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ORIGINAL PAPER

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Optimisation of media and cultivation conditions for L(+)(*S*)-lactic acid production by *Lactobacillus casei* NRRL B-441

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Abstract Process variables and concentration of carbon in media were optimised for lactic acid production by *Lactobacillus casei* NRRL B-441. Lactic acid yield was inversely proportional to initial glucose concentration within the experimental area (80–160 g l^{-1}). The highest lactic acid concentration in batch fermentation, 118.6 g l^{-1}, was obtained with 160 g l^{-1} glucose. The maximum volumetric productivity, 4.4 g l^{-1} h^{-1} at 15 h, was achieved at an initial glucose concentration of 100 g l^{-1}. Similar lactic acid concentrations were reached with a fedbatch approach using growing cells, in which case the fermentation time was much shorter. Statistical experimental design and response surface methodology were used for optimising the process variables. The temperature and pH optima for lactic acid production were 35 °C, pH 6.3. Malt sprout extract supplemented with yeast extract (4 g l^{-1}) appeared to be an economical alternative to yeast extract alone (22 g l^{-1}) although the fermentation time was a little longer. The results demonstrated both the separation of the growth and lactic acid production phases and lactic acid production by non-growing cells without any nutrient supplements. Resting *L. casei* cells converted 120 g l^{-1} glucose to lactic acid with 100% yield and a maximum volumetric productivity of 3.5 g l^{-1} h^{-1}.

Introduction

Lactic acid has traditionally been employed in the food industry as a preservative, pH regulator and taste-enhancing additive. However, the synthesis of biodegradable polylactic and acrylic acids is a significant growing market area for pure lactic acid. Poly-L(+)-lactides are currently used for high cost medical implants; and, in order to expand the markets for large scale applications of biodegradable polymers, it is necessary to minimise the bioprocess costs and optimise the process conditions.

Raw materials represent a large part of the bioprocess costs for bulk chemicals. Lactic acid productivity by *Lactobacillus* sp. has been shown to be directly proportional to nitrogen supplementation (Chiarini et al. 1996; Yoo et al. 1997). Yeast extract especially, which is commonly used as a nutritive supplement for lactic acid bacteria, is a costly source of nitrogen and growth factors. According to Tejayadi and Cheryan (1995), the cost of yeast extract alone formed about 38% of the total production costs, when unpurified 85% lactate was produced from whey by *L. bulgaricus* using 10 g l^{-1} yeast extract as a nitrogen source. The use of renewable byproducts from agriculture, such as malt sprouts, could markedly lower the production costs. Further, the use of immobilised, non-growing cells would be an elegant solution for improving process profitability. With resting, non-growing cells, lactic acid can be produced from glucose without any nitrogen supplementation.

Batch fermentation is still the most commonly used method in industrial lactic acid production, although it is limited to relatively low productivity due to end-product inhibition (Borzani et al. 1993). Recently, a lot of effort has been targeted to developing continuous lactic acid production processes, mostly by using cell-recycle reactors (Zhang and Cheryan 1994; Tejayadi and Cheryan 1995; Olmos-Dichara et al. 1997).

L. casei is a homofermentative *Lactobacillus* strain which produces L(+)-lactic acid in excess to D(–)-lactic acid (Rogosa 1974). In our previous work (Hujanen and Linko 1996) we screened 17 lactic acid bacteria for their ability to produce lactic acid from glucose and selected *L. casei* NRRL B-441 for further studies. We also demonstrated the use of malt sprout extract as an inexpensive

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and effective nitrogen source for lactic acid production in shake-flask cultures. One objective of the present work was to optimise the batch production of lactic acid by *L. casei* NRRL B-441 in bioreactors using malt sprout extract as the main nitrogen source and varying concentrations of glucose as the carbon source. Also, the effect of carbon concentration in media was studied. Due to the large amount of solid particles in sprout extract, which made dry weight analysis difficult, yeast extract was used as nitrogen source during the temperature and pH optimisation using response surface methodology and statistical experimental analysis. Lactic acid production by non-growing cells from glucose without nutrient supplements and the suitability of the fed-batch technique in lactic acid production were also investigated.

Materials and methods

Micro-organism and culture conditions

L. casei NRRL B-441 was used throughout the study. The stock culture was maintained at –80 °C in Nunc cryoampules on plastic beads in MRS medium (Difco, USA) supplemented with 10% glycerol. Precultures for bioreactor cultivations were grown in MRS broth at 37 °C. At least three precultures were cultivated before fermentation in the bioreactor. Inocula were centrifuged before use in order to remove the MRS medium and the cell pellet was suspended to sterile medium and transferred to the bioreactor.

Media

MRS medium (Difco, USA) was used in all precultures. The basic medium for fermentations consisted of (per litre of distilled water) 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.05 g $MnSO_4 \cdot 4\ H_2O$, 0.5 g sodium acetate, 1.5 g KH_2PO_4, 1.5 g K_2HPO_4 and varying amounts of glucose, yeast extract and/or malt sprout extract. In the response surface optimisation experiments, the glucose amount was 100 g l^{-1} and the yeast extract concentration was 10 g l^{-1}. When lactic acid was produced by resting cells, the production medium consisted of (per litre of distilled water) 40 g glucose, 0.01 g $MgSO_4$ and 0.01 g MnSO4.

Preparation of malt sprout extract

Malt sprouts (0.04 g nitrogen g^{-1} dry malt sprouts; Raisio Group, Finland) were agitated at 215 rpm with distilled water for 30 min at 28 °C. The resulting mixture was then autoclaved (20 min, 121 °C) and the liquid was collected by filter press. Based on our earlier results (Hujanen and Linko 1994), 53.8 g dry malt sprouts l^{-1} (corresponding to the nitrogen content of 22 g yeast extract l^{-1}) were used per 100 g glucose l^{-1}.

Bioreactor cultivations

Cultivations with growing cells were carried out in a Biostat MD (B. Braun Melsungen, Germany) stirred-tank reactor with a working volume of 1.5 l. The pH was maintained at the desired value by the addition of 6 M NH_3 or NaOH. The cultures were not aerated and the stirring speed was 200 rpm. In the optimisation experiments, yeast extract was used as the only nitrogen source.

Lactic acid production with resting *L. casei* cells was studied in a Biostat Q multiple fermentor unit. Before the lactic acid production phase, the bacteria were cultivated for 24 h in 1 l MRS to reach a sufficient biomass. This was then centrifuged and washed once with sterile water. The biomass concentration used was 5.8 g l^{-1}. The fermentation was performed at optimum conditions (35 °C, pH 6.3). The initial glucose concentration was 20 g l^{-1} and 50 g glucose l^{-1} was added twice when the glucose concentration of media fell below 5 g l^{-1} (monitored by a glucose analyzer; YSI, Ohio, USA). Fermentations were made in duplicate.

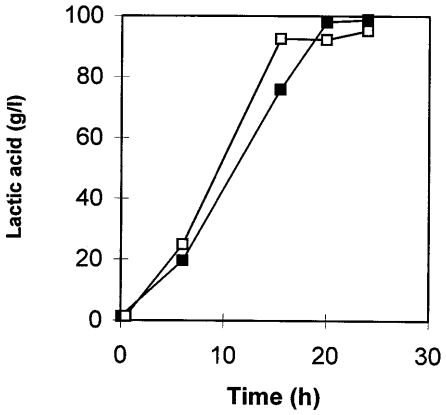

Fig. 1 Production of lactic acid by *Lactobacillus casei* in a 2-l bioreactor with either □ yeast extract (22 g l^{-1}) or ■ malt sprout extract supplemented with 4 g yeast extract l^{-1}, as the nitrogen source

Experimental design and methods

A central composite circumscribed 2^2 experimental design was used, with two variables, temperature and pH, four star points and four replicates at the central point, resulting in a total amount of 12 experiments. Modde for Windows, version 4.0 (Umetri, Sweden) software was used for statistical experimental design, for analysis of the results and for drawing the response surface. The model was fitted using a multiple linear regression.

Analytical methods

Lactic acid and glucose were determined by HPLC using an Aminex HPX-87H^+ cation-exchange column (BioRad Laboratories, USA) at a column temperature of 40 °C with UV- and IR-detectors. The eluent was 0.5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. The injection volume of the sample was 20 µl. When samples contained sprout extract, residual glucose was determined according to Nelson (1944). Biomass was determined as dry weight by centrifuging 10 ml of fermentation media, washing once with distilled water and drying at 115 °C overnight. The consumption of glucose and the production of lactic acid was also monitored by a YSI 2700 Select Biochemistry Analyzer (YSI, Ohio, USA).

Results

Effect of nitrogen source and growth factors

Malt sprout extract was compared to yeast extract as the main source of nitrogen and growth factors when lactic acid was produced by *L. casei* NRRL B-441 with 100 g glucose l^{-1} as the carbon source. The results are presented in Fig. 1. The maximum lactic acid concentration with the malt sprout medium was 98.8 g l^{-1} as compared to 95.3 g l^{-1} with yeast extract (22 g l^{-1}) as the only nitro-

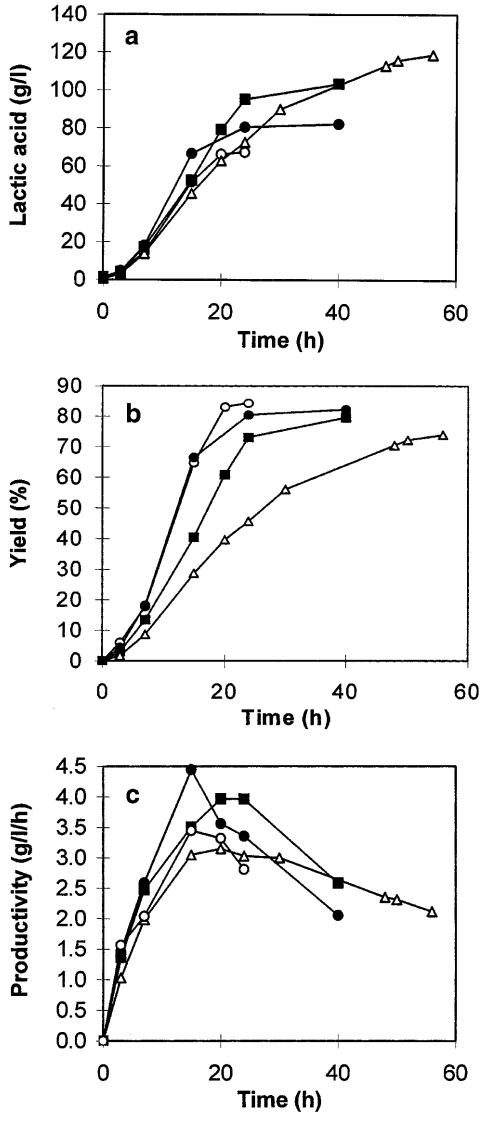

Fig. 2a–c Lactic acid (**a**) concentration, (**b**) yield and (**c**) volumetric productivity (grams per litre per hour, *g/l/h*) as a function of time with different concentrations of initial glucose: ○ 80 g l^{-1}, ● 100 g l^{-1}, ■ 130 g l^{-1} and Δ 160 g l^{-1}

gen source. However, the consumption of glucose was a little slower during the last 40 g l^{-1} of glucose consumed; and the production rate of lactic acid in the beginning was a little lower than in the case of yeast extract medium. Nevertheless, in both cases the maximum lactic acid concentration was obtained after 24 h cultivation, when all of the glucose had been consumed.

Effect of initial glucose concentration in batch fermentations

The batch production of lactic acid by *L. casei* NRRL B-441 was studied using malt sprout extract as the main nitrogen source and varying concentrations of glucose as the carbon source. Figure 2a shows that an increase in the initial glucose level resulted in an increase in the final lactic acid concentration. A maximum of 118.6 g lactic acid l^{-1} was obtained with 160 g glucose l^{-1}, resulting in a 74% yield. Figure 2b shows the yield as a function of time in five typical fermentations with different concentrations of initial glucose. The highest yield of 84% was achieved with an initial concentration of 80 g glucose l^{-1}. The final yield was almost inversely proportional to the initial substrate concentration within the experimental area. When glucose concentration was 120 g l^{-1} or less, a yield of at least 80% could be achieved. However, the product yield varied only slightly.

Figure 2c illustrates the volumetric productivity as a function of time. The highest volumetric productivity (4.4 g l^{-1} h^{-1}) was reached at 15 h with 100 g l^{-1} initial glucose. In most cases, the highest productivity was achieved in 15 h. Thereafter, the productivity started to decrease gradually; and with 130 g glucose l^{-1} and 160 g glucose l^{-1} maximum productivity was obtained at 20 h.

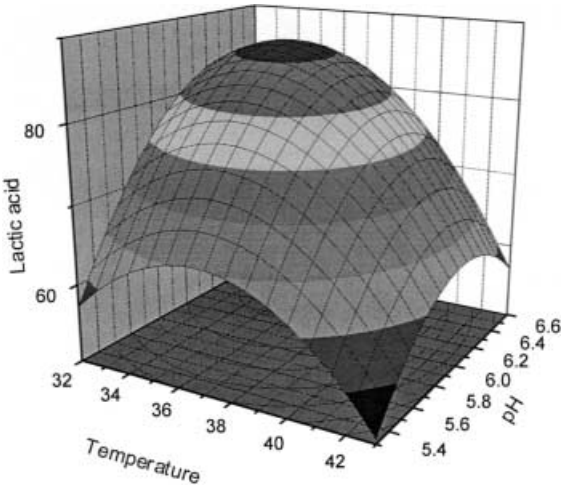

Fig. 3 Lactic acid concentration as a function of pH and temperature and represented as a response surface

Optimisation of pH and temperature by response surface methodology

The multiple regression equation for the concentration of lactic acid at 25 h cultivation, *Y*, by *L. casei* NRRL B-441 is the following, using pH and temperature (*T*)as variables:

$$Y=83.275+5.506\ \mathrm{pH}-4.242\ T-2.45\ \mathrm{pH}T-5.22\ \mathrm{pH}^2-4.544\ T2$$

Based on the results, a quadratic model for final lactic acid concentration was calculated. A contour plot produced according to the model (R^2=0.848, Q^2=0.601) is presented in Fig. 3. Both pH and temperature significantly affected the production of lactic acid by *L. casei* NRRL B-441. As can be seen from the contour plot, the optimal values of the independent variables investigated were approximately pH 6.3 and a temperature of 35 °C.

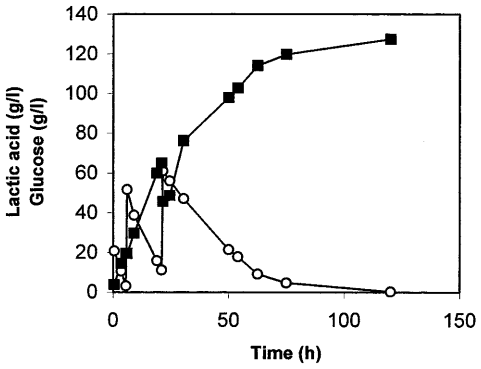

Fig. 4 Lactic acid production by resting cells of *L. casei* NRRL B-441. Concentration of glucose (○)and lactic acid (■)

Fed-batch fermentation with resting cells

The use of immobilised resting cells could markedly improve the economy of the lactic acid production process. Because the nitrogen source is the most expensive substrate, its omission from media at the lactic acid production phase would bring substantial savings. The production of lactic acid from pure glucose without nutrient supplement with resting *L. casei* NRRL B-441 cells is demonstrated in Fig. 4. Each result shown is the average of two fermentations. Approximately all glucose could be converted to lactic acid without expensive growth supplements. The maximum volumetric productivity of the process was 3.53 g l^{-1} h^{-1}; and the total fermentation time was 120 h. The method for preparing pure lactic acid with resting cells is patented (Viljava and Koivikko 1997).

Discussion

Göksungur and Güvenç (1997) studied alternatives for yeast extract as a nitrogen source in lactic acid production and showed malt sprout to be the best, from seven alternative nitrogen sources studied. However, malt sprouts yielded a markedly lower final lactic acid concentration than did yeast extract. In our experiments, the amount of yeast extract could be reduced from 22 g l^{-1} to 4 g l^{-1} without any decrease in lactic acid concentration in the presence of the more economical malt sprout extract.

Theoretically, in homolactic fermentation 2 mol lactic acid are produced from 1 mol glucose. However, 100% conversion is rarely achieved (Buchta 1983). Initial glucose concentrations as high as 200 g l^{-1} have been used in some industrial lactic acid fermentations as a carbon source. Goncalves et al. (1991) used glucose concentrations up to 300 g l^{-1} in studies on the substrate and product inhibition kinetics of *L. casei* ssp. *rhamnosus.* In more recent studies, the initial amount of glucose employed has usually been less than 100 g l^{-1}. However with higher initial glucose levels, the final lactic acid concentration in the medium is higher and the product requires less concentration. In the present work, the effect of initial glucose concentration on lactic acid production by *L. casei* was examined within the range 80–160 g l^{-1}, using malt sprouts as the main nitrogen source in 2-l bioreactors. The final lactic acid concentration increased with an increase in the initial glucose level. The highest lactic acid concentration in batch fermentation, 118.6 g l^{-1}, was obtained with 160 g glucose l^{-1} (84% yield). The results are in good agreement with Göksungur and Güvenç (1997), who studied lactic acid production from beet molasses with initial sugar concentrations of 28.2–107.2 g l^{-1}. In those experiments, the final lactic acid levels increased with increasing sugar level. Goncalves et al. (1991) studied substrate inhibition kinetics in lactic acid production using a glucose range of 50–340 g l^{-1}. They claimed that final lactic acid concentration increased with increasing initial glucose concentration, up to 200 g l^{-1}. A maximum of 140 g lactic acid l^{-1} was reached with 200 g initial glucose l^{-1}, resulting in a 70% yield.

As can be seen from Fig. 2a, the fermentation time also increased with the increase in glucose concentration. With 160 g initial glucose l^{-1}, the time required for the maximum lactic acid concentration was 56 h, in comparison to the 30 h and 24 h for initial glucose levels of 100 g l^{-1} and 80 g l^{-1}, respectively. In the present work, at each glucose concentration studied, practically all substrate was consumed by the end of the fermentation.

It is important not only to achieve a maximum substrate utilisation but, more importantly, a high yield, in order to minimise feedstock costs. The yields obtained using malt sprout extract as the main nitrogen source were in good agreement with the results of other research groups using yeast extract. For example, Vaccari et al. (1993) obtained product yields of 82–85% with *L. casei*, when the initial glucose concentrations varied over 20–120 g l^{-1} and 30 g yeast extract l^{-1} was used as the nitrogen source.

In our study, the productivities were calculated from the start of the cultivation (lactic acid concentration divided by fermentation time). The maximum productivity with yeast extract alone was 6.0 g l^{-1} h^{-1} at 16 h fermentation time, in comparison to the 4.9 g l^{-1} h^{-1} at 16 h obtained with malt sprout extract. Maximum volumetric productivity with resting cells was 3.5 g l^{-1} h^{-1}. Specific productivities with growing cells and malt sprout extract could not be determined, due to solid particles in the malt sprout extract. However, the maximum specific productivity of resting *L. casei* cells was 0.6 g lactic acid g^{-1} cells h^{-1}.

The effect of temperature and pH on lactic acid production by *L. casei* NRRL B-441 in the batch fermentations was studied using response surface methodology. According to the regression model, the maximum lactic acid concentration was reached at pH 6.3 and a temperature of 35 °C. The optimum temperature defined by the model is slightly lower than was reported earlier (Hujanen and Linko 1996). Also, Oh et al. (1995) determined 35 °C as the optimum growth temperature for

L. casei, using response surface methodology and glucose as a carbon source. In their study of five factors, the incubation temperature most strongly affected the growth of *L. casei*. In contrast, the results differ slightly from those by Rincon et al. (1993), who optimised the fermentation of whey by *L. casei*. They found the maximum lactic acid production rate at pH 5.4, 38 °C. This is probably due to differences in the media and carbon sources used.

Lactic acid bacteria are very fastidious microorganisms. They are known to have limited biosynthetic ability, thus requiring multiple amino acids and vitamins for growth (Rogosa 1974). These growth factors are usually supplied by a complex nitrogen source like yeast extract. The part of nutrients that is not bound to biomass has to be removed from the product during a downstream process. However, the growth phase of lactic acid bacteria can be separated from the actual fermentation, to establish discrete growth and production phases. Lactic acid could also be produced by resting *L. casei* cells from pure glucose without growth supplements. All of the glucose, 120 g l^{-1}, in the production phase was converted to lactic acid. The production of lactic acid by resting cells with refreshing cycles has recently been patented (Viljava and Koivikko 1997).

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