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Potential of solid state fermentation for production of L(+)-lactic acid by *Rhizopus oryzae*

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Abstract Production of L(+)-lactic acid by *Rhizopus oryzae* NRRL 395 was studied in solid medium on sugar-cane bagasse impregnated with a nutrient solution containing glucose and $CaCO_3$. A comparative study was undertaken in submerged and solid-state cultures. The optimal concentrations in glucose were 120 g/l in liquid culture and 180 g/l in solid-state fermentation corresponding to production of L(+)-lactic acid of 93.8 and 137.0 g/l, respectively. The productivity was 1.38 g/l per hour in liquid medium and 1.43 g/l per hour in solid medium. However, the fermentation yield was about 77% whatever the medium. These figures are significant for L(+)-lactic acid production.

Introduction

Lactic acid is common in nature, being found in man, animals, plants and micro-organisms. Lactic acid is widely used in the food industry as a preservative or taste-enhancing additive. Ferrous salts and the various L(+)-lactic acid salts are used in the pharmaceutical industry for their therapeutic qualities (Soccol 1992). L(+)-lactic acid can be polymerised to form polylactic acid (PLA), a polymer used in the manufacture of new biodegradable plastics (Vert et al. 1991, 1992). The latter are increasingly used in surgery for sutures (Vert 1991; Holland et al. 1986; Claes 1992). They could play an increasing role in industry in the near future because of environmental problems (Cooke 1990; Vert and Guerin 1992). They could be considered as a substitute for plastics manufactured from petroleum derivatives. Lactic acid is also used in the textile and tanning industries (Butcha 1983).

Only the L(+) form of lactic acid is metabolised in animal and human cells (Nielsen and Veibel). This is due to the fact that only L(+)-lactate dehydrogenase is synthesised by these cells. As a result, consumption of large quantities of D(−)-lactic acid will result in accumulation in the blood and hence hyper-acidity of urine and/or decalcification (Kandler 1982).

Lactic acid is prepared by culturing lactic bacteria or filamentous fungi (such as *Rhizopus*) in liquid medium (Lockwood 1975; Soccol 1992). The main purpose of the work described here was to characterise L(+)-lactic acid production by *R. oryzae* cultured on solid medium on a natural support impregnated with nutrients solution. Bagasse, a by-product of sugar-cane processing, was used as the supporting material for solid-state fermentation.

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Materials and methods

Micro-organisms

R. oryzae NRRL 395 was the main fungal strain used for the production of L(+)-lactic acid: it was kindly supplied by Dr. Kurtzman [Northern Regional Research Center (NRRL), US Department of Agriculture (USDA), Peoria, Ill., USA]. The strain was maintained by successive subcultures on potato dextrose (PDA) medium (Difco Laboratories, Detroit, Mich, USA).

Sporangiospore production

Sporangiospores were produced on PDA medium in petri dishes after 8 days of culture at 28°C. They were collected with a platinum spatula and suspended in tubes containing 20 ml sterile physiological saline solution with 0.01% Tween 80 and glass balls to extract as many spores as possible during Vortex agitation.

Culture on solid medium

Preparation of the support

Bagasse, a ligno-cellulose residue of juice extraction from sugar cane, was used as the support for solid-state fermentation. It was crushed, moulded, carefully washed, dried at 60°C and finally sieved. Only the 0.8–2.0 mm fraction was kept for use. It was then dry sterilised at 110°C for 24 h.

Preparation of the inoculum

Inoculum was prepared with the medium described by Lockwood et al. (1936). It was modified according to our requirements for certain experiments. The composition was as follows in g/l: glucose, 100; $(NH_4)_2SO_4$, 1.35; KH_2PO_4, 0.3; $ZnSO_4 \cdot 7H_2O$, 0.04; $MgSO_4 \cdot 7H_2O$, 0.25. The culture medium was placed in 250-ml erlenmeyer flasks (20 ml per flask) and autoclaved at 110°C for 30 min. Then 160 µl vitamins and trace elements solution according to Soccol (1992) was added. The pH was adjusted to 6.0 with a solution of 1 M NaOH. Each flask was incubated with a sporansgiopore suspension (10^7 spores/g glucose) and placed in a shaking incubator for 15 h at 120 rpm (G-25 Shaker incubator, New Brunswick Scientific, Edison, N.J., USA).

Fermentation

The standard fermentation medium was prepared in 1-l erlenmeyer flasks and contained 20% glucose. The medium was autoclaved at 110°C for 30 min and the pH was adjusted to 7.5 with a solution of 1 M NAOH. Each 120-g wet weight aliquot fermented (with a water content of approximately 70%) consisted of 10 g bagasse, 10 g $CaCO_3$, 80 ml fermentation medium and 20 ml inoculum. The mixture was blended using a blade beater to obtain a homogeneous fraction. Impregnated support (not more than 0.45 g/cm^3) was placed in glass column reactors (4 cm in diameter and 20 cm high). The columns containing 80 g fermenting medium were placed in an experimental set-up described by Raimbault and Alazard (1980) (Fig. 1). Fermentation proceeded for 96 h at 35°C with aeration of 1.2 l/h per column.

Culture in liquid medium

Submerged culture in flasks

The following medium composition was used for this type of culture (g/l): glucose, 120; $(NH_4)_2SO_4$, 3.02; $MgSO_4 \cdot 7H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.04; KH_2PO_4, 0.15. The medium was placed in 250-ml flasks (50 ml per flask) and autoclaved at 110°C for 30 min. The pH was adjusted to 6.0 with a solution of 1 N NaOH. Then 400 µl of the vitamins solution and 400 µl of the trace elements solution described previously were added. Flask inoculation was performed with a spore suspension and adjusted to obtain 10^7 spores per flask. The flasks were then placed in a shaking incubator (G-25 Shaker Incubator, New Brunswick Scientific). Fermentation was performed in two stages. The first phase consisted of sporangiospores germination and mycelium formation under the following conditions: duration 24 h, temperature 35°C, agitation 140 rpm. After 24 h, 4 g previously sterilised $CaCO_3$ was added to each erlenmeyer flask. Different conditions were applied to the second stage of fermentation: 48 h at 35°C with an increase in agitation (250 rpm).

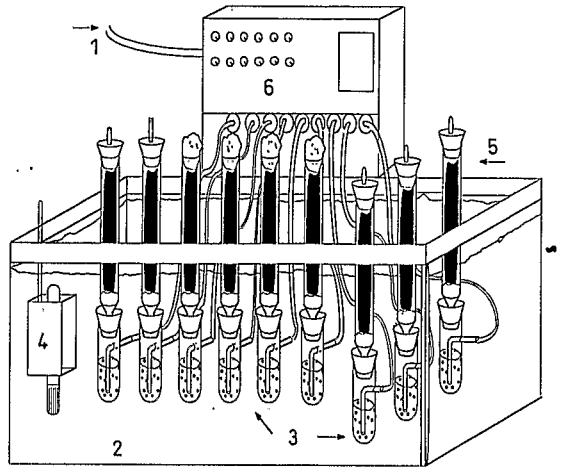

Fig. 1. Diagram of the inoculation device used for solid-state fermentation: *1*, air inlet; *2*, temperature-controlled vessel; *3*, humidifier; *4*, heating system; *5*, reactors; *6*, air-flow control

Submerged culture in a fermentor

Fermentor inoculum was prepared as for fermentation on solid medium. Incubation lasted for 24 h in most cases. The fermentation medium for production of L(+)-lactic acid consisted of (in g/l): glucose, 120; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.04. The medium was sterilised in the fermentor tank at 110°C for 30 min. The pH was adjusted to 7.0 with 1 M NaOH. The fermentation medium was always inoculated with 10% of the previously prepared starter culture and 6% $CaCO_3$. Fermentation was performed in a 2-l fermentation unit (LSL-Biolafitte Saint-Germain-en-Laye, France) for 72 h at 35°C with an aeration rate of 1.25 (1/l per minute) and agitation at 400 rpm.

Analyses

All kinetics were determined by taking a sufficiently large sample (either a predetermined quantity in liquid fermentation or a set of columns in the case of solid fermentation) for the required analyses.

In solid fermentation, the fermented material of each column was mixed with a solution of 1 M H_2SO_4. It was then placed in a press cell with 0.5–1.5 mm orifices over the whole of its surface. A hydraulic press (500 to 2,100 kg/cm^2) was used to extract concentrated liquid consisting mainly of L(+)-lactic acid, unfermented sugar, other metabolites formed (such as fumaric acid) and salts. The insoluble fraction contained the support, calcium sulphate and mycelium biomass. The biomass produced was determined by estimation of sample dry weight.

Samples of liquid fermentation were processed and analysed in the same way whatever the fermentation type. Fermented medium was strained on 200-µm mesh nylon bolter-sieve. The filtrate was centrifuged at 6,000 rpm for 10 min. Lactic acid, ethanol and fumaric acid were determined in the supernatant. The rest of the material retained by the sieve (mycelium biomass and $CaCO_3$) was washed with a solution of 2 M HCl.

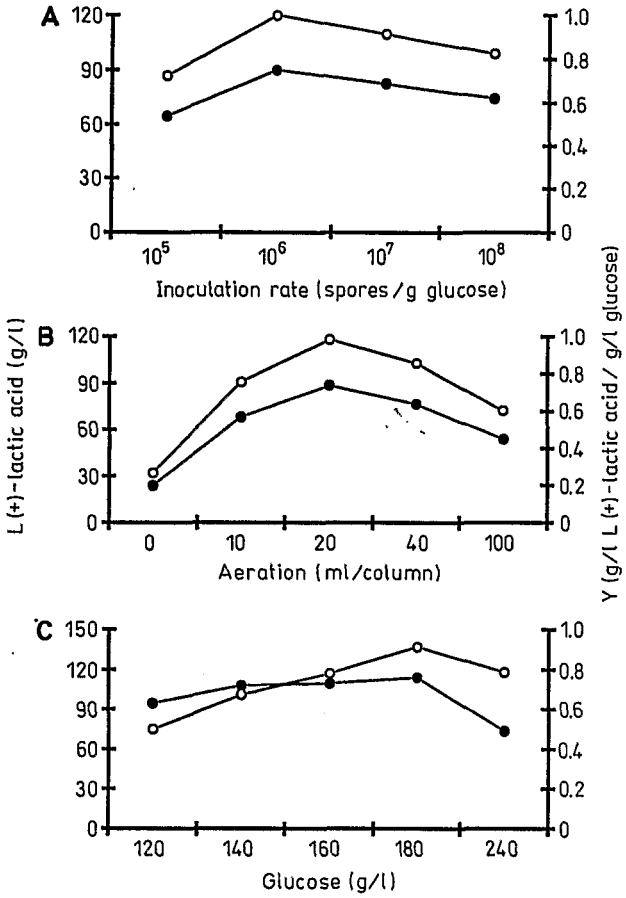

Fig. 2A–C. Effect of the various culture conditions on L(+)-lactic acid production (○) and productivity (●) *R. oryzae* NRRL 395 in solid-state fermentation. **A** Effect of inoculation rate. **B** Effect of aeration rate. **C** Effect of glucose concentration

L(+)-Lactic acid and fumaric acid were determined by HPLC using an Aminex HPX 87H column (Bio-Rad Laboratories, Paris, France) and a specific eluant according to the method developed in the laboratory (Giraud et al. 1991). Glucose was determined with the dinitrosalicylic acid (DNS) method (Miller 1959).

Results

The influence of inoculation rate on L(+)-lactic acid production by *R. oryzae* NRRL 395 cultured in solid-state fermentation has been tested. The optimal inoculation rate appeared to be 2×10^6 spores/g glucose. Under these conditions, production of L(+)-lactic acid reached 120 g/l with complete glucose uptake and 75% yield. There is a parallel between inoculation rate and production of L(+)-lactic acid. Any variation in inoculation rate caused similar variation in L(+)-lactic acid production (Fig. 2A).

In solid-state fermentation, aeration of the moistened medium is important. It controls both humidity of the solid support and heat-release of the metabolism, and

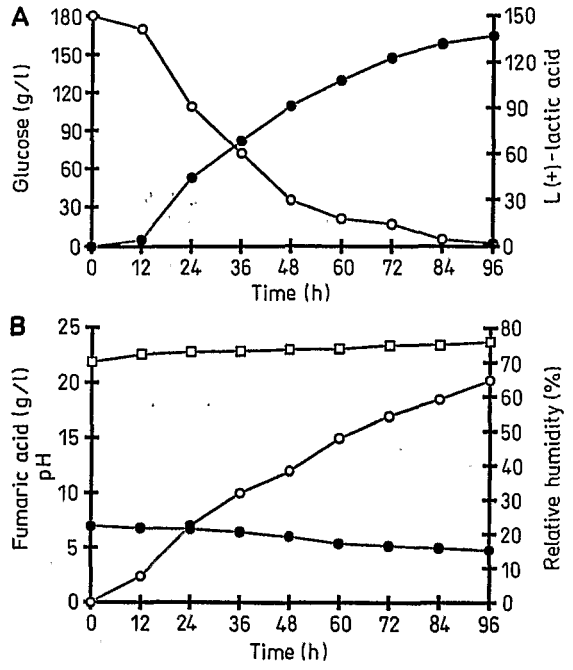

Fig. 3A, B. Kinetics of L(+)-lactic acid production by *R. oryzae* NRRL 395 in solid-state fermentation. **A** Changes in glucose uptake (○) and L(+)-lactic acid formation (●) with time. **B** Changes in relative humidity (□), pH (●) and fumaric acid formation (○) during fermentation

provides the O_2 required for growth. Maximal L(+)-lactic acid production was obtained with aeration of 20 ml/min (Fig. 2B). However, aeration of 100 ml/min per column caused a significant reduction (approximately 35%) of lactic acid production; this is related to an increase in the aerobic respiration rate of the fungus. A significant proportion of glucose was not taken up during fermentation without aeration and the L(+)-lactic acid yield decreased.

Figure 2C shows the influence of glucose concentration on L(+)-lactic acid production in solid-state fermentation. Production of L(+)-lactic acid was maximal (137 g/l) with a 180 g/l initial glucose concentration; the yield was 76%. However, increasing glucose to 240 g/l led to a decrease in L(+)-lactic acid production. Because of the partial consumption of glucose in the culture medium, a glucose concentration of about 160 g/l gave 117 g/l of L(+)-lactic acid with a fermentation yield of approximately 74%. In constrast, if the glucose concentration fell to 120 g/l, L(+)-lactic acid production fell to 75 g/l with a lower fermentation yield (63%).

The changes with time of different parameters during L(+)-lactic acid production by *R. oryzae* NRRL 395 in solid-state fermentation was analysed. Figure 3A shows the changes in glucose consumption and L(+)-lactic acid production, whereas Fig. 3B shows the variation in both pH and moisture content of the fermentation medium. The pH fell significantly during fermentation by more than two units from an initial value of 6.96 to

a final value of 4.80. A slight increase in relative humidity was observed throughout fermentation; it reached 70–76% after 96 h of culture. Similar results have been described for different biological material (Narahara et al. 1982; Oriol et al. 1988a, b). The fumaric acid concentration reached 20.24 g/l after 96 h; both L(+)-lactic acid and fumaric acid productivities were maximal during the first 24 h of fermentation.

The results obtained in submerged fermentation are reported in Fig. 4. A low production of fumaric acid (1.27 g/l) and the production of ethanol (1.68 g/l), which was not detected in the solid-state fermentation, are noteworthy.

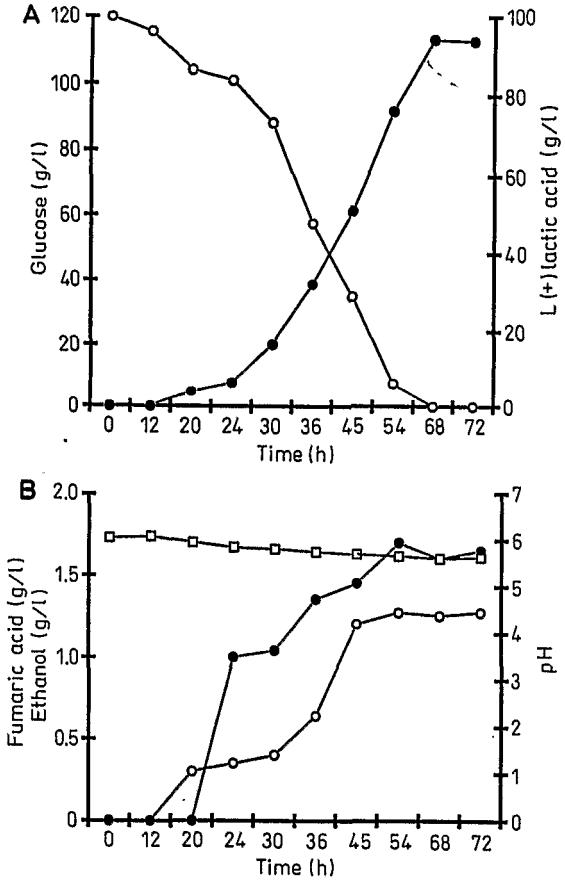

Fig. 4A, B. Kinetics of L(+)-lactic acid production by *R. oryzae* NRRL 395 in submerged culture. **A** Changes in glucose uptake (○) and L(+)-lactic acid formation (●) with time. **B** Changes in pH (□), fumaric acid (○) and ethanol formation (●) during fermentation

Discussion

The main results obtained with the different types of fermentor used in the study on the L(+)-lactic acid production are shown in Tables 1 and 2. The highest fermentation yields (78%) were obtained in liquid fermentation in 2-l fermentors and in solid-state fermentation on an impregnated support (77%). The lowest yield (74%) was obtained in erlenmeyer flasks.

Fermentation in solid medium gave a very high final L(+)-lactic acid concentration (137 g/l) whereas submerged culture in flasks and a 2-l fermentor gave only 89 and 93.8 g/l, respectively. The high level in solid medium is explained by the use of higher substrate concentrations (mainly glucose), which is hardly possible in submerged fermentation. The yield was comparable with some 15% higher productivity. In spite of its longer duration (96 h), fermentation in solid medium gave the highest L(+)-lactic acid production rate (1.43 g/l per hour) followed by the 2-l fermentor (1.38 g/l per hour) and finally the flask fermentation (1.24 g/l per hour).

Fumaric acid production was important in solid fermentation (20.24 g/l). It was much lower in submerged fermentation in flasks (2.1 g/l) and in 2-l fermentors (1.27 g/l). The culture conditions caused synthesis of this organic acid. Closer investigation of the biochemical

Table 1. Comparison of physical (aeration and agitation) and chemical (pH) conditions in relation to fungal biomass production by *Rhizopus oryzae* NRRL 395 in different fermentations

Fermentation type	Time (h)	Aeration (v/v/h)	Agitation (rpm)	pH (final)	Biomass (g/l)
Flask	72.00	ND	250.00	5.60	3.16
Fermenter (2-l)	68.00	75.00	400.00	5.63	3.49
Solid-state fermentation (column)	96.00	4.80	0.00	4.80	ND

ND, Not determined

Table 2. Comparison of metabolite production by *R. oryzae* NRRL 395 in different fermentors

Fermentor type	Time (h)	Glucose (g/l)		L(+)-lactic acid (g/l)	Fumaric acid (g/l)	Ethanol (g/l)	Y	r (g/lh)
		Initial	Final					
Flask	72.00	120.00	0.00	89.00	2.1	2.35	0.74	1.24
Fermentor (2-l)	68.00	120.00	0.00	93.80	1.27	1.65	0.78	1.38
Solid-state fermentation column	96.00	180.00	2.50	137.00	20.24	0	0.77	1.43

Y, yield [g/l L(+)-lactic acid/g/l glucose]; r, producivity; V, volume

processes generating this acid would be necessary for a better understanding of the metabolic pathways involved. Lactate-dehydrogenase activity is related to the presence of its substrate, pyruvic acid, and also to that of the cofactors that are indispensable in the reaction it catalyses. The absence of reduced cofactors (such as $NADH_2$) or the degree of cytochrome oxidation may limit the reaction. Other metabolic pathways are involved in ethanol production.

The final pH was practically the same for fermentation in flasks and 2-l fermentors (5.60 and 5.63, respectively). It was nevertheless lower (approximately 4.8) in solid fermentation. Membrane processes are involved in proton translocation between the cell medium and the outside medium. The nature of the proton-pumping activities concerned should be characterised in relation to L(+)-lactic acid production. This type of approach has been used in numerous studies of lactic bacteria, for example.

Ethanol was not produced during solid fermentation. Presence of even small amounts of ethanol in liquid fermentation in flasks (2.35 g/l) and in 2-l fermentors (1.27 g/l) indicates a change in the fungal metabolism in liquid fermentation.

This preliminary study on L(+)-lactic acid production requires a physiological and biochemical approach for a better understanding of the contribution of the enzymes of the metabolic pathway (or pathways) leading to the synthesis of L(+)-lactic acid. An NAD^+-dependent lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) catalysing the reduction of pyruvate into lactate is certainly one of the fundamental enzymes involved. Better understanding of the formation of both ethanol and fumaric acid are required. The latter is often formed by a shuttle between pyruvic acid and oxaloacetic acid involving a carboxylation reaction. One of the major advantages of solid-state fermentation in comparison with submerged fermentation is that higher substrate concentrations can be used (especially glucose).

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