Appl Microbiol Biotechnol (1996) 45:307–313

ORIGINAL PAPER

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Effect of temperature and various nitrogen sources on L(+)-lactic acid production by *Lactobacillus casei*

Received: 25 September 1995/Accepted: 24 October 1995

Abstract Two homofermentative strains, *Lactobacillus casei* NRRL B-441 and *Lactobacillus casei* subsp. *rhamnosus* NRRL B-445 were selected for further study from 17 lactic acid bacterial strains screened for lactic acid production. The effect of temperature on lactic acid production with the selected strains was investigated by adapting both strains to four different temperatures. The production of L(+)-lactic acid by both strains was most efficient at 37°C, although with *L. casei* the highest lactic acid concentration was obtained at 41°C. The maximal volumetric productivity with *L. casei* was 4.1 $g l^{-1} h^{-1}$ and with *L. casei* subsp. *rhamnosus* 3.5 $g l^{-1} h^{-1}$. The composition of the medium was studied in order to replace the costly yeast extract with less expensive sources of nitrogen and amino acids. From 11 different nitrogen sources investigated at 37°C, barley malt sprouts (88 $g l^{-1}$ lactic acid in 66 h) and grass extract (74 $g l^{-1}$ lactic acid in 73 h) were the best economic alternatives. The effect of different combinations of yeast extract, peptone and malt sprouts was further studied by using statistical experimental design, and an empirical second-order polynomial model was constructed on the basis of the results. With the right combination most of the yeast extract could be substituted by barley malt sprouts for efficient lactic acid production. A method for extraction of nutrients and growth factors from malt sprouts is also described.

Introduction

Lactic acid, the first biotechnically produced chemical, has a wide range of applications in food, pharmaceutical and cosmetics industries. Because of its asymmetric carbon atom lactic acid exists in three forms and is a good substrate for organic syntheses (Mattey 1992). Recently, a great interest in biodegradable lactide polymers has accelerated research on the production of pure L(+)- or D(−)-lactic acid as bulk raw material, and efforts have been made to enhance the productivity and economy of the L(+)- or D(−)-lactic acid production processes.

Strain screening and development for industrial lactic acid fermentation have been studied recently by Tsai et al. (1993) and Venus et al. (1992). Monteagudo et al. (1993) determined the best nutrient medium for the production of L-lactic acid from the beet molasses. The variables used in the study were concentrations of yeast extract, peptone, Tween 80, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$ and K_2HPO_4/KH_2PO_4. Among them yeast extract and peptone were found to affect the cell concentration significantly. The optimum amount of nitrogen in the medium for producing lactic acid by *Lactobacillus casei* has been previously reported by Hujanen and Linko (1994). Borzani et al. (1993) has studied the effect of initial yeast autolysate concentration on the production of lactic acid on whey medium by *Lactobacillus bulgaricus*. A comprehensive review of the use of immobilized lactic acid bacteria in lactic acid production has been published by Linko (1985).

In the present paper 17 lactic acid bacterial strains were screened for lactic acid production, and 2 were selected for further studies. Their optimum temperature for lactic acid production was investigated, different sources of nitrogen and growth factors were tested under the optimal conditions, and the combinations of the three most promising nitrogen sources, yeast extract, peptone and malt sprouts, were further examined using a central composite statistical experimental design. Since solid sprouts would be difficult to handle in continuous fermentations, a method for producing sprout extract was also developed.

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Materials and methods

Microorganisms

Seventeen homofermentative lactic acid bacterial strains of the genus *Lactobacillus*: *L. casei* NRRL B-441; *L. casei* subsp. *rhamnosus* ATCC 393, NRRL B-445, TKK-105-8-5; *L. plantarum* TKK-105-9-8, TKK-105-9-9, NRRL B-531, ETH B-4257, ATCC 8014, DSM 20314; *L. helveticus* TKK 105-13-1, *L. fermentum* TKK-105-2-1 and NRRL B-1932; *L. lactis* TKK-105-14-1; *L. amylovorus* DSM 20532 and NRRL B-4542; and *L. delbrueckii* ATCC 9649, were screened for their efficiency in the production of lactic acid. Bacteria were stored in deMan, Rogosa and Sharpe (MRS) broth with glycerol at $-80°C$, and precultures were grown in MRS broth at 37°C for 16 h. At least three generations of precultures were required before fermentation. The inoculum was usually centrifuged before use. When the effect of temperature was investigated, the inoculum was adapted to the cultivation temperature during five generations.

Media

Medium in the screening experiment contained, per litre of distilled water, 70 g glucose, 10 g trypticase, 5 g yeast extract, 3 g tryptose, 3 g K_2HPO_4, 3 g KH_2PO_4, 1 g sodium acetate, 200 mg cysteine hydrochloride, 50 g $CaCO_3$, 5 ml salt solution and 1 ml Tween 80. The salt solution contained per 100 ml distilled water: 11.5 g $MgSO_4 \cdot 7H_2O$, 0.68 g $FeSO_4 \cdot 7H_2O$ and 2.4 g $MnSO_4 \cdot 4H_2O$.

The basic medium contained per litre of distilled water: 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$, 0.5 g sodium acetate, 1.5 g KH_2PO_4, 1.5 g K_2HPO_4 and various amounts of glucose, calcium carbonate, yeast extract or another source of nitrogen and growth factors.

When the effect of nitrogen sources was examined, the initial glucose concentration was 100 g l^{-1} and 1.0 ml vitamin solution containing (per 100 ml 20% ethanol) 0.2 g vitamin B_6, 0.1 g niacin, 0.1 g calcium pantothenate, 0.1 g riboflavin, and 0.1 g folic acid was used. The tested nitrogen sources were yeast extract, malt sprouts, peptone, grass extract, N-Z-case plus, corn-steep liquor, N-Z-amine YT, casein hydrolysate, distillers waste, a mixture of $(NH_4)_2HPO_4$ and $NH_4H_2PO_4$ (1 : 2) and urea. The nitrogen contents analysed are given in Table 1. The quantity of nitrogen in the medium was 0.22%, which has been previously shown to be optimal for lactic acid production by *L. casei* when yeast extract is used as the nitrogen source (Hujanen and Linko 1994). In experiments at different temperatures the initial glucose concentration was 90 g l^{-1} and the yeast extract concentration 22 g l^{-1}. The experiments were carried out in triplicate.

Screening

The screening was done in three replicates in a working volume of 20 ml in 30-ml test-tube shake cultures. The medium used for all strains was a modified MRS solution with 70 g l^{-1} glucose. Lactic acid produced was neutralized by $CaCO_3$. The cultivation temperature was 37°C, the shaking speed 150 rev min^{-1} and the inoculum size 10%. The inoculum was not centrifuged. The cultivation conditions and medium were same for all strains and not optimized for individual strains.

Fermentations

Cultivations were carried out in 100 ml working volume in 250-ml conical flasks. Lactic acid was neutralized by an equivalent amount of $CaCO_3$. The cultivation temperatures used were 30°C, 37°C, 41°C and 45°C, generally at 37°C, unless stated otherwise. The shaking speed was 150 rev min^{-1}. The inoculum size was 20% and the inoculum was centrifuged before use.

Statistical experimental design

The statistical experimental design (Box and Hunter 1957) with six star points and four replicates at the centre-point was used to find the optimal concentrations of the three selected nitrogen sources. The three independent variables used at five different levels were yeast extract (X_1; 0, 0.7, 2.5, 4.3 and 5 g l^{-1}), peptone (X_2; 0, 0.7, 2.5, 4.3 and 5 g l^{-1}) and malt sprouts (X_3; 0, 15.8, 53.8, 91.8 and 107.6 g l^{-1}) concentrations. Statistical examination of the results and a response surface study were carried out by using the statistical programme package SYSTAT 5.01 (Systat Inc., Evanston, Ill. USA). The polynomial models used were of the form $Y = B_0 + \Sigma B_i x_i + \Sigma\Sigma B_{ij} x_i x_j$.

Preparation of the malt sprout extract

The effect of grinding the malt sprouts for lactic acid production was investigated by using ground and unground sprouts directly in the medium or the malt sprout extract after one or two extractions with water. Extraction was carried out by shaking the malt sprouts with water for 30 min at 28°C at a shaking speed of 215 rev min^{-1}. The resulting mixture was autoclaved for 20 min at 121°C, 1.1 atm (111.4 kPa) and the liquid was separated by filter press.

Analytical methods

Lactic acid was analysed by HPLC with an Aminex HPX-87H^+ cation-exchange column (BioRad Laboratories, USA) using a column temperature of 65°C, 0.04 M H_2SO_4 as the eluent at a flow rate of 0.8 ml min^{-1}, and a UV detector (Dr. Herbert Knauer Wissenschaftliche Geräte KG, Germany). Glucose was determined according to Nelson (1944) and nitrogen by the Kjeldahl method using the Kjeltec system 1026 (Tecator, Höganäs, Sweden).

Results

Screening

Seventeen homofermentative strains were screened for their lactic acid production at 37°C. In this screening the best lactic-acid-producing strains were *L. casei* NRRL B-441, *L. casei* subsp. *rhamnosus* NRRL B-445 and *L. plantarum* TKK-105-9-9, each with a 100% yield. Also the strains *L. casei* subsp. *rhamnosus* ATCC 393 (yield 95%), *L. plantarum* NRRL B-531 (yield 97%) and *L. plantarum* ATCC 8014 (yield 87%) showed good potential as lactic acid producers at 37°C. *L. casei* NRRL B-441 and *L. casei* subsp. *rhamnosus* NRRL B-445 were selected for further research because of their excellent lactic acid production potential.

Effect of temperature

L. casei NRRL B-441 and *L. casei* subsp. *rhamnosus* NRRL B-445 were adapted to four different temperatures

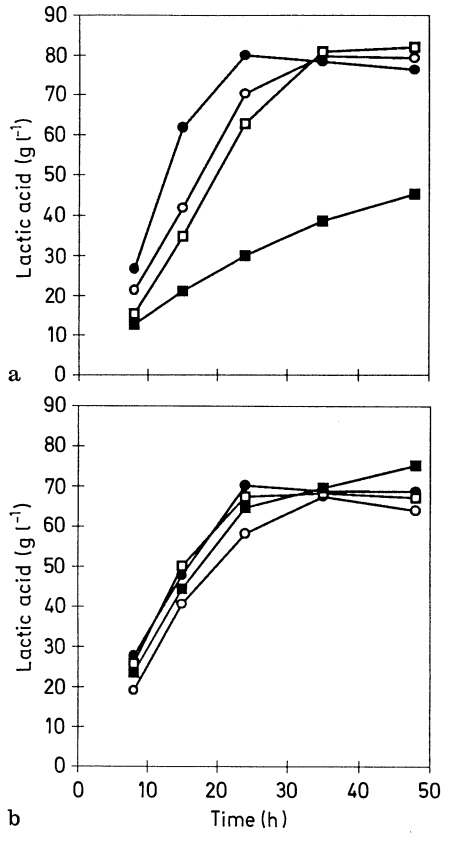

Fig. 1a, b Lactic acid production at different temperatures by (**a**) *L. casei* and (**b**) *L. casei* subsp. *rhamnosus*; ○ 30°C, ● 37°C, □ 41°C, ■ 45°C

during five culture generations. Figure 1 gives the time course of the production of lactic acid with both strains. As can be seen, the best production temperature for *L. casei* was 37°C, at which the highest maximum volumetric productivity of lactic acid, 4.1 $g l^{-1} h^{-1}$, was obtained. The highest lactic acid concentration was 80 $g l^{-1}$, produced in 24 h. With this strain at 41°C the final concentration of lactic acid was still slightly higher, 82 $g l^{-1}$ in 48 h. When adapted to 45°C the *L. casei* inoculum did not grow. However, after cultivation of the inoculum at 37°C, lactic acid could be subsequently produced at 45°C, with a lactic acid concentration of 45 $g l^{-1}$ reached in 48 h. The residual glucose concentration was less than 1 $g l^{-1}$ in 35 h with *L. casei* grown at temperatures up to 41°C. With *L. casei* subsp. *rhamnosus* the highest final lactic acid concentration of 75 $g l^{-1}$ was reached at 45°C in 48 h. The maximal volumetric productivity at this temperature was 3 $g l^{-1} h^{-1}$. However, the maximum productivity of 3.5 $g l^{-1} h^{-1}$ was highest at 37°C. At this temperature 70 $g l^{-1}$ lactic acid was reached in 24 h. Consequently, 37°C appeared to be the best temperature for lactic acid production by both strains, *L. casei* NRRL B-441 and *L. casei* subsp. *rhamnosus* NRRL B-445.

Table 1 Lactic acid concentration from 100 $g l^{-1}$ glucose at 37°C produced by *Lactobacillus casei* using different sources of nitrogen and growth factors. The nitrogen content of all media was 0.22%

Nitrogen source		Lactic acid concentration ($g l^{-1}$)		
	N(%)	24 h	48 h	66 h
Yeast extract	9.8	78	100	92
Malt sprouts	4.0	28	65	88
Peptone	14.1	9	60	79
Grass extract	1.9	29[a]	44[b]	74[c]
N-Z-case plus	13.2	13	37	67
Corn-steep liquor	3.5	26	39	59
N-Z-amine YT	12.4	16	34	48
Casein hydrolysate	10.0	16	23	44
Distillers' waste	4.6	21	33	43
$(NH_4)_2HPO_4/NH_4H_2PO_4$ (1:2)	16.2	2	2	3
Urea	46.7	2	2	2

[a] 22 h reaction time
[b] 46 h reaction time
[c] 73 h reaction time

Effect of nitrogen source

From the 22 nitrogen sources examined for their nitrogen content 11 were chosen for test fermentations on the basis of their nitrogen quantity and costs. The lactic acid produced by *L. casei* NRRL B-441, using selected nitrogen sources, with the nitrogen content of the medium was kept at 0.22%, are shown in Table 1. As could be expected, when *L. casei* was cultivated in a medium containing only the inorganic nitrogen source and vitamins, no lactic acid was produced. With 100 $g l^{-1}$ glucose and malt sprouts as nitrogen source a satisfactory lactic acid concentration of 88 $g l^{-1}$ was obtained in 66 h as compared to 100 $g l^{-1}$ in 48 h with yeast extract. Thus malt sprouts were regarded as the best alternative to yeast extract as the source of nitrogen and growth factors. With 1.5% peptone (corresponding to 2.2% yeast extract) lactic acid production was lower (79 $g l^{-1}$ in 66 h) than with medium containing 5.4% malt sprouts (88 $g l^{-1}$ in 66 h). Grass extract, a waste material from silage production, also gave satisfactory results, 74 $g l^{-1}$ lactic acid in 73 h.

Since malt sprouts as the only nitrogen source resulted in a slow rate of lactic acid production, the three best nitrogen sources from earlier experiments were chosen as independent variables for a statistical experimental design. To investigate whether the expensive nitrogen sources, yeast extract and peptone, could be replaced by the cheaper malt sprouts, the quantities of yeast extract and peptone were kept quite low, within the range of 0–5 $g l^{-1}$. The lactic acid yield was over 90% with all combinations of independent variables, except in the case where no malt sprouts were added and the total nitrogen content was only 0.06%. Table 2 presents the resulting second-order models and Fig. 2 illustrates

Table 2 Polynomial models for the form $Y = B_0 + \Sigma B_i x_i + \Sigma\Sigma B_{ij} x_i x_j$ obtained (see text for x_i)

Parameter	Equation number				
	1 Y_{h12}	2 Y_{h20}	3 Y_{h30}	4 Y_{h47}	5 Y_{h96}
Constant	21.677***	42.309***	55.974***	79.868***	79.309***
x_1	2.879*	6.734**	9.372***	9.590*	0.112
x_2	1.256	2.014	4.255*	4.698	− 0.226
x_3	0.622	3.201	6.085**	10.066*	5.147
x_1x_2	0.025	− 0.838	− 1.063	− 4.713	− 1.400
x_2x_3	− 0.625	− 0.988	− 1.488	− 0.863	− 0.500
x_1x_3	− 0.825	− 2.313	− 3.713	− 3.613	− 2.725
x_1^2	− 0.141	1.713	− 0.712	− 1.829	3.960
x_2^2	2.559*	2.563	1.413	2.948	3.285
x_3^2	− 2.517	− 3.764	− 2.938	− 5.880	− 2.242
R	0.872	0.908	0.950	0.880	0.540
R^2	0.760	0.824	0.902	0.774	0.292
P	0.080	0.029*	0.004**	0.066	0.922

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

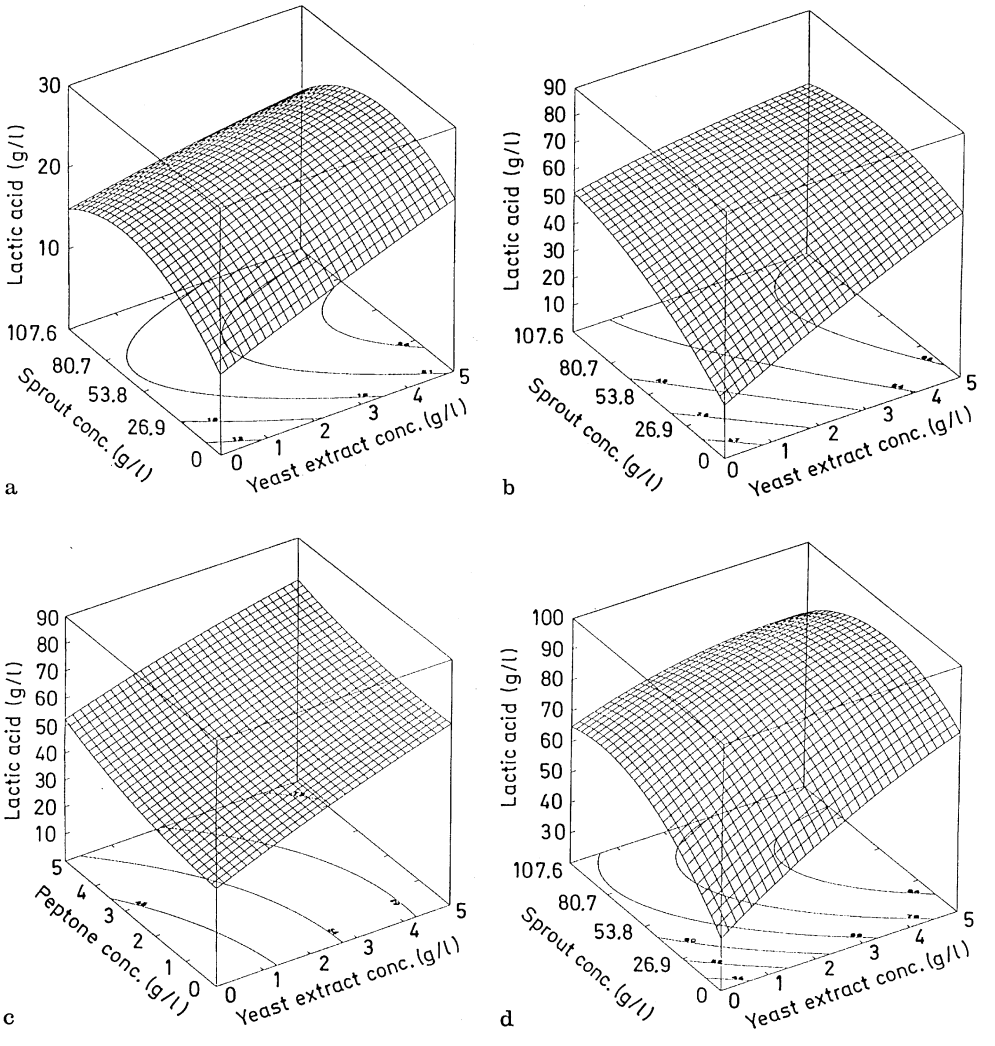

Fig. 2a–d Lactic acid concentration as a function of yeast extract concentration and (**a**) concentration of malt sprouts at 12 h, (**b**) concentration of malt sprouts at 30 h, (**c**) concentration of peptone at 30 h and (**d**) concentration of malt sprouts at 47 h fermentation time

the three-dimensional response surfaces showing the expected concentration of lactic acid as a function of two independent variables with the third kept at a constant centre-point level.

In the early stages of fermentation the yeast extract concentration was clearly the most significant factor affecting the production of lactic acid. Figure 2a shows that, at 12 h of fermentation, the lactic acid concentration increased with an increasing yeast extract concentration. Further, an increase in the yeast extract concentration had an almost linear effect on lactic acid concentration up to 30 h of cultivation (Fig. 2b, c). At 30 h the fermentation had reached a steady state and at this time the polynomial model expressed the situation very accurately ($P < 0.01$, Table 2). All the independent variables were significant ($P < 0.05$). Yeast extract was still the most significant variable ($P < 0.001$) but sprout concentration was also very significant ($P < 0.05$). As can be seen from Fig. 2b an increase in the concentration of sprouts decreased the effect of yeast extract concentration on lactic acid production. Further, Fig. 2c shows that, even if the medium contained 5 $g l^{-1}$ peptone, the amount of yeast extract was still quite important.

According to Fig. 2d the lactic acid production more than doubled in 47 h of cultivation when the malt sprout concentration was increased from 0 to 80 $g l^{-1}$ and when the medium had no yeast extract.

Preparation of sprout extract

Because malt sprouts contained only 4% nitrogen, a 54 $g l^{-1}$ concentration was needed to obtain a nitrogen content of 0.22%. The resulting medium was too heterogeneous and would cause problems in downstream processing. For this reason a method for extracting nutrients from sprouts was developed. As can be seen from Fig. 3, although yeast extract alone resulted in the highest initial lactic acid production rate, malt sprouts with a single-step extraction gave the highest final lactic acid concentration of 107 $g l^{-1}$ in 69 h. If the same amount of sprouts was either extracted or used as they were, the sprout extract gave a higher lactic acid level. Figure 4 demonstrates the production of lactic acid in a medium in which most of the yeast extract had been substituted by malt sprout extract or grass extract. The final lactic acid concentration was even higher when sprout extract (100 $g l^{-1}$) or grass extract (103 $g l^{-1}$) was used together with 0.4% yeast extract, as compared with yeast extract used as the only source of nitrogen and growth factors at the 2.2% level (95 $g l^{-1}$). However, the volumetric productivity of 3.8 $g l^{-1} h^{-1}$ was highest when 2.2% yeast extract alone was used. Figure 5 shows that there was no marked difference between the use of whole and ground sprouts. Consequently, the grinding of sprouts could be omitted.

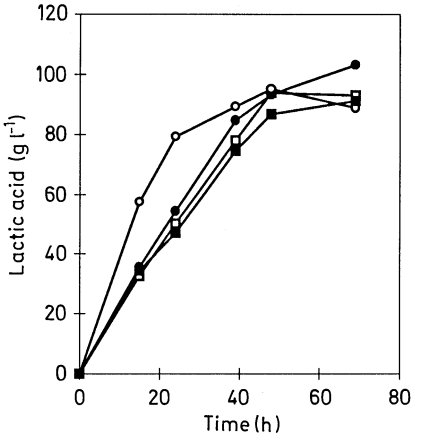

Fig. 3 Effect of extraction of sprouts on lactic acid production by *L. casei*. ○ Yeast extract 2.2%, ● one extraction, □ two extractions, ■ sprouts in the medium

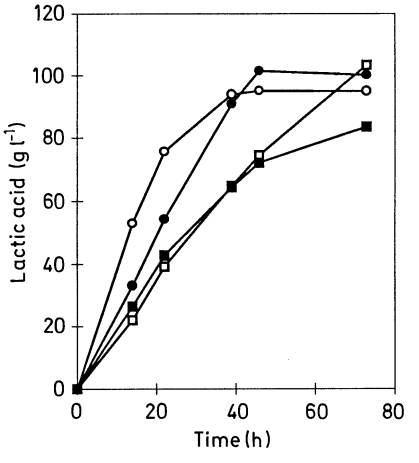

Fig. 4 Effect of nitrogen source on lactic acid production by *L. casei*. ○ Yeast extract 2.2%, ● malt sprout and yeast extract 0.4%, □ grass extract and yeast extract 0.4%, ■ 0.4% yeast extract

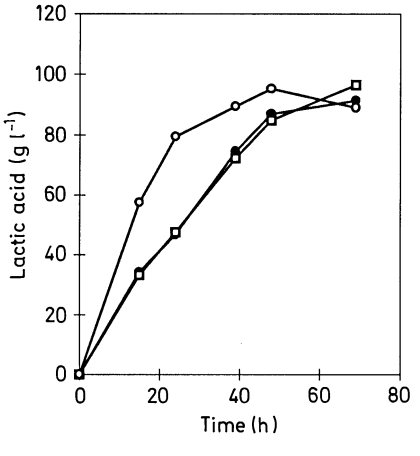

Fig. 5 Effect of grinding of sprouts on lactic acid production by *L. casei*. ○ Yeast extract 2.2%, ● ground malt sprouts, □ whole malt sprouts

Discussion

Different lactic acid bacterial strains differed in their temperature requirements, but, in general, results obtained agreed well with the data published earlier. As can be seen from Fig. 1a, b the best temperature for the production of lactic acid by *L. casei* NRRL B-441 and *L. casei* ssp. *rhamnosus* NRRL B-445 was 37°C. Demirci et al. (1993) also reported 37°C to be the best growth temperature for *L. casei* ATCC 11443. According to Kandler and Weiss (1986) and Demirci et al. (1993) *L. casei* does not grow at 45°C. This was confirmed in the present work, but if the inoculum was first cultivated at 37°C and lactic acid fermentation was carried out at 45°C the cells remained viable and produced 45 $g l^{-1}$ lactic acid in 48 h (Fig. 1a). On the other hand, Tuli et al. (1985) found no significant influence of temperature between 40°C and 50°C on the conversion efficiency of immobilized *L. casei* in whey permeate medium. They also reported a decreased lactic acid formation at 35°C. According to the present work, the importance of the cultivation temperature appeared to be much greater for *L. casei* NRRL B-441 than *L. casei* subsp. *rhamnosus* NRRL B-445.

The results clearly showed that lactic acid production was markedly influenced by the type and initial concentration of the nitrogen source (Figs. 2, 4). Inexpensive food- and feed-processing byproducts, such as malt sprouts and grass extract, showed a good potential as sources of nitrogen and growth factors for lactic acid production by *L. casei*. Casein hydrolysate alone was not sufficient, in good agreement with earlier results (Vahvaselkä and Linko 1987). The nutritional requirements of *Lactobacilli* have been widely discussed already by Rogosa et al. (1961), who emphasized the importance of amino acids and vitamins for cell growth, and more recently by Vahvaselkä and Linko (1987) and by Amrane and Prigent (1994). Amrane and Prigent (1994) stressed the importance of yeast extract in the preculture medium. They supposed that the main contributions of yeast extract are the purine and pyrimidine bases, and vitamins of the B group. Whey alone is a poor nitrogen source for *Lactobacilli*, and different supplementations, such as yeast extract (Vahvaselkä and Linko 1987), mustard oil cake (Tuli et al. 1985) and corn-steep liquor (Amrane and Prigent 1994), have been proposed. Aeschliman and von Stockar (1991) proposed supplementation of the whey-based medium with 10 $g l^{-1}$ yeast extract for high system conversion and high final lactic acid concentration. They reported a decrease in lactic acid productivity by 25% with *L. helveticus* when the 60% yeast extract was replaced by skim milk in the production medium. Vahvaselkä and Linko (1987) also studied the kinetics of lactic acid fermentation on milk ultrafiltrate by *L. helveticus* and the effects of various growth supplements on lactic acid formation. They, too, substituted malt sprout extract for yeast extract as a growth supplement, but only a slight effect on lactic acid formation was observed when milk ultrafiltrate was used as the substrate.

Malt sprout extract and grass extract are nutritious waste products of the food industry and therefore could be considered as feasible, inexpensive alternative nitrogen sources for lactic acid fermentation by *L. casei* NRRL B-441 on glucose. As shown in Fig. 4, with 2.2% yeast extract the lactic acid concentration increased linearly up to 22 h, with sprout extract and 0.4% yeast extract up to 46 h, and with grass extract supplemented with 0.4% yeast extract up to 73 h. In a response-surface study, based on a statistical experimental design, the polynomial models obtained expressed the effects of the different nitrogen sources as the independent variables quite accurately. Especially at 30 h of cultivation the chosen variables (yeast extract, peptone and malt sprout extract) were very significant, as can be seen from their P values (the level of significance) in Table 2. Clearly yeast extract exhibited the most significant effect on lactic acid production, especially at the beginning of growth, with the lactic acid concentration increasing nearly linearly with an increase in yeast extract level (Fig. 2). The importance of yeast extract at the early stages of fermentation was probably caused by the amino acids and vitamins in yeast extract that are crucial for growth (Rogosa et al. 1961). Our previous work (Hujanen and Linko 1994) also confirmed the importance of yeast extract in the early stages of lactic acid fermentation. However, if the medium contained a sufficient quantity of malt sprouts as a source of nitrogen and growth factors, the amount of yeast extract could be reduced from 22 $g l^{-1}$ to 4 $g l^{-1}$ without a decrease in lactic acid production, although a higher yeast extract concentration increased the initial rate of fermentation (Fig. 4). For the convenient use of malt sprouts in fermentation they were extracted with water. This water extract was an even better source of nitrogen and growth factors than malt sprouts (Fig. 3). Also grinding of the malt sprouts could be omitted, because there was no marked difference between ground and whole sprouts, as can be seen from Fig. 5.

In the present work we have demonstrated that it is possible to substitute most of the expensive yeast extract with less expensive waste products without a decrease in lactic acid production.

Acknowledgements The authors are grateful to the Academy of Finland for financial support.

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