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Solid-state fermentation for L-lactic acid production from agro wastes using *Lactobacillus delbrueckii*

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Abstract

A viable process based on a low cost production media is desired to enhance the economics of fermentative production of L-lactic acid. Attempts were made to exploit two agro-industrial wastes, cassava bagasse and sugarcane bagasse, as a raw material and inert solid support using solid-state fermentation (SSF). Gelatinised cassava bagasse was enzymatically hydrolysed and starch hydrolysate containing reducing sugar was used to moisten the inert sugarcane bagasse, which was used as the solid support for SSF. This substrate was supplemented with 0.5 g/5 g support NH_4Cl and yeast extract. SSF was carried out in 250 ml Erlenmeyer flasks at 37 °C using *Lactobacillus delbrueckii* as inoculum. Key parameters such as initial moisture content and initial sugar were optimised statistically by response surface methodology. A maximum of 249 mg/gds L-lactic acid was obtained after 5 days of fermentation under the optimised conditions with a conversion efficiency of about 99% of the initial reducing sugars.

Keywords: L(+)-Lactic acid; Solid-state fermentation; Cassava bagasse hydrolysate; Sugarcane bagasse; *Lactobacillus delbrueckii*; Response surface methodology

1. Introduction

Lactic acid has got one of the prime positions in the family of carboxylic acids because of its applications in food and non-food industries. It is used as a preservative and acidulant in foods. Calcium lactate is a good dough conditioner while sodium lactate is both conditioner and emulsifier. Lactic acid is being used as the pH controller in wine making. In dairy industry, lactic acid is used for the production of cheese. Its salts with calcium, sodium, potassium, zinc, iron, etc. find applications in pharmaceutical, cosmetic and food industries. It is used as the raw material for different organic acids such as propinoic acid, acetic acid, acrylic acid, etc. Technical grade lactic acid is extensively used in leather tanning industries for deliming. As a precursor for the biodegradable plastic, poly lactic acid (PLA), lactic acid has got much consideration [1–3]. From chemical synthesis only the racemic mixture of the L(+) and D(−) enantiomers is obtained. Fermentation has the added advantage of producing biologically active L(+) form or D(−) or DL lactic acid [3].

One of the main obstructions in the large-scale production of lactic acid is the cost of the raw material. Application of agro-industrial waste residues in bioprocesses provides an alternative way to replace the refined and costly raw materials and the bulk use of such materials will help to solve many environmental hazards [2]. Cassava (*Manihot esculenta* Crantz) ranks as the world's sixth important food crop. A large part of cassava is used in industries to obtain starch and the industrial application of cassava generates solid residue in the form of bagasse [4,5]. Cassava bagasse traps a large part of starch in its fibrous matter. The utilization of starchy materials in the place of the expensive refined sugar is economical [6]. According to Pandey et al., SSF carried out on inert solid material would provide good fermentation conditions and the purity of the product would be relatively high [7]. Sugarcane bagasse is one of the largest cellulosic agro-industrial by-products, which was used as the inert support for the production of various metabolites [7–9]. Nampoothiri and Pandey [8] reported the production of L-glutamic acid in which sugarcane bagasse was impregnated with a medium containing glucose, urea, mineral salts and vitamins. Similarly Soccol et al. [9] checked the potential of sugarcane bagasse as inert support, which is impregnated with glucose and calcium carbonate medium for the production of lactic acid from a strain of *Rhizopus oryzae*. The present paper shows results

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with a bacterial culture of *L. delbrueckii* for producing lactic acid by SSF, using inert sugarcane bagasse which was impregnated with the sugar solution obtained from another agro residue, the cassava bagasse. The key process parameters required for SSF were optimised.

2. Materials and methods

2.1. Microorganism and inoculum preparation

Lactobacillus delbrueckii NCIM 2025, a homofermentative L(+) lactic acid producing bacterium was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The culture was maintained in MRS agar (Hi-media, Mumbai) stabs at 4 °C and subcultured fortnightly. The inoculum for the experiments was prepared from fresh MRS slants. A loopful of culture was inoculated into 25 ml MRS medium in 250 ml Erlenmeyer flask and incubated at 37 °C for overnight. The 18 h old bacterial culture having 10^9 CFU/ml was used as the inoculum.

2.2. Enzymes

Hydrolysis of cassava bagasse (contains approximately 50% starch) is carried out using the commercially available thermostable α-amylase (Termamyl, 5000 IU/ml) and glucoamylase (AMG, 2000 IU/ml) obtained from Rashesh and Co., Mumbai, India.

2.3. Enzymatic hydrolysis of cassava bagasse

Cassava bagasse is obtained from Varalakshmi Starch Industries Ltd., Salem, Tamil Nadu, India. Gelatinisation of cassava bagasse (7.5 g/100 ml) is performed at 100 °C for 15 min followed by liquefaction with alpha amylase (90 °C, pH 5 for 30 min) and further saccharification with glucoamylase (60 °C, pH 4 for 70 min). The hydrolysate obtained was filtered through a muslin cloth and the clear hydrolysate containing reducing sugar was used as the sole carbon source and the moistening medium for SSF.

2.4. Solid-state fermentation

Five grams of inert (sugar free – continuous washing with distilled water) sugarcane bagasse taken in 250 ml Erlenmeyer conical flask is moistened with concentrated cassava bagasse hydrolysate to get desired level of moisture and sugar. Before moistening the substrate, the hydrolysate was further supplemented with minimum amount of a nitrogen source, NH_4Cl (0.5 g/5 g support) and the growth promoter, yeast extract (0.5 g/5 g support). To avoid the problem of pH decrease as lactic acid accumulation starts, 2.5 g/5 g support $CaCO_3$ was also added into it as the buffering agent. The moistened solid substrate was thoroughly mixed and was autoclaved at 121 °C for 15 min. The sterile solid medium was inoculated with 18 h old cells of *L. delbrueckii* and after thorough mixing, the flasks were incubated at 37 °C in an environmental chamber (Sanyo, Japan) for 5 days.

2.5. Optimisation of process parameters

The initial moisture level of the substrate for the bacterial culture in SSF is very crucial. The inert sugarcane bagasse (5 g) was moistened with a minimum amount of concentrated cassava starch hydrolysate based medium in such a way that the initial reducing sugar in it was in all cases 250 mg/gds. Different moisture levels (43, 48, 52, 56, 60, 64, 68, 72, 76 and 80) were further adjusted by adding required amount of distilled water. The SSF medium with varying moisture levels were inoculated with *L. delbrueckii* and fermentation was carried out at 37 °C for 5 days.

To optimise the initial reducing sugar concentration, 5 g of sugarcane bagasse was moistened with various volumes of concentrated cassava bagasse hydrolysate based medium. In all cases the set moisture was maintained at 72%. In cases where low volumes of hydrolysate were used (low sugar concentration), further moistening was carried out using distilled water. SSF was carried out as it is described earlier.

To study the influence of inoculum size on lactic acid production, SSF was carried out using different volumes of inoculum (1, 1.5, 2, 2.5, 3, 3.5 and 4 ml). One millilitre of the 18 h old inoculum contains 10^9 CFU and moisture level was kept constant.

To find the influence of initial pH of the medium, the pH of the moistening medium was adjusted to varying pH (4, 5, 6, 6.5, 7, 7.5, 8, 9 and 10) and was used to wet the substrate. Care was taken not to add any $CaCO_3$ in the SSF medium. However, a simultaneous attempt was also made where the minimum amount of calcium carbonate was also used in the medium irrespective of the pH of the moistening medium. Later, the concentration of calcium carbonate required for effective accumulation of lactic acid was also optimised using different concentration of calcium carbonate (0.5–3 g, i.e. 20, 40, 60, 80, 100, 120% of the reducing sugar) in the medium. Since the particle size matters the surface area of support as well as the mass transfer, it is one of the key factors in the solid-state fermentation. SSF was carried out using sugarcane bagasse of different sizes and a combination of them (<0.1, 0.1–0.2, 0.2–0.5, 0.5–1.5, 1.5–3 mm, and combination of all the five sizes in 1:1:1:1:1 ratio). All the experiments were done in triplicates and the data were presented with the standard deviation.

Apart from the above single parameter optimisation studies, response surface methodology (central composite design) was employed for the optimisation of two crucial factors such as reducing sugar and moisture level on lactic acid production. Central composite surface design made to require five levels and coded as −1.4, −1.0, 0, +1.0, and +1.4. Response surface generated using statistical software STATISTICA (StatSoft Inc., USA). Summary of the variable used for the central composite design for the optimisation of moisture and reducing sugar are shown in Table 1.

2.6. Analytical methods

Standard acid hydrolysis method [10] was adopted to determine the total starch content in cassava bagasse. From the fermented solid mass, the lactic acid formed was extracted with 1 M H_2SO_4. For that, to the fermented matter 100 ml of 1 M H_2SO_4 was added and placed in shaker for 30 min with a rotation of 200 rpm. During the process, lactic acid was liberated from its salt form, the calcium lactate. The whole content was centrifuged at 8000 rpm for 15 min. In the clear supernatant, L-lactic acid was estimated by colorimetric method of Barker and Summerson [11] and is expressed as mg/gram dry fermented matter (mg/gds). The amount of reducing sugar was determined by the Dinitrosalicylic acid method [12].

3. Results and discussion

Cassava bagasse was enzymatically hydrolysed and that hydrolysate was used as the sole carbon source for lactic acid production. The hydrolysate was supplemented with minimum amount of yeast extract and the ammonium chloride because the lactic acid bacteria generally have complex nutrient requirements to synthesis amino acids and vitamins for growth and fermentation [13,14]. A homofermentative L(+)-enantiomer producing bacterium, *L. delbrueckii* NCIM 2025, is used for lactic acid production. In general, homo fermentative organisms have the conversion efficiency of 1 mol glucose to

Table 1
Summary of variables for the central composite design of initial sugar and moisture level

Factor	Value of the factor for coded value				
	−1.4	−1.0	0	+1.0	+1.4
Moisture content (%)	43	48	60	72	77
Reducing sugar (mg/gds)	90	130	230	330	370

2 mol lactic acid [15]. World's one of the largest agricultural waste, sugarcane bagasse, was used as the inert support to grow the lactic acid bacteria. This material was free of available sugar and contains about 50% cellulose, 25% hemicellulose and 25% lignin [7]. According to Pandey et al. [7] SSF carried out on inert support materials has been regarded as one of the future developments of SSF systems. There were not many reports on lactic acid production by solid-state fermentation and also by using bacterial cultures. Some studies reported with amylolytic lactobacilli for conversion of starch and some work with fungal cultures [6,9,15]. Soccol et al. [9] reported around 75% conversion in SSF using *R. oryzae* NRRL 395.

Since initial sugar and substrate moisture are the two major factors for the growth and production, these parameters were optimised initially by single parameter optimisation. Different concentrations of reducing sugar were provided to the medium obtained through cassava bagasse hydrolysates. Total reducing sugar present in the whole solid medium was kept at different levels such as 1, 1.5, 2, 2.5, 3 and 3.5 g. The highest conversion based on initial sugar to lactic acid was took place in 2.5 g (250 mg/gds) reducing sugar concentration (Fig. 1). The reducing sugar concentration was kept at 250 mg/gds for the further studies. In SSF, the intensity of microbial growth generally depends on the initial moisture level and it indirectly affects the production titre. In general, fungal cultures required low moisture level (20–70%) compared to the requirement of a bacterial culture, which is higher than 70% [3]. As it is evident from Fig. 1, among the different moisture levels kept for the optimisation, 72% was proved as the best for lactic acid production. Statistical experimental layouts can be adopted at various phases of an optimisation process for finding optimal conditions for targeted results. Central composite design is one of the popular choices in statistical designs. Response surface methodology has by now been established as a convenient method for developing optimum processes with precise conditions and has minimized the cost of production of many a process with efficient process parameters [16,17]. Later, we tried the response surface methodology to compare these two variables together and to see the interaction of them in different levels. Interestingly, as it is shown in Fig. 2 and in Table 2 the values are comparable to our single parameter study.

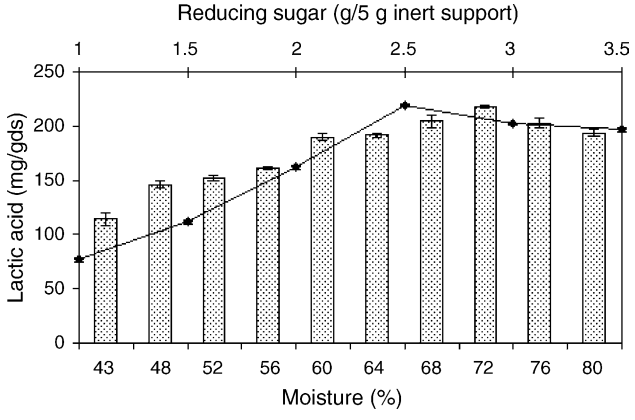

Fig. 1. Optimisation of reducing sugar concentration (◆) and moisture level (▨).

Table 2
Experimental design (central composite design) used to optimise the initial sugar and moisture level

Standard run	Moisture content (%)	Reducing sugar (mg/gds)	Lactic acid (mg/gds)
1	48	130	97
2	48	330	210.5
3	72	130	41.5
4	72	330	222
5	43	230	54
6	77	230	33
7	60	92	42
8	60	370	130
9	60	230	232
10	60	230	219.5

Inoculum size and incubation time were also checked for the best conversion. As optimised in the submerged fermentation 18 h old inoculum was taken for the SSF studies. Different volumes (1, 1.5, 2, 2.5, 3, 3.5 and 4 ml) of 18 h old inoculum where 1 ml contains 10^9 CFU were used to study the effect of initial biomass for effective utilization of sugar to convert it to lactic acid. In the case of submerged fermentation (SmF), the inoculum level varies according to the initial sugar content used in liquid fermentation. In general practice, a low-level sugar medium needs 2–3% (v/v) and higher level needs 5–10% inoculum. Naveena et al. [18] used 3.5 ml of inoculum of 24 h old *L. amylophilus* GV6 to convert 10 g of wheat bran with 54.2% starch. In our studies we found 3 ml inoculum was the best and levels higher than 3 ml has no favourable effect (data not shown). The incubation temperature affects the viability of the culture and the effect of incubation temperature on lactic acid production was studied by incubating the flasks at various temperatures (25, 30, 37, 44, 51 °C). Table 3 shows the maximum production of 238.8 mg/gds at 37 °C. However, the culture showed equally good production (234.35 mg/gds) at 30 °C. In solid-state fermentation, the metabolic heat generation will also be added up during the course of fermentation and it may or may not be good for the culture. However, *L. delbrueckiii* is known to produce lactic acid over a wide range of temperature, 30–42 °C [19]. Similarly, the effect of pH was tested at various pH values, from 4 to 10, with and without buffering. The pH of the moistening medium was adjusted with

Table 3
Optimisation of incubation temperature and pH during the fermentation

Effect of temperature		Effect of pH	
Temperature (°C)	Lactic acid (mg/gds)	pH	Lactic acid (mg/gds)
25	196.44	4	136.8
30	234.35	5	196.4
37	238.8	6	214.8
44	86.15	6.5	237.5
51	78.71	7	197.7
		7.5	199.2
		8	192.3
		9	194
		10	175

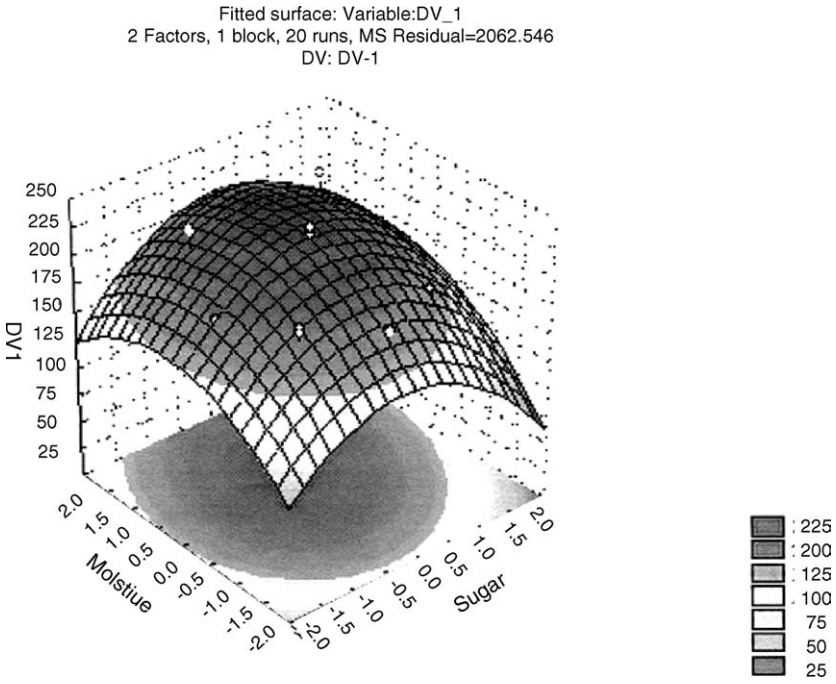

Fig. 2. Effect of reducing sugar concentration and moisture level on the production of lactic acid.

1N $Ca(OH)_2$ and 1N HCl. In the absence of buffering, the pH decreased to less than 3.5 within three days of fermentation and that low pH resulted in low lactic acid production (data not shown). When the pH set in the above range together with $CaCO_3$ mixed in the solid mass, pH remains constant since it readily converted to Ca-lactate. The set pH, 6.5 was proved to be the optimum for the lactic acid production (237.5 mg/gds) as listed in Table 3. From pH 7 to 9 the yield was quite stable between 192.3 and 199.2 mg/gds. pH 4 (136.8 mg/gds) and 10 (175 mg/gds) are the most unfavourable conditions. Later, we optimised the level of calcium carbonate requirement for maximum accumulation of calcium lactate and it was found to be 30–50% of the initial substrate level i.e. 1.5–2.5 g/5 g sugarcane bagasse. Naveena [15], reported that high calcium carbonate concentration inhibit the lactic acid production.

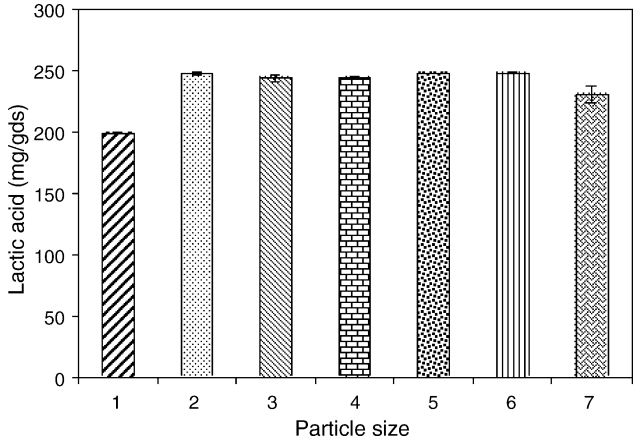

Fig. 3. Optimisation of particle size. (1) <0.1 mm (▨); (2) 0.1–0.2 mm (⬚); (3) 0.2–0.5 mm (▧); (4) 0.5–1.5 mm (▤); (5) 1.5–3 mm (▩); (6) 0.5–1.5 + 1.5–3 mm (1:1) (▥) (7) <0.1 + 0.1–0.2 + 0.2–0.5 + 0.5–1.5 + 1.5–3 mm (1:1:1:1:1) (▨).

An important parameter in SSF is the particle size of the substrate used. Too less and too high sizes were not suited. With smaller particle, the available surface area for the microbial growth is larger but the inter particle space and hence the porosity becomes less. Although they provide large surface area, extremely small particle sizes are also not favourable as they reduce mass transfer [8]. In our studies, the particle sizes ranging from 0.1 to 3 mm supported the growth of the culture (visual observation) as well as lactic acid production (Fig. 3) with nearly 99% conversion. In the case of bigger particle the water holding capacity is very low but it reduces the diffusion of oxygen.

Major success of any bioprocess depends on the duration of fermentation and it means the time requirement for the complete utilization of the source material and the corresponding accumulation of the desired product. A time course study was conducted under all the optimised conditions. As shown in Fig. 4, in current SSF system, it took just 120 h for the complete

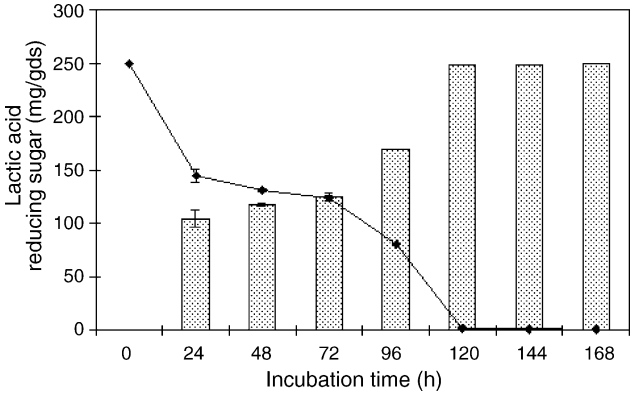

Fig. 4. Time course study shows the production of lactic acid (⬚) and the consumption of reducing sugar (◆).

utilization of the sugar and to get the maximum titre of (249.1 mg/gds) of lactic acid.

4. Conclusion

L. delbrueckii was able to grow in a solid support such as sugarcane bagasse and it effectively utilized the sugar available in the medium used for moistening the substrate. Cassava hydrolysate prepared from the bagasse was the sole carbon source used with minimum supplementation of ammonium salt and yeast extract. Under the optimised conditions, the strain produced up to 249.1 mg lactic acid/gds with a conversion efficiency of more than 99% of the total sugar available to lactic acid. This process offers a cost effective, eco friendly technology to scale up lactic acid production. We also successfully demonstrated that not only the filamentous fungi but also the well-known lactic acid bacteria like *L. delbrueckii* can be used for solid-state fermentation, which is now globally accepted for the value addition of agroresidues.

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