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ORIGINAL ARTICLE

Polyurethane foam as an inert carrier for the production of L(+)-lactic acid by *Lactobacillus casei* under solid-state fermentation

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Keywords
cassava bagasse, lactic acid, *Lactobacillus casei*, polyurethane foam, solid-state fermentation.

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Abstract

Aim: Production of L-lactic acid in solid-state fermentation (SSF) using polyurethane foam (PUF) as inert support moistened with cassava bagasse starch hydrolysate.

Methods and Results: PUF impregnated with cassava bagasse starch hydrolysate as major carbon source was used for the production of L-lactic acid using *Lactobacillus casei* in solid-state condition. The key parameters such as reducing sugar, inoculum size and nutrient mixture were optimized by statistical approach using response surface methodology. More than 95% conversion of sugars to lactic acid from 4 g reducing sugar per gram dry support was attained after 72 h when the inert substrate was moistened with 6·5 ml of nutrient solution and inoculated with $1{\cdot}5 \times 10^9$ CFU of *L. casei*. While considering the lactate yield based on the solid support used, a very high yield of 3·88 g lactic acid per gram PUF was achieved.

Conclusion: PUF acted as an excellent inert support for *L. casei* and provided a platform for the utilization of starchy waste hydrolysate in a lower reactor volume.

Significance and Impact of the Study: This is a cost effective cultivation of lactic acid bacteria for producing lactic acid from agro based waste products such as cassava bagasse. This is the first report on the exploitation of PUF as an inert support for lactate production under SSF.

Introduction

Lactic acid is a chemical having interesting applications in food, textile and pharmaceutical industries. It is used as a raw material for lactate ester, propylene glycol, 2,3-pentanedione, propanoic acid, acrylic acid, acetaldehyde and dilactide in chemical industries. It has attained a high demand as it is the precursor for polylactic acid, a biodegradable plastic (Rojan *et al.* 2005; John *et al.* 2006a, 2006b). Solid-state fermentation (SSF) techniques are suitable for the production of commercial products such as enzymes, organic acid, *etc.* because of their valuable advantages such as high yields, low energy consumption, low environmental impact of the process and differential expression of metabolites. The use of an inert support with an almost constant physical structure and impregnated with a liquid medium, throughout the process facilitates reproducible and detailed studies in SSF, which will eventually be the basis for efficient process development, control strategies and reactor design (Lareo *et al.* 2006). The use of inert materials such as hemp, perlite (Weber *et al.* 1999), polyurethane foam (PUF) (Murado *et al.* 1997), polymeric resin (Christen *et al.* 1995), sugarcane bagasse (Rojan *et al.* 2005; John *et al.* 2006a) and polystyrene (Gautam *et al.* 2002) for SSF is well documented. Usually, inert supports are used for the fungal cultures, but there are reports on bacterial cultures used in SSF for value addition (Prabhu and Chandrasekaran 1995; Nampoothiri and Pandey 1996; Rojan *et al.* 2005; John *et al.* 2006a). Cassava (*Manihot esculenta* Crantz) ranks

the fourth among staple crops in the world and is consumed by more than 800 million people (Elkholy and El-tantawy 2000). Cassava bagasse, a solid residue generated from the cassava starch industry contains nearly 50% starch (Pandey and Soccol 2000). The utilization of starchy materials instead of expensive refined sugars is most economical (Vishnu *et al.* 2002). In this study, cassava bagasse hydrolysate was used as carbon source and PUF as an inert support for lactic acid production. Response surface optimization is more advantageous than the traditional single parameter optimization in that it saves time, space and raw material.

Materials and methods

Micro-organism

Homofermentative *Lactobacillus casei* NCIMB 3254 (National Collection of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland) used in the present study was maintained in MRS agar stabs at 4°C and was subcultured fortnightly. *L. casei* grown in MRS medium (18-h old; 10^9 CFU ml^{-1}) was aseptically centrifuged (at 10 000 *g*) and re-suspended in fresh MRS broth to get $1{\cdot}5 \times 10^9$ CFU ml^{-1} and was used as the inoculum.

Media for lactic acid fermentation

Cassava bagasse, a solid residue generated from the cassava starch industry contains nearly 50% starch as the major carbon source. The bagasse was subjected to enzymatic hydrolysis using commercially available (Rashesh and Co., Mumbai, India) α-amylase (Termamyl, 5000 IU ml^{-1}) and glucoamylase (AMG, 2000 IU ml^{-1}) and the resultant hydrolysate contained the reducing sugar necessary for the fermentation process (Rojan *et al.* 2005; John *et al.* 2006a). In our previous experiments, we optimized parameters such as α-amylase and glucoamylase concentration, time for saccharification, *etc.* and the optimized conditions were used for almost complete conversion of cassava starch to reducing sugar. More than 95% starch hydrolysis was achieved by optimized conditions (7·5%, w/v cassava bagasse liquefied with α-amylase, 1000 IU at 90°C for 30 min and saccharified by glucoamylase, 400 IU at 60°C for 90 min). The reducing sugar concentration in the medium was estimated by dinitrosalicylic acid method using glucose as standard. The hydrolysate was further enriched with a nutrient mixture (g l^{-1}) of NH_4Cl:$MnCl_2$:yeast extract (Himedia, Mumbai, India) in the ratio 5:0·01:5. For pH stabilization, $CaCO_3$ (60%, w/w of reducing sugar) was used as buffering agent. Twelve millilitres of the above medium was used for soaking 1 g of PUF cubes (0·5 × 0·5 × 0·5 cm) without any leaking out from the sponge and was used as the support for *Lactobacillus* to grow in solid-state condition. PUF cubes used in the study had uniform shape and were able to hold nutrient solution uniformly. The constant volume of moistening medium (12 ml) at higher concentration of sugar or nutrient was achieved by concentrating either the hydrolysate alone or both the hydrolysate and nutrient mixture together using a rotary evaporator (Büchi, Switzerland). Fermentation was carried out in 250 ml Erlenmeyer conical flasks at 37°C for 4 days.

Optimization of lactic acid fermentation using central composite design

Response surface methodology was used to optimize the key process parameters for enhanced L(+)-lactic acid production using central composite design (CCD). 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 process parameters chosen for optimization were the concentration of reducing sugar, concentration of nutrient mixture and the inoculum size. The experimental design used for the study is shown in Table 1. A total of 20 experiments

Table 1 Central composite design showing the levels of parameters and actual lactic acid yield from gram reducing sugar and the total lactic acid per inert support

Run	Reducing sugar concentration (g) in hydrolysate	Inoculum volume (ml)	Nutrient mixture volume (ml)	LA yield (gram lactic acid per gram reducing sugar)	LA yield (gram lactic acid/ inert support)
1	2·00	1·00	3·00	0·75	1·5
2	4·00	1·50	6·50	0·947	3·75
3	2·00	1·00	10·00	0·84	1·68
4	4·00	1·50	12·39	0·955	3·82
5	6·00	1·00	3·00	0·42	2·52
6	0·64	1·50	6·50	0·96	0·62
7	7·36	1·50	6·50	0·38	2·82
8	4·00	2·34	6·50	0·97	3·88
9	4·00	0·66	6·50	0·55	2·21
10	4·00	1·50	0·61	0·55	2·21
11	4·00	1·50	6·50	0·96	3·84
12	6·00	1·00	10·00	0·47	2·82
13	2·00	2·00	3·00	0·86	1·72
14	6·00	2·00	10·00	0·43	2·58
15	4·00	1·50	6·50	0·956	3·83
16	6·00	2·00	3·00	0·38	2·32
17	4·00	1·50	6·50	0·855	3·42
18	4·00	1·50	6·50	0·87	3·48
19	4·00	1·50	6·50	0·872	3·49
20	2·00	2·00	10·00	0·945	1·89

Values are mean of three sets of fermentations.

were carried out. Various volumes of inoculum was added (as in the design) to 1 g of hydrolysate moistened and nutrients nourished PUF and mixed well using a sterilized spatula to get uniform distribution of micro-organism. All experiments were carried out in triplicate and the average of L(+)-lactic acid yield obtained was taken as the dependent variable or response.

Analytical methods

Lactic acid was estimated according to the colorimetric method of Barker and Summerson (1941) after extraction with 1 mol H_2SO_4. The amount of reducing sugar was determined by dinitrosalicylic acid method (Miller 1959). Lactic acid yield was calculated on the basis of gram lactic acid produced from gram reducing sugar (gram lactic acid per gram reducing sugar) and also gram lactic acid produced from gram dry inert support (gram lactic acid per gram inert support).

Scanning electron microscopy

Growth distribution of *L. casei* on PUF was examined using a scanning electron microscope (JEOL JSM 5600LV, 115 Japan). The fermented sample (72 h) was adequately dried and mounted on a brass stud followed by a mild gold coating (0·01 μm) and was subjected to electron microscopy at an accelerating voltage of 10 kV.

Results

CCD was used for the optimization of lactic acid production by *L. casei* from cassava bagasse starch hydrolysate under SSF using PUF as the inert support. The ANOVA of the quadratic regression model of CCD indicated that the model was highly significant, as the *F* values for the model were 9·61 and 7·66 in case of gram lactic acid per gram reducing sugar and gram lactic acid per gram inert support, respectively. The probability values after each of the *F* values of the model were 0·0007 and 0·0019, which also confirmed that the model was highly statistically significant.

Figure 1 shows the interactive effect of reducing sugar concentration and inoculum size on lactic acid fermentation. The results showed that for the particular model, the lactate accumulation increased corresponding to an increase in reducing sugar concentration up to 4 g in the medium, after which it declined. Figure 2 shows the interaction between the reducing sugar and the concentration of nutrient mixture. Although a linear increase in lactic acid production occurred with the increase in nutrient solution level (Fig. 2), the reducing sugar concentration beyond 4 g was inhibitory for lactic acid

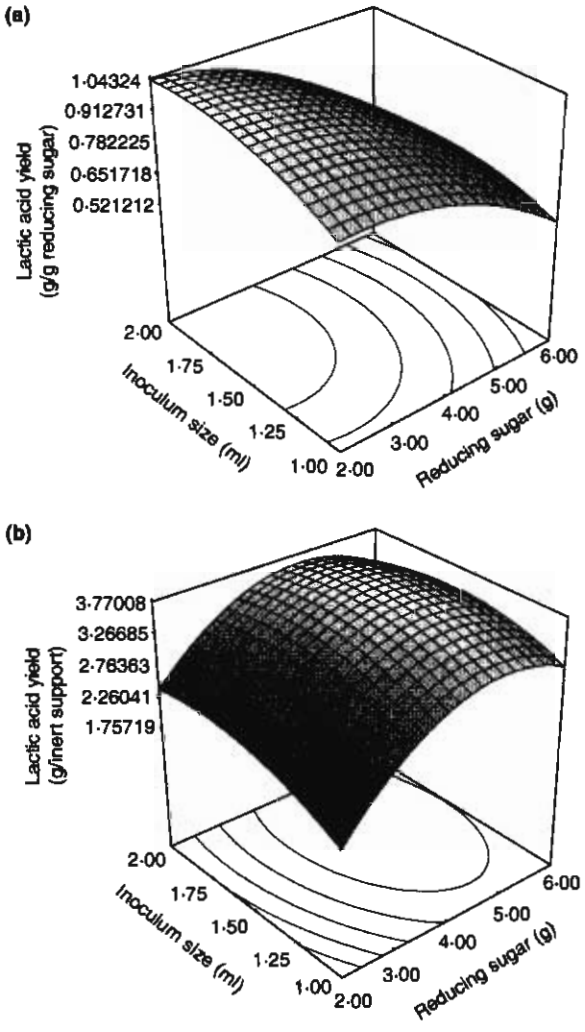

Figure 1 Influence of reducing sugar and inoculum volume on lactic acid production. (a) Lactic acid yield (g g^{-1} reducing sugar), (b) total lactic acid yield (g g^{-1} inert support). Total volume of medium used in each run kept as 12 ml by concentrating the cassava starch hydrolysate and nutrient mixture to get appropriate concentration.

accumulation as is the case with inoculum size (Fig. 1). Results presented in Fig. 3 shows the interaction between the concentration of nutrient mixture and inoculum size. Higher concentration of inoculum always necessitated a higher nutrient concentration, and thus lactic acid yield increased with increase in either of the above two variables. However, no significant increase in lactic acid yield was observed at highest levels of inoculum size and nutrient mixture. From all the response surface graphs, it was concluded that 1·5 ml of 18-h-old inoculum, which contains $1·5 \times 10^9$ CFU ml^{-1}, a reducing sugar concentration of 4 g and 6·5 ml of nutrient mixture were optimum for the maximum lactate yield.

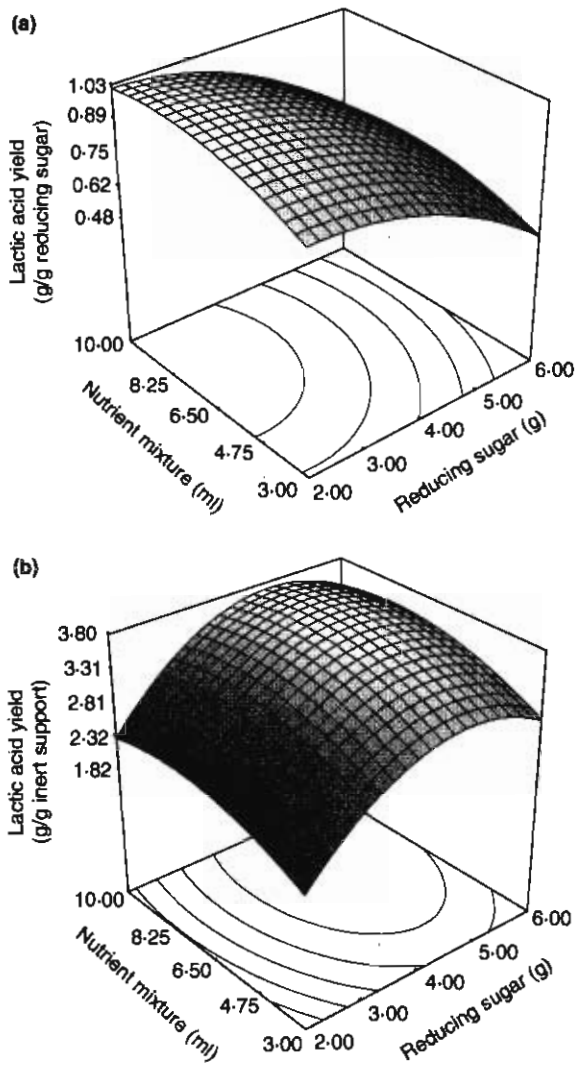

Figure 2 Influence of reducing sugar and nutrient mixture volume on lactic acid production. (a) Lactic acid yield (g g^{-1} reducing sugar), (b) total lactic acid yield (g g^{-1} inert support). Total volume of medium used in each run kept as 12 ml by concentrating the cassava starch hydrolysate and nutrient mixture to get appropriate concentration.

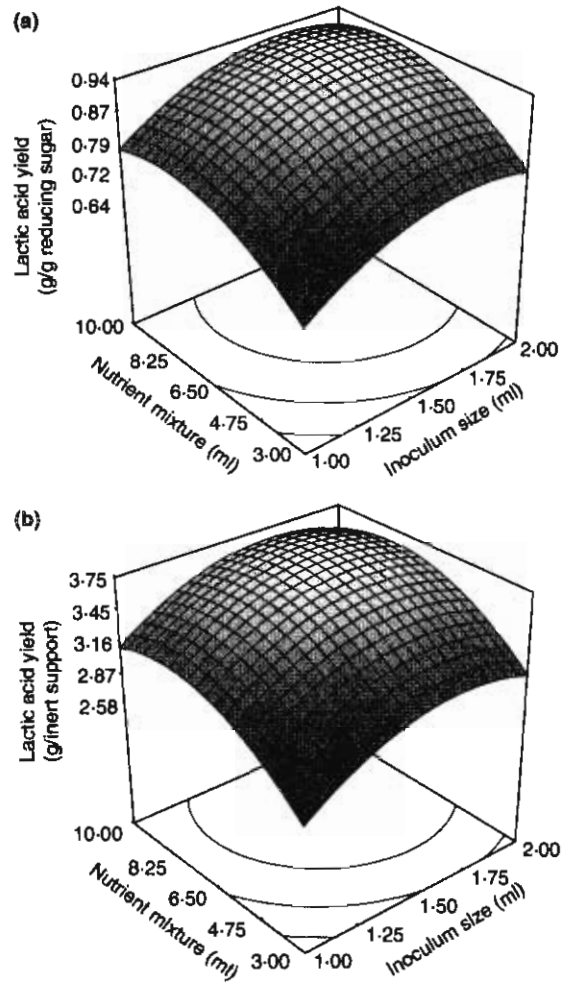

Figure 3 Influence of inoculum volume and nutrient mixture volume on lactic acid production. (a) Lactic acid yield (g g^{-1} reducing sugar), (b) total lactic acid yield (g g^{-1} inert support). Total volume of medium used in each run kept as 12 ml by concentrating the cassava starch hydrolysate and nutrient mixture to get appropriate concentration.

The scanning electron micrograph studies of fermented support showed almost uniform distribution of *L. casei* (Fig. 4) indicating that PUF cubes could provide a platform for uniform distribution of the micro-organism.

Discussion

There were hardly any cyanogenic or harmful volatile compounds in cassava bagasse as it is mostly removed during the starch extraction process. There may not be any considerable loss of reducing sugars from the hydrolysate, or some volatile amino acids present in the yeast extract used in the nutrient mixture during the mild evaporation process under vacuum and low temperature (60°C) using rotary evaporator. Initial reducing sugar level, inoculum size and the nitrogen source are key factors influencing growth and lactic acid production, and these parameters were noted initially by single parameter optimization (data not shown). Lactic acid bacteria generally have complex nutrient requirements for growth and fermentation (Rojan *et al.* 2005; John *et al.* 2006a, 2006b). Nutrient mixture containing yeast extract and NH_4Cl played a positive role in enhancing lactic acid yield as it contained the major nitrogen source and other growth stimulating factors. The growth promoters in

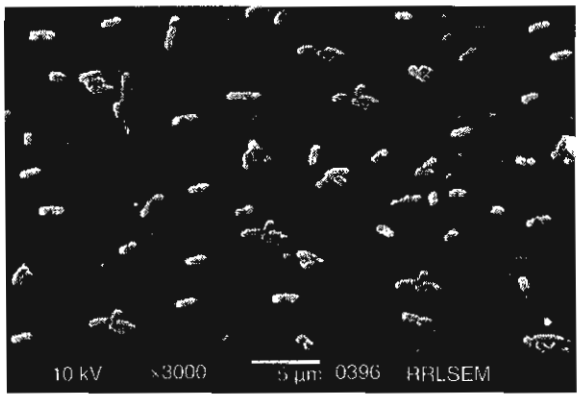

Figure 4 Scanning electron micrograph showing the distribution of bacteria on polyurethane foam at 72 h of incubation.

yeast extract, mainly vitamin B, help in the growth and hence the production of lactic acid (Nancib *et al.* 2005). However, there was a limit beyond which no further increase in lactate yield was observed, which was in confirmation with our former studies (Rojan *et al.* 2005; John *et al.* 2006a). John *et al.* 2006b reported that the lower concentration of $MnCl_2$ had positive influence on growth and lactic acid production by *L. casei*, and hence in the current nutrient mixture a low concentration of $MnCl_2$ was also used.

Soccol *et al.* (1994) reported a better production of lactic acid by *Rhizopus oryzae* under SSF using sugar cane bagasse as support in comparison to routine submerged fermentation (SmF). In SSF, lactate yield of 0·077 g g^{-1} corn fibre and 0·18 g g^{-1} wheat bran were obtained using *Lactobacillus amylophilus* GV6 (Naveena *et al.* 2005). The lactic acid yields while using *L. casei* or *Lactobacillus delbrueckii* were more than 0·95 g g^{-1} reducing sugar, after 120 h, when sugar cane bagasse impregnated with cassava starch hydrolysate was used in SSF. The yields obtained in these works range between 0·5 and 0·58 g g^{-1} sugarcane bagasse (Rojan *et al.* 2005; John *et al.* 2006a). For lactate production, there was a feed back inhibition of the product and negative influence of initial higher reducing sugar concentration as it affects the osmotic potential of the cells and develops glucose repression. The catabolite repression was reported to act at higher concentration of glucose in SmF system during the production of exopectinase. This was solved by SSF where polyurethane was used as an inert support (Diaz-Godinez *et al.* 2001). In this study, we could achieve a similar yield of 0·95–0·97 g lactic acid per gram reducing sugar like our previous reports. However, a very interesting observation was that while considering the lactate yield based on the support used, a very high yield of 3·88 g lactic acid per gram inert support was achieved. It was mainly due to the efficiency of the PUF cubes to hold greater amounts of reducing sugars per gram dry support than inert sugar cane bagasse. It might be due to the characteristic water-retaining properties of the PUF. Hence it can be concluded that PUF is a better choice as an inert support system for lactic acid production in SSF. It provides uniform dispersal of the inoculum and resulted in higher yield from the simple media. In SSF, the size of the reactor can be reduced and it needs only simple design. As the process is based on the utilization of starch from cassava bagasse, an agro industrial residue, it assures a value added, economically viable and ecofriendly process.

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