Biotechnol Lett (2010) 32:517–520
DOI 10.1007/s10529-009-0187-y

ORIGINAL RESEARCH PAPER

Strain improvement of *Lactobacillus lactis* for D-lactic acid production

D. S. Joshi · M. S. Singhvi · J. M. Khire · D. V. Gokhale

Received: 1 October 2009 / Revised: 26 November 2009 / Accepted: 30 November 2009 / Published online: 24 December 2009

Abstract Three mutants, isolated by repeated UV mutagenesis of *Lactobacillus lactis* NCIM 2368, produced increased D-lactic acid concentrations. These mutants were compared with the wild type using 100 g hydrolyzed cane sugar/l in the fermentation medium. One mutant, RM2-24, produced 81 g lactic acid/l which was over three times that of the wild type. The highest D-lactic acid (110 g/l) in batch fermentation was obtained with 150 g cane sugar/l with a 73% lactic acid yield. The mutant utilizes cellobiose efficiently, converting it into D-lactic acid suggesting the presence of cellobiase. Thus, this strain could be used to obtain D-lactic acid from cellulosic materials that are pre-hydrolyzed with cellulase.

Keywords Cellobiose utilization · *Lactobacillus lactis* · D-Lactic acid production · Mutant · Sucrose (cane sugar)

Introduction

Lactic acid and its derivatives are widely used in the food, pharmaceutical and cosmetic industries (VickRoy 1985). It is also a major raw material for the production of polylactic acid (PLA) that is a biodegradable, environmentfriendly polymer which could be a substitute for synthetic plastics derived from petroleum feedstocks. Fermentative production of lactic acid offers the great advantage of producing optically pure L-or D-lactic acid depending upon the strains selected for fermentation. The optical purity of lactic acid is crucial for the physical properties of PLA. Though L-lactic acid can be polymerized to give crystalline (PLLA) suited to commercial uses (Sodegard and Stolt 2002), its application is limited by its low melting point. Complexing PLLA with poly D-lactic acid (PDLA), however, increases the melting point thus presenting an attractive solution to the heat sensitivity of PLA (Tsuji and Fukui 2003). This finding has increased the importance of the synthesis of D-lactic acid. Presently, L-lactic acid is widely used in the food and pharmaceutical industries and hence its production by fermentation is well established (Yu and Hang 1989). However, fermentation of sugars to D-lactic acid is little studied and its microbial productivity is not well known (Zhou et al. 2003). Therefore, we decided to investigate D-lactic acid fermentation with a view to obtaining improved strains capable of producing D-lactic acid with enhanced productivities.

India is one of the largest producers of cane sugar (more than 20 million tonnes). During this process, large amounts of molasses are generated as a byproduct which contains 40–60% (w/v) sucrose.

D. S. Joshi · M. S. Singhvi · J. M. Khire · D. V. Gokhale (✉)
NCIM Resource Center, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411 008, Maharashtra, India
e-mail: dv.gokhale@ncl.res.in

In addition, approx. 45 million tonnes of bagasse are generated. All these byproducts can be converted to value-added products such as lactic acid. *Lactobacillus lactis* NCIM 2368 is a homofermentative D-lactic acid-producing strain. Mutants of it were selected after UV mutagenesis showing greater acid formation zones on a selected medium. This paper describes the production of D-lactic acid from sucrose, molasses and cellobiose using one of these mutants.

Materials and methods

Microorganisms and growth media

Lactobacillus lactis NCIM 2368 and its mutants were grown on medium consisting of 100 g hydrolyzed cane sugar/l; 10 g yeast extract/l; and 45 g $CaCO_3$/l. The basic fermentation medium was the same as the growth medium. Molasses and cellobiose were also used as carbon sources at 10% (w/v) for D-lactic acid production. The cane sugar and molasses were hydrolyzed by adding 1 ml 20% (v/v) H_2SO_4 to 100 ml sugar solution. The acidified sugar solution was heated in boiling water bath for 20 min (Kadam et al. 2006). The ingredients of the media were separately added after sterilization at 121°C for 20 min and the pH of fermentation and growth media was adjusted to 7.0 prior to sterilization.

Lactic acid production in shake-flasks

An overnight culture (~5 ml) was transferred to 100 ml growth medium in 250 ml screw-cap conical flasks and then shaken (150 rpm) at 42°C for 24 or 48 h. This culture was inoculated at 5% (v/v) into fermentation media. Fermentation experiments were carried out in screw-cap conical flasks containing 100 ml fermentation medium and also shaken at 150 rpm. Culture samples were centrifuged at 1000×*g* for 10 min and the supernatant was analyzed for sugar and lactic acid and pH measurement. The supernatant was acidified with equal volume of 1 M HCl where free acid is liberated. Cell mass, reducing sugar concentration and lactic acid in samples were determined as described earlier (Kadam et al. 2006). The presence of L-lactic acid was analyzed by a L-lactate oxidase enzyme kit (Randox Laboratories, UK) and the D-lactic acid content was calculated by subtracting L-lactic acid values from total lactic acid estimated by HPLC equipped with UV or RI detectors. An ion exclusion column (Aminex HPX-87H) was used with 4 mM H_2SO_4 as a mobile phase at 0.6 ml/min. An injection volume of the sample was 50 µl.

Mutagenesis and mutant selection

Lactobacillus lactis was grown in 30 ml MRS liquid medium with 0.1% $CaCO_3$ at 37°C under stationary conditions for 24 h. Ten ml of the culture was centrifuged at 1000×*g* for 10 min, the supernatant was decanted and the cell pellet was washed three times with saline. The cells were resuspended in 10 ml saline; the total viable count was adjusted 10^7/ml. Ten ml of the diluted sample in a 9 cm Petri dish was irradiated with a UV lamp at 254 nm at 6 cm for 20 min. The samples were serially diluted in sterile saline and survivors determined by spreading 0.1 ml of diluted samples on a agar medium containing 100 g cane sugar/l; 10 g yeast extract/l; 10 g $CaCO_3$/l and 20 g agar/l. The viable counts were determined after anaerobic incubation at 42°C until the distinct colonies appeared with zones of acid production.

Results and discussion

Mutants were isolated by exposing the 10 ml cell suspension (10^8 cells) to UV irradiation for 17 min which gave approx. 99% killing. Selection was based on rapid growth as well as a wider zone of acid production. About 10 colonies showing greater acid production were tested for lactic acid production in shake-flasks. One mutant, MI-2, with higher lactic acid production capacity was further treated with UV irradiation which created such further mutants. Among the seven mutants, RM-2 was selected and further mutated by exposing it to UV-irradiation for 20 min which resulted in selection of mutant RM2-24 showing enhanced D-lactic acid production in shake-flasks (Table 1) and was therefore selected for further studies.

Lactic acid production from different substrates using RM2-24 was performed in fermentation medium with 10% (w/v) substrate (Table 2). RM2-24 produced lactic acid more efficiently than the parent strain. Lactic acid production using molasses

Table 1 Comparison of D-lactic acid production by parent (NCIM 2368) and mutant strains of *L. lactis* in production medium containing hydrolyzed cane sugar (10%), yeast extract (1%) and $CaCO_3$ (4.5%)

Organism	Cell mass (g/l)		D-Lactic acid (g/l)	
	24 h	48 h	24 h	48 h
NCIM 2368	2.6	3.2	16.3 (0.68)	26 (0.54)
M1-2	4.4	4.3	25 (1.04)	47.5 (0.98)
RM2	4.5	5.6	34.5 (1.43)	49 (1.02)
RM2-24	4.4	5	52 (2.17)	81 (1.68)

The cultures were grown anaerobically for 48 h at 42°C with shaking (150 rpm). The values given are the average of three independent experiments. The numbers in the parenthesis show lactic acid productivities (g/l h)

Table 2 Effect of different substrates on cell growth and D-lactic acid production by *L. lactis* RM2-24

Carbon sources	Cell mass (g/l)		D-Lactic acid (g/l)	
	24 h	48 h	24 h	48 h
Glucose	4	4.5	51.5 (2.12)	78 (1.62)
Hydrolyzed cane sugar	4.4	4.7	52 (2.17)	81 (1.68)
Molasses	5.1	5.5	50 (2.08)	70 (1.45)
Cellobiose	3.7	5	50 (2.08)	80 (1.66)

The cultures were grown anaerobically for 48 h at 42°C with shaking (150 rpm). The numbers in the parenthesis show lactic acid productivities (g/l h). The values given are the average of three independent experiments

was comparatively less probably due to the presence of non-fermentable sugars. The maximum D-lactic acid production from cellobiose suggested the presence of high cellobiase activity in the mutant strain. These are the highest productivity and efficiency values reported so far for lactic acid production from cellobiose. There are few reports on production of D-lactic acid from unpolished rice (Lu et al. 2009), defatted rice bran (Tanaka et al. 2006) and from waste cardboard (Yanez et al. 2005). Recently, a recombinant strain of *Corynebacterium glutamicum* capable of producing lactic acid from the mixture containing cellobiose, glucose and xylose was reported (Sasaki et al. 2008). Previously, we reported the L-lactic acid production from bagasse derived cellulose using another mutant *L. delbrueckii* Uc-3 (Adsul et al. 2007a) which showed the presence of a cell-bound cellobiase (Adsul et al. 2007b).

Table 3 Effect of initial hydrolyzed cane sugar concentration on fermentation time required, lactic acid produced, and D-lactic acid productivity

Sucrose (g/l)	Cell mass (g/l)		D-Lactic acid (g/l)	
	48 h	96 h	48 h	96 h
50	4.1	–	40.5 (0.85)	–
100	4.7	–	81 (1.68)	–
150	3.5	4.1	65.5 (1.36)	110 (1.15)
200	3.6	4.2	60 (1.25)	108 (1.13)

The cultures were grown anaerobically for 48 h at 42°C with shaking (150 rpm). The numbers in the parenthesis show lactic acid productivities (g/l h)

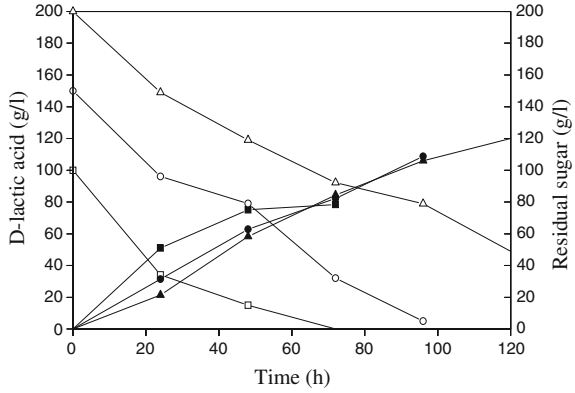

Fig. 1 Effect of different initial concentrations of hydrolyzed cane sugar on D-lactic acid production by *Lactobacillus lactis* RM2-24. *Closed symbols*: L-lactic acid production and *open symbols*: residual sugar (*open square*, *closed square*) 100 g/l, (*open circle*, *closed circle*) 150 g/l (*open triangle*, *closed triangle*) 200 g/l

Lactic acid production by RM2-24 was studied using various concentrations of hydrolyzed cane sugar. As shown in Table 3, the mutant produced increasing amounts of lactic acid with increasing sugar concentrations up to 200 g/l. Maximum lactic acid production (120 g/l) was with 200 g cane sugar/l with a productivity of 1 g/l h (Fig. 1). The optical purity of d-lactic acid derived from all substrates was 98%. Okino et al. (2008) constructed a genetically modified strain of *Corynebacterium glutamicum* which produced 120 g D-lactic acid/l with higher productivity. However, this organism produced succinic and acetic acid along with D-lactic acid. Calabia and Tokiwa (2007) reported 104 g D-lactic acid/l from molasses with productivity of 1.48 g/l h. Our strain, RM2-24, produced only D-lactic acid with

comparable yields without the formation of succinic and acetic acids, as confirmed by HPLC.

Conclusion

Lactobacillus lactis RM2-24 is a promising strain for the production of D-lactic acid from molasses and hydrolyzed cane sugar. It also utilizes cellobiose efficiently and converts it into lactic acid. Thus bottlenecks, like feed-back inhibition by glucose and cellobiose, are removed leading to complete conversion of cellulosic substrates to D-lactic acid. This study shows the potentiality of such a strain for producing commodity chemicals from renewable resources.

Acknowledgement We acknowledge the financial support from NMITLI Division, Council of Scientific and Industrial Research (CSIR), New Delhi, India.

References

Adsul MG, Varma AJ, Gokhale DV (2007a) Lactic acid production from waste sugar cane bagasse derived cellulose. Green Chem 9:58–62

Adsul MG, Khire JM, Bastawade KB, Gokhale DV (2007b) Lactic acid production from cellobiose and cellotriose by *Lactobacillus delbrueckii* mutant Uc-3. Appl Environ Microbiol 73:5055–5057

Calabia BP, Tokiwa Y (2007) Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by *Lactobacillus delbrueckii*. Biotechnol Lett 29: 1329–1332

Kadam SR, Patil SS, Bastawade KB, Khire JM, Gokhale DV (2006) Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production. Proc Biochem 41:120–126

Lu Z, Lu M, He F, Yu L (2009) An economical approach for D-lactic acid production utilizing unpolished rice from aging paddy as major nutrient source. Bioresour Technol 100: 2026–2031

Okino S, Suda M, Fujikura K, Inui M, Yukawa H (2008) Production of D-lactic acid by *Corynebacterium glutamicum* under oxygen deprivation. Appl Microbiol Biotechnol 78:449–454

Sasaki M, Jojima T, Inui M, Yukawa H (2008) Simultaneous utilization of D-cellobiose, D-glucose and D-xylose by recombinant *Corynebacterium glutamicum* under oxygen deprived conditions. Appl Microbiol Biotechnol 81: 691–699

Sodegard A, Stolt M (2002) Properties of lactic acid based polymers and their correlation with composition. Prog Polym Sci 27:1123–1163

Tanaka T, Hoshina M, Tanabe S, Sakai K, Ohtsubo S, Taniguchi M (2006) Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation. Bioresour Technol 97:211–217

Tsuji H, Fukui I (2003) Enhanced thermal stability of poly (lactide)s in the melt by enantiomeric polymer blending. Polymer 44:2891–2896

VickRoy TB (1985) Lactic acid. In: Moo-Young M (ed) Comprehensive biotechnology, vol 3. Pergamon Press, New York, pp 761–776

Yanez R, Alonso JL, Parajo JC (2005) D-Lactic acid production from waste cardboard. J Chem Technol Biotechnol 80: 76–84

Yu R, Hang Y–D (1989) Kinetics of direct fermentation of agricultural commodities to L(+)-lactic acid by *Rhizopus oryzae*. Biotechnol Lett 11:597–600

Zhou S, Causet TB, Hasona A, Shanmugan KT, Ingram LO (2003) Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *Escherichia coli* W3110. Appl Environ Microbiol 69:399–407