied, previously, one parameter at a time (data not shown). Based on these experiments, these five independent variables were chosen for the further optimization studies using a response surface methodology (RSM). A Box-Behnken [11] design with five variables at three levels and a total of 46 runs were used for the study. The three levels of each variable were coded as −1, 0 and +1, which corresponded to the lower middle and higher values, respectively. For individual parameters these were, cassava bagasse concentration (50, 100, 150 g/L), enzyme mixture concentration (5, 12.5, 20 mL/L), yeast extract concentration (2.5, 7.5, 12.5 g/L), NH_4Cl concentration (1, 3, 5 g/L) and inoculum size (1×10^9, 3×10^9, 5×10^9 CFU). The software Design-Expert (Version 6.0.6, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis and quadratic model building. The response surface graphs were obtained using the software to understand the effect of variables individually and in combination, and to determine their optimum levels. The experimental setup of RSM is shown in Table 1.

2.5. Analysis

Samples were withdrawn after 60 h of incubation and treated with 1 M H_2SO_4 to release lactic acid from medium as it is formed as calcium lactate with buffering agent, $CaCO_3$. Lactic acid extracted out from medium and the extract was diluted to the required level with distilled water and the amount of total lactic acid was estimated according to the colorimetric method of Barker and Summerson [12] and was expressed as mg/mL of the fermentation medium. The amount of reducing sugar was determined by the 3,5 dinitro salicylic acid method [13]. Starch was estimated by the method described by Nampoothiri et al. [14] using aqueous iodine solution as reagent. The color developed was measured using a UV spectrophotometer (Shimadzu, Japan) at 620 nm. All the represented values are means of three replicates ±S.D.

3. Results and discussion

Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material. There were a total of 46 runs for optimizing the five individual parameters in the current Box-Behnken design. In our previous study [6], we optimized the incubation time (60 h for 15.5% cassava bagasse). When higher concentration of cassava bagasse use, it takes more time to get maximum yield and it reduces the productivity (yield/h). Also, 60 h was taken as the incubation time in current study as there was no more increase in the yield after 60 h. The current design was applied to the production of lactic acid from cassava bagasse by simultaneous saccharification and fermentation using mixed culture of lactobacilli. The data were analyzed by multiple regression analysis using the Design Expert software and the following polynomial equation was derived to represent lactic acid yield as a function of the independent variables tested.

$$\begin{aligned} Y = {} & 48.57 + 18.89X_1 + 4.44X_2 + 5.43X_3 + 2.92X_4 \\ & + 8.44X_5 + 0.83X_1^2 - 4.29X_2^2 - 4.30X_3^2 - 0.69X_4^2 \\ & - 6.26X_5^2 + 4.25X_1X_2 + 0.65X_1X_3 + 1.20X_1X_4 \\ & + 8.18X_1X_5 - 2.63X_2X_3 + 2.05X_2X_4 + 0.95X_2X_5 \\ & - 1.10X_3X_4 + 8.08X_3X_5 - 0.52X_4X_5 \end{aligned} \quad (1)$$

Table 1
Predicted and lactic acid yields for individual runs of the RSM design

Run order	Cassava bagasse (g/L)	Enzyme mixture (ml/L)	Yeast extract (g/L)	NH_4Cl (g/L)	Inoculum size (CFU/100 mL) $\times 10^9$	LA (g/L) (predicted value)	LA (g/L) (actual value)
1	100	12.5	2.5	5	3	42.62	42.5
2	150	12.5	12.5	3	3	70.51	68.5
3	100	12.5	7.5	5	1	37.07	38.3
4	100	20	7.5	3	5	52.31	50.5
5	100	20	7.5	3	1	33.52	40.8
6	100	12.5	7.5	3	3	49.02	47.6
7	100	12.5	7.5	3	3	49.02	49.5
8	100	12.5	7.5	5	5	52.91	54.3
9	100	12.5	12.5	5	3	51.27	54.5
10	100	5	7.5	3	1	26.52	32.3
11	50	12.5	2.5	3	3	21.88	21.5
12	50	20	7.5	3	3	26.85	26.2
13	150	5	7.5	3	3	55.75	53.5
14	150	20	7.5	3	3	73.14	78.2
15	100	12.5	7.5	1	5	48.12	48.3
16	100	5	7.5	3	5	41.52	38.2
17	100	12.5	7.5	3	3	49.02	47.5
18	100	12.5	12.5	1	3	47.63	48.9
19	150	12.5	2.5	3	3	58.36	62.8
20	100	12.5	2.5	1	3	34.58	32.5
21	50	12.5	12.5	3	3	31.43	24.6
22	50	5	7.5	3	3	26.46	18.5
23	100	12.5	7.5	1	1	30.18	30.2
24	100	12.5	12.5	3	5	59.5	57.2
25	100	20	7.5	1	3	42.61	32.3
26	100	12.5	2.5	3	5	32.5	31.1
27	50	12.5	7.5	5	3	31.07	32.4
28	100	20	7.5	5	3	52.54	46.3
29	150	12.5	7.5	3	5	78.19	78.9
30	100	5	7.5	1	3	37.82	36.3
31	100	5	2.5	3	3	27.03	28.4
32	100	12.5	7.5	3	3	48.11	46.5
33	100	12.5	2.5	3	1	31.76	28.6
34	150	12.5	7.5	1	3	63.02	66.9
35	50	12.5	7.5	3	5	24.06	30.6
36	100	5	7.5	5	3	39.56	42.1
37	100	12.5	7.5	3	3	48.11	48.6
38	150	12.5	7.5	5	3	71.26	67.9
39	100	5	12.5	3	3	43.13	48.5
40	50	12.5	7.5	3	1	23.52	22.9
41	100	12.5	12.5	3	1	26.46	22.4
42	100	20	12.5	3	3	46.77	52.1
43	100	20	2.5	3	3	41.17	42.5
44	100	12.5	7.5	3	3	48.11	51.7
45	50	12.5	7.5	1	3	27.64	36.2
46	150	12.5	7.5	3	1	44.96	38.5

where Y is the predicted lactic acid yield and X_1, X_2, X_3, X_4 and X_5 are the coded values for cassava bagasse, enzyme mixture, yeast extract, NH_4Cl and inoculum size, respectively.

The experimental data were statistically analyzed using the Fischer's statistical test for analysis of variance (ANOVA) and the results are shown in Table 2. The ANOVA of the quadratic regression model indicated that the model was highly significant, as the F-value for the model was 14.61. There was only 0.01% chance that the "Model F-value" this large could occur due to noise. The Prob > F-value of the model was 0.0001, which also confirmed that the model was highly significant. The coefficient estimate and the corresponding Prob > F-values suggested that all the independent variables studied had a significant effect on lactic acid production. The analysis also showed that there were significant interactions between cassava bagasse and inoculum size and yeast extract and inoculum size.

Increase in the concentration of cassava bagasse positively influenced lactic acid production, as the starch in bagasse gets converted to hexoses in presence of amylase enzyme mixture. Usually, for the double step fermentation gelatinized starchy substrate is treated first with α-amylase (liquefaction) and then with glucoamylase (saccharification) at elevated temperatures for faster liberation of the hexoses. Although during simultaneous saccharification and fermentation is slower than the former,

Table 2
Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob > F
Model	9025.06	20	451.25	14.61	<0.0001
X_1	5711.58	1	5711.58	184.94	<0.0001
X_2	315.95	1	315.95	10.23	0.0039
X_3	470.89	1	470.89	15.25	0.0007
X_4	136.31	1	136.31	4.41	0.0463
X_5	1140.75	1	1140.75	36.94	<0.0001
X_1^2	5.94	1	5.94	0.19	0.6649
X_2^2	160.74	1	160.74	5.20	0.0317
X_3^2	161.37	1	161.37	5.23	0.0314
X_4^2	4.18	1	4.18	0.14	0.7163
X_5^2	341.82	1	341.82	11.07	0.0028
$X_1\,X_2$	72.25	1	72.25	2.34	0.1392
$X_1\,X_3$	1.69	1	1.69	0.055	0.817
$X_1\,X_4$	5.76	1	5.76	0.19	0.6697
$X_1\,X_5$	267.32	1	267.32	8.66	0.0071
$X_2\,X_3$	27.56	1	27.56	0.89	0.3542
$X_2\,X_4$	16.81	1	16.81	0.54	0.4678
$X_2\,X_5$	3.61	1	3.61	0.12	0.7354
$X_3\,X_4$	4.84	1	4.84	0.16	0.6957
$X_3\,X_5$	260.82	1	260.82	8.45	0.0077
$X_4\,X_5$	1.10	1	1.10	0.036	0.8517
Residual	741.21	24	30.88		
Lack of fit	724.98	20	36.25	8.94	0.0231
Pure error	16.23	4	4.06		
Corrected total	9775.67	45			

the lactic acid production is faster due to the lower temperatures of operation. Also, there is no negative influence of the high reducing sugar concentrations as the sugars gets converted into lactic acid as soon as it is liberated. Under these conditions the enzymes may not show their optimum level of activity, but the available activity is expected to help the controlled release of sugars, which is very crucial for the fermentation. Thus, even at a very high starch concentration, feed back inhibition due to higher reducing sugar levels is prevented. In the case of simultaneous saccharification and fermentation, the conversion percentage of starch to reducing sugar is also higher than separate hydrolysis and fermentation. Linko and Javanainen [15] reported 66% of conversion from starch to sugar during the saccharification at 37 °C on barley starch after 24 h. When the temperature was elevated to 60 °C there was only a slight increase, and at 24 h the yield was only 69%. Our previous work also proved temperature at 37 °C had better results in the conversion of starch to lactic acid using SSF [6].

The results of the study showed that the cassava bagasse concentration in the medium was the most significant single parameter which influenced lactic acid production followed by inoculum concentration and yeast extract concentration (Table 2; Eq. (1)). The interactions between cassava bagasse concentration and enzyme loading, and that between cassava bagasse concentration and inoculum density also had significant effects. Response surfaces were plotted using Design Expert software to study the effects of parameters and their interactions on lactic acid production.

In the current study, a 15% concentration of cassava bagasse was selected as the higher level since any further increase in concentration resulted in an undesirable increase of viscosity, causing it to solidify readily and negatively affecting the distribution of enzymes and inoculum. There was a linear increase in the yield of lactic acid with increase in the concentration of cassava bagasse as it was the sole carbon source (Fig. 1). The effect of enzyme loading was also independent like that of substrate concentration and regardless of substrate concentration the highest enzyme loading gave the highest yields. However, at the lowest substrate loading, enzyme needed was compara-

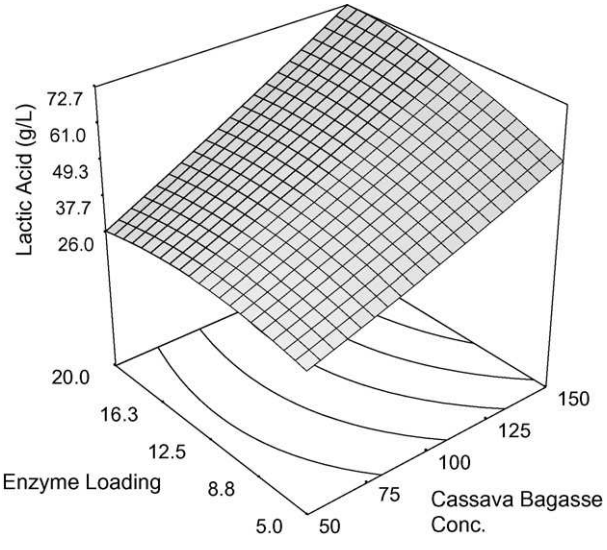

Fig. 1. Effect of cassava bagasse and enzyme mixture concentration on lactic acid production.

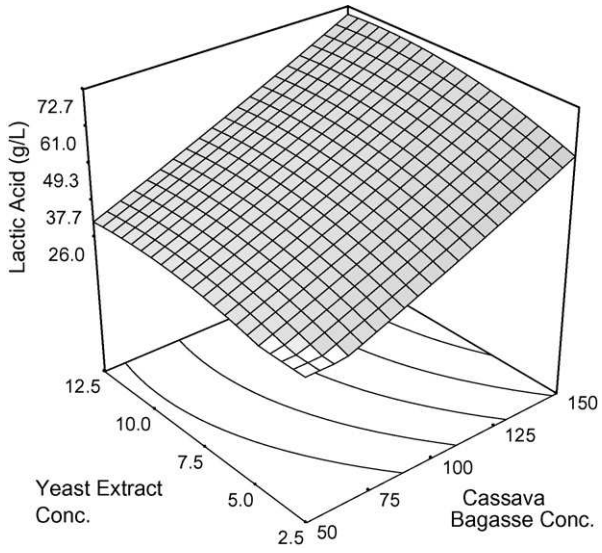

Fig. 2. Effect of cassava bagasse and yeast extract concentration on lactic acid production.

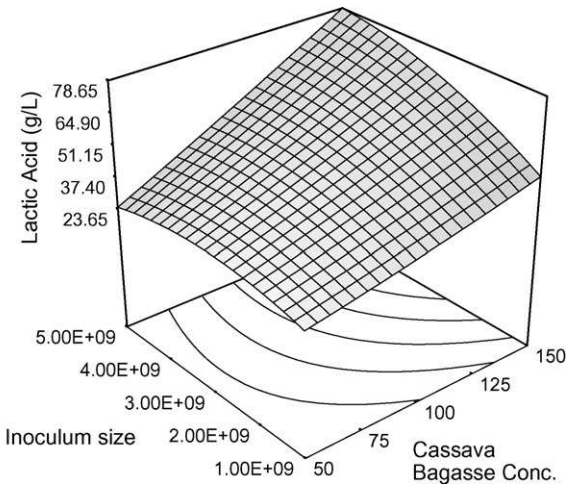

Fig. 3. Effect of cassava bagasse concentration and inoculum size on lactic acid production.

tively lower. This relationship is as expected since it directly follows from the fact that a higher enzyme concentration may be required for hydrolyzing more substrate and thus increasing the level of fermentable sugars in the medium available to the organisms.

The results presented in Fig. 2, indicated that the yeast extract concentration positively influenced lactic acid production independent of the concentration of cassava bagasse in the medium. Lactic acid bacteria generally have complex nutrient requirements for the growth and fermentation [2,3]. The growth promoters in the yeast extract, mainly vitamin B help in the growth and hence the production of lactic acid [16]. Regardless of cassava bagasse concentration, an increase in lactic acid production was obtained with increased yeast extract concentration up to the "+0.5" level of this parameter.

As in the case of yeast extract, NH_4Cl, the inorganic nitrogen source used had a positive impact on the lactic acid production. But the influence was not as pronounced as in the case of yeast extract. At both the lower and higher levels of cassava bagasse in the medium, a higher NH_4Cl concentration resulted in better yield, probably indicating its effect on promoting growth of the organism (data not shown). Thus, it may be said that an increased concentration of substrate supported better growth and production and in the process necessitated the increased supply of yeast extract and NH_4Cl which served as nitrogen sources. A higher loading of enzyme apparently became necessary at higher bagasse concentrations for saccharifying the increased amount of substrate.

Results presented in Fig. 3 shows the interaction between the concentration of cassava bagasse and inoculum size. Higher concentration of substrate always necessitated the requirement of a higher inoculum concentration, while at lower levels of substrate concentration, the yield increased initially with increase in inoculum concentration and then decreased. When there is lesser substrate in the medium, the addition of more inoculum probably result in a competition, with the result that growth and productivities might be affected which could be the reason for this observation. At higher substrate concentrations, inoculum had an additive effect with increases in cell number yielding more of the product.

The interaction between inoculum concentration and yeast extract was significant in SSF for production of lactic acid. The necessity of the nutrients is higher when a higher inoculum size is used since *Lactobacilli* are fastidious organisms requiring complex nutrients. At a lower inoculum concentration, the demand on yeast extract for lactic acid production was lesser and for an inoculum concentration up to $\sim 3 \times 10^9$ CFU/ml, a yeast extract concentration of less than 10 g/L could support growth and maximal lactic acid (~49 g/L). However, and increased inoculum concentration placed a corresponding increase in demand on yeast extract and the highest productivities (~57 g/L) was achieved with a yeast extract concentration of 11–12.5 g/L (Fig. 4). With an increase in number of the viable cells, it is expected that there would be a corresponding increase in demand for nutrients, which could explain this observation.

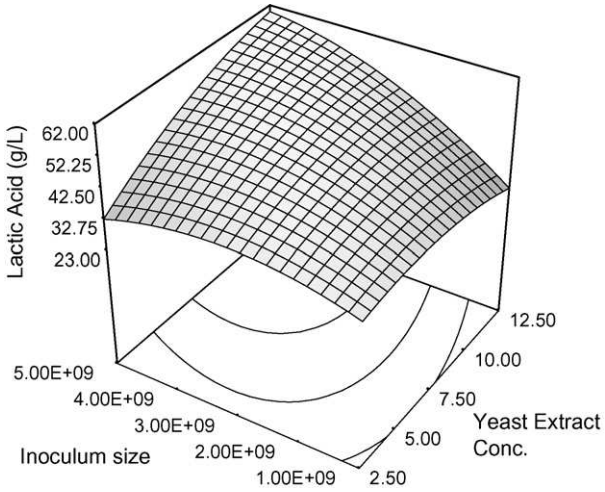

Fig. 4. Effect of inoculum and yeast extract concentration on lactic acid production.

The software Design-Expert (Version 6.0.6, Stat-Ease Inc., Minneapolis, USA) was used for experimental design, data analysis and quadratic model building. The response surface graphs were obtained using the software to understand the effect of variables individually and in combination, and to determine their optimum levels for maximal lactic acid production. In the current study, all the individual parameters significantly affect the production, as is the interactions between substrate concentration and inoculum concentration and yeast extract concentration and inoculum concentration. At 15% (w/v) cassava bagasse, the inoculum size, enzyme concentration and nutrient concentration positively influence in the production of lactic acid. When the validation experiment was performed, the maximum lactic acid yield was of 81 g/L (against 78.9 g/L in the initial experiment) when 15% (w/v) cassava bagasse was treated with 12.5 mL/L enzyme mixture and nourished with 7.5 g/L yeast extract and 3 g/L NH_4Cl. The inoculum size used was of 3×10^{10} CFU/L.

Woiciechowski et al. [17] studied the hydrolysis of cassava bagasse starch by acid and enzymatic hydrolysis. They reported both methods were quite efficient when considering one or other parameter like the percentage of hydrolysis, time and cost of the chemicals and energy consumption. Although acid hydrolysis is time saving and cost effective, there will be a neutralizing step after acid hydrolysis and which will create the unnecessary increase of salts in the medium and it will affect the growth and production of lactic acid. So, the enzymatic hydrolysis is better as it yields a high percentage of reducing sugars from cassava bagasse. But the enzymatic hydrolysis of 150 kg cassava bagasse required US$ 2470 as power for long time saccharification and cost of enzymes. Simultaneous saccharification reduces the cost of energy consumption for the liquefaction and saccharification and thus it is cost effective and time saving process for the lactic acid production.

4. Conclusion

A cost effective and eco friendly method was adopted for value addition of the agro industrial waste product cassava bagasse, which contains huge amount of entrapped starch. A single step conversion of cassava starch present in the bagasse to L-lactic acid is demonstrated using a mixed culture employing two lactobacilli. The tedious and expensive preparation of separate starch hydrolyzate either by enzymatic or chemical method is avoided in simultaneous saccharification and fermentation process. The process also can use comparatively larger amounts of initial raw material, since there is a controlled release of sugar, which can be utilized immediately by the fermenting microorganisms. The mixed culture could effectively replace single cultures to increase the production rate or lactic acid yield.

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References

[1] S.R. Kadam, S.S. Patil, K.B. Bastawde, J.M. Khire, D.V. Gokhale, Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production, Proc. Biochem. 41 (2006) 120–126.
[2] P.J. Rojan, K.M. Nampoothiri, A.S. Nair, A. Pandey, L(+)-Lactic acid production using *Lactobacillus casei* in solid-state fermentation, Biotechnol. Lett. 27 (2005) 1685–1688.
[3] R.P. John, K.M. Nampoothiri, A. Pandey, Solid-state fermentation for L-lactic acid production from agro waste using *Lactobacillus delbrueckii*, Proc. Biochem. 41 (2006) 759–763.
[4] H. Elkholy, A. Eltantawy, The world of cassava production: an overview, J. Root Crops 26 (2000) 1–5.
[5] R. Anuradha, A.K. Suresh, K.V. Venkatesh, Simultaneous saccharification and fermentation of starch to lactic acid, Proc. Biochem. 35 (1999) 367–375.
[6] R.P. John, K.M. Nampoothiri, A. Pandey, Simultaneous saccharification and fermentation of cassava bagasse for L(+)lactic acid production, Appl. Biochem. Biotechnol. 134 (2006) 263–272.
[7] E. Bautista, R.S. Dahiya, M.L. Speck, Identification of compounds causing symbiotic growth of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in milk, J. Dairy Res. 33 (1966) 299–307.
[8] N.J. Moon, G.W. Reinbold, Commensalism and competition in mixed cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, J. Milk Food Technol. 39 (1976) 337.
[9] K.B. Lee, Comparison of fermentative capacities of lactobacilli in single and mixed culture in industrial media, Proc. Biochem. 40 (2000) 1559–1564.
[10] K.P. Tiwari, A. Pandey, N. Mishra, Lactic acid from molasses by mixed population of lactobacilli, Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Zweite Naturwiss Abt Mikrobiol Landwirtsch Technol Umweltschutzes 134 (1979) 544–546.
[11] G.E.P. Box, D.W. Behnken, Some new three level designs for the study of quantitative variables, Technometrics 2 (1960) 455–475.
[12] S.B. Barker, W.H. Summerson, The colorimetric determination of lactic acid in biological materials, J. Biol. Chem. 138 (1941) 535–554.
[13] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1959) 426–429.
[14] K.M. Nampoothiri, R.R. Singhania, C. Sabarinath, A. Pandey, Fermentative production of gellan using *Sphingomonas paucimobilis*, Proc. Biochem. 38 (2003) 1513–1519.
[15] Y.Y. Linko, P. Javanainen, Simultaneous liquefaction, saccharification and lactic acid fermentation on barley starch, Enzyme Microb. Technol. 19 (1996) 118–123.
[16] A. Nancib, N. Nancib, D. Meziane-Cherif, A. Boudenbir, M. Fick, J. Boudrant, Joint effect of nitrogen sources and B vitamin supplementation of date juice on lactic acid production by *Lactobacillus casei* subsp. *rhamnosus*, Biores. Technol. 96 (2005) 63–67.
[17] A.L. Woiciechowski, S. Nistsche, A. Pandey, C.R. Soccol, Acid and enzyme hydrolysis to recover reducing sugars from cassava bagasse: an economic study, Braz. Arch. Biol. Technol. 45 (2002) 393–400.