D-(−)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-2†4

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Lactobacillus lactis mutant RM2-24 utilizes cellobiose efficiently, converting it into D-(−)-lactic acid. Cellobiose-degrading enzyme activities were determined for whole cells, cell extracts and disrupted cells. Aryl-β-glucosidase activity was detected in whole cells and disrupted cells, suggesting that these activities are confined to the cells. The mutant produced 80 g l^{-1} of lactic acid from 100 g l^{-1} of cellobiose with 1.66 g l^{-1} h^{-1} productivity. Production of D-lactic acid from different cellulose samples was also studied. The cellulose samples at high concentration (10%) were hydrolyzed by cellulase enzyme preparation (10 FPU g^{-1} cellulose) derived from *Penicillium janthinellum* mutant EU1 generated in our own laboratory. We obtained a maximum 72% hydrolysis, yielding glucose and cellobiose as the main end products. Lactic acid was produced from these cellulose samples by simultaneous saccharification and fermentation (SSF) in a media containing a cellulase enzyme preparation derived from *Penicillium janthinellum* mutant EU1 and cellobiose utilizing *Lactobacillus lactis* mutant RM2-24. A maximum lactic acid concentration of 73 g l^{-1} was produced from a concentration of 100 g l^{-1} of bagasse-derived cellulose, the highest productivity and yield being 1.52 g l^{-1} h^{-1} and 0.73 g g^{-1}, respectively. Considering that bagasse is a waste material available in abundance, we propose to use this biomass to produce cellulose and then sugars, which can be fermented to valuable products such as ethanol and lactic acid.

Introduction

Lactic acid and its derivatives have been widely used in food, pharmaceutical, cosmetic and industrial applications.[1] It has a potential to become a commodity chemical for production of biodegradable polymers, oxygenated chemicals, plant growth regulators and special chemical intermediates. It has been receiving great attention as a feedstock for manufacture of polylactic acid (PLA), a biodegradable polymer used as environmentally friendly biodegradable plastic. Lactic acid is manufactured either chemically or by microbial fermentation. Chemical synthesis always results in racemic mixture of lactic acid, which is a major disadvantage. Microbial fermentation offers the advantage in both utilization of renewable carbohydrates and production of pure L- or D-lactic acid depending on the strain selected. The physical properties of PLA depend on the isomeric composition of lactic acid. Poly(L-lactide) (PLLA) obtained by polymerization of L-lactic acid or L-lactide has a melting temperature of 175 °C. The melting point of this polymer can be increased by blending with poly (D-lactide) (PDLA) in a solvent. Recently, it was found that the polymer blend of PLLA and PDLA produces a stereo-complex with melting temperature around 230 °C.[2] This finding has attracted more attention to the production of D-lactic acid.

Cellulosic substances are abundantly available resources of renewable biopolymer which can be utilized as a feedstock for producing a number of bulk chemicals like ethanol or lactic acid through fermentation processes. The production of such value-added products from renewable feedstocks is a present need, and there is a demand to make it an economically and environmentally feasible process. Such bioprocesses involving cellulosic residues not only provide alternative substrates but also help solve their disposal problems. With the advent of biotechnological innovations, mainly in the area of enzyme technology and fermentation technology, many new avenues have opened up for their exploitation as value-added products. Currently, optically pure lactic acid is produced mainly from corn starch. However, the use of agro-waste materials for lactic acid production appears to be more attractive because they do not then have any impact on the human food chain.

Unfortunately, the process for converting cellulosic material into lactic acid is not yet feasible due to the high cost of cellulase enzymes involved in cellulose hydrolysis[3,4] and also to the use of fastidious organisms.[5] The utilization of cellulosic wastes such as cardboard and corn cobs as substrates for lactic acid fermentation, simultaneous saccharification and fermentation (SSF) has been considered to be a promising approach.[6] However, there have been many technical problems – for instance, the enzymes of cellulose hydrolysis are inhibited by the intermediate products such as cellobiose, and lactic acid biosynthesis is inhibited by lactic acid.[7] Many investigations have been carried out to relieve such inhibitions, for example, *in situ* product removal technology

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has been applied to the SSF process, but this needs large electric energy or high-level equipment.[8,9] Cellulase inhibition by glucose and cellobiose during the hydrolysis of cellulosic material by cellulases is the main bottleneck, which significantly slows down the rate of hydrolysis. The addition of β-glucosidase at the beginning of SSF is recommended for the removal of cellobiose inhibition, but sometimes it does not work because of rapid deactivation of the enzyme.[10] Therefore, it is advantageous to use a lactic acid producing strain that has the ability to utilize both glucose and cellobiose efficiently.[5] It is known that some *Lactobacillus* strains utilize cellobiose as a carbon source,[11] but very little information is available about lactic acid production from cellobiose.

We have previously reported the production of L-lactic acid from sugarcane bagasse cellulose with high productivity and yield using a *Lactobacillus* mutant created in our laboratory.[12] In this paper, we describe the efficient utilization of cellobiose and cellulosic materials by *Lactobacillus lactis* mutant RM2-24 for lactic acid production. The mutant was isolated following UV mutagenesis and selected on the basis of a greater zone of acid formation on a sucrose-based medium. We also report the aryl-β-glucosidase activity (involved in cellobiose utilization) that this mutant displays.

Experimental

Chemicals

p-Nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-β-D-cellobioside (pNPC), *p*-nitrophenyl-β-D-galactopyranoside (pNPgal), 3,5-dinitrosalicylic acid and Sigma cellulose were obtained from Sigma-Aldrich Co., St Louis, MO, USA. Avicel PH-101 was obtained from Fluka Chemie GmbH. Solka Floc SW44 was purchased from Brown Co., Berlin, NH. The α-cellulose with 0.18% lignin and 98% cellulose was prepared from sugarcane bagasse in our laboratory.[12]

Strain information and cellulase production

Mutant EU1 was isolated by exposing conidia of *Penicillium janthinellum* NCIM 1171 to UV irradiation. The procedure of generation of mutant EU1 and its crude enzyme preparation have already been reported earlier.[13] The mutant was maintained on potato dextrose agar (PDA) and sub-cultured once every three months. PDA contained (per liter) extract from 200 g potatoes, glucose (20.0 g), yeast extract (1.0 g), and agar (20.0 g). Enzyme production was carried out in a 250 ml Erlenmeyer flask, with 70 ml of production medium containing 1% (w/v) cellulose-123 powder and 2.5% wheat bran.[13] *Lactobacillus lactis* mutant RM2-24, producing lactic acid with high productivity, was isolated by UV mutagenesis.[14] It was maintained in liquid MRS medium supplemented with 0.1% $CaCO_3$.

Lactic acid production in shake flasks

Lactic acid production from cellobiose in shake flasks was performed as reported earlier.[14] The saccharification of cellulosic substrates and simultaneous saccharification and fermentation were carried out using *Penicillium janthinellum* mutant EU1 cellulase, as per the methodologies reported earlier.[12]

Enzyme assay

β-Glucosidase (β-D-glucosideglucohydrolase; E.C. 3.2.1.21) activity was estimated according to the method described earlier[13] using pNPG as substrate. The total of assay mixture (1 ml) consisting of 0.9 ml of substrate and 0.1 ml of suitably diluted enzyme was incubated at 50 °C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm after developing the color with 2 ml of sodium carbonate (2%). One unit of enzyme activity is equivalent to one μmol of *p*-nitrophenol generated per minute. The other substrates used were pNPC and pNPgal.

Analytical methods

Cell growth was measured by spectrophotometrically using Spectrometer-117 (Systronics, Mumbai, India) at a wavelength of 660 nm. Reducing sugar concentration was determined by DNS method. The amounts of glucose and lactic acid were determined using a high-performance liquid chromatography (HPLC) system (Dionex India Ltd.) equipped with UV- or RI-detectors.[15] An ion exclusion column (Aminex, HPX-87H, Biorad, Hercules, CA) was used at a temperature of 30 °C with 0.008 N H_2SO_4 as a mobile phase at flow rate of 0.6 ml min^{-1}. An injection volume of the sample was 50 μl. The presence of L-(+)-lactic acid was analyzed by an 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.[14]

Results and discussion

The profile of growth (optical density) and lactic acid production from cellobiose is shown in Fig. 1. The maximum amount of lactic acid was produced within 48 h of fermentation, with an increase in optical density from 0.6 to 11 and a decrease in pH from 6.5 to 4.8. The maximum (80 g l^{-1}) amount of lactic acid was produced from 100 g l^{-1} of cellobiose with 1.66 g l^{-1} h^{-1} productivity and 0.8 g g^{-1} yield. These are the highest productivity and efficiency values reported so far for the production of lactic acid from cellobiose. This strain, therefore,

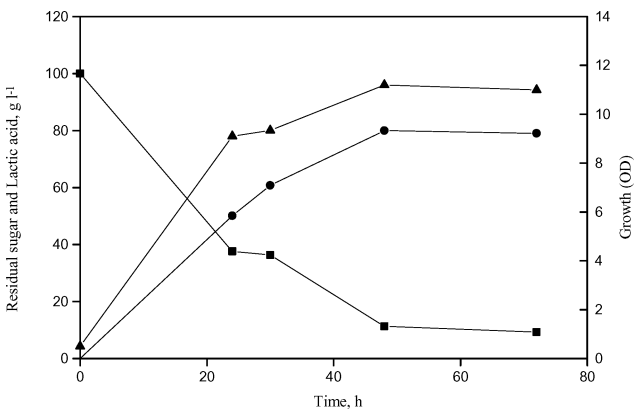

Fig. 1 Profile of lactic acid production (●), residual sugar (■) and growth (▲) during fermentation by *Lactobacillus lactis* mutant RM2-24 using cellobiose (100 g l^{-1}).

Table 1 Detection of aryl-β-glucosidase activity from *Lactobacillus lactis* mutant RM2-24 grown in different sugar substrates[a]

		Enzyme activity (U g^{-1} of cells) grown in:			
Enzyme source	Substrate	Glucose	Lactose	Hydrolyzed sucrose	Cellobiose
Cells	pNPG	2.88	7.56	2.7	23.4
	pNPgal	5.18	6.6	5.1	4.41
	pNPC	ND	1.215	ND	6.075
Sonicated cells	pNPG	ND	4.02	ND	10.5
	pNPgal	2.1	5.01	2	2.48
	pNPC	ND	0.81	ND	3.79

[a] The cells were harvested at the onset of stationary phase for the determination of enzyme activity. ND, not detected.

is proven to be highly efficient for the conversion of cellobiose to lactic acid, and could be exploited at a commercial level. Previously, we reported the production of L-(+)-lactic acid from cellobiose by *Lactobacillus delbrueckii* mutant Uc-3.[16]

This mutant strain, RM2-24, was observed to utilize cellobiose, indicating the presence of cellobiose-degrading enzymes. Therefore, we attempted to detect the cellobiose-degrading enzymes by using pNPG, pNPC and pNPgal as substrates. The cells were grown in different sugars at 42 °C and harvested at the late-exponential phase by centrifugation. After centrifugation, the supernatant and cells were used for an analysis of aryl-β-glucosidase and aryl-β-galactosidase activities. We could not detect any enzyme activity in the supernatant. The cells were washed three times with phosphate buffer (50 mM, pH 7.0) and resuspended in the same buffer. The suspension was used for analyzing cell-bound glucosidase and galactosidase activities.

Table 1 presents the data for all the enzyme activities in the mutant strain grown in liquid medium with different sugars. All activities were detected in lactose and cellobiose grown cells. However, a higher level of aryl-β-glucosidase was observed in cellobiose-grown cells. Other than cellobiose, lactose-grown cells also exhibited all activities. When cells were grown in glucose and a hydrolyzed sucrose-based medium, aryl-β-glucosidase and aryl-β-galactosidase activities were detected in small amounts. There was no activity detected using pNPC as substrate. These results show that the aryl-β-glucosidase or aryl-β-galactosidase gene could be constitutively expressed, and enhancement in activity was observed when the mutant was grown in either cellobiose or lactose, respectively. Activity with pNPC as substrate was detected only when the culture was grown in cellobiose or lactose. However, the induction in lactose-grown cells is less than that in cellobiose-grown cells.

None of these activities were detected in fermented broth, suggesting the intracellular location of these enzymes. To check the intracellular location of these enzymes, cells were subjected to sonication (SONICS Vibra cell; model VC 130) in phosphate buffer (pH 7.0, 50 mM) containing 0.5 mM EDTA and 1 mM dithiothreitol. The sonication was performed at 60% amplitude (125 μm) for 3 min by using a 2 mm probe under cold conditions. Almost 90% of the cells were disrupted by this method. The supernatant and sonicated cell debris were analyzed for all above-mentioned enzyme activities. No activities were detected in the supernatant, showing that enzymes are not located in the cytoplasm. However, all activities (for lactose- and cellobiose-grown cells) were detected in sonicated cells (Table 1), suggesting that the enzymes could be bound to the cell wall/membrane. The activity levels were low compared to those of the intact cells, which could be due to the inactivation of the enzymes during sonication. It is noteworthy that aryl-glucosidase activities were higher in the *Lactobacillus lactis* mutant RM2-24 compared to those observed in *Lactobacillus delbrueckii* mutant Uc-3 reported earlier.[16]

Experiments were performed to evaluate the hydrolysis of various cellulose samples using cellulase enzyme preparations derived from *Penicillium janthinellum* EU1. Hydrolysis of the most crystalline substrate, Avicel, was always lower than that of other cellulose samples due to its microcrystalline structure which prevents easy access to the enzyme. However, 71% hydrolysis was obtained in case of bagasse-derived cellulose, indicating its greater accessibility towards cellulase enzyme (Fig. 2). Solka-Floc and Sigma cellulose were hydrolyzed to approximately 50%, probably due to the more amorphous nature of these two substrates.

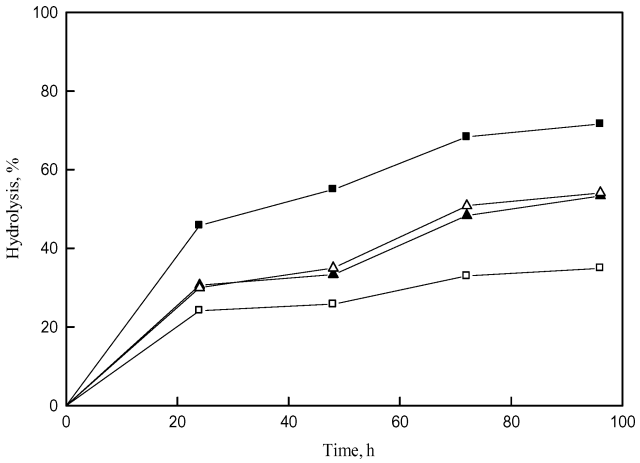

Fig. 2 Profile of hydrolysis of Avicel (□), baggasse cellulose (■), Solka-Floc (▲) and Sigma cellulose (△) by mutant (EU1) enzyme preparations. The hydrolysis was carried out using 10% substrate concentrations with mutant enzyme preparations (10 FPU g^{-1}).

SSF experiments were performed under the selected conditions (42 °C, pH 6.5) because the organism used in this fermentation is a mutant of *Lactobacillus lactis* (RM2-24) and cannot grow at temperatures more than 42 °C. We carried out the SSF at pH 6.5, at which the cellulases used were active with retention of more than 50% activity. SSF experiments were performed in production media containing cellulases (10 FPU g^{-1} of substrate). The pH of the fermentation broth dropped to 5.4 within 24 h (Fig. 3), which is the pH at

Table 2 Lactic acid production by *Lactobacillus lactis* mutant RM2-24 using different cellulosic substrates

	Yield (g l^{-1})			Productivity (g l^{-1} h^{-1})		
Substrates	24 h	48 h	72 h	24 h	48 h	72 h
α-Cellulose	47.92	73.31	73.1	1.99	1.52	1.01
Sigma cellulose	17.78	17.94	20.8	0.74	0.37	0.28
Solka-Floc	30.96	31.13	32.62	1.29	0.64	0.45
Avicel PH-101	15.88	17.11	17.33	0.66	0.35	0.24

Table 3 Comparison of recent data with the present work on SSF production of D-(−)-lactic acid from cellulosic substrates

Substrate (g l^{-1})	Microorganism	Enzyme (FPU g^{-1})	Fermentation time (h)	Maximum lactic acid concentration (g l^{-1})	Yield (% g g^{-1})	Productivity (g l^{-1} h^{-1})	Ref.
Filter paper (33)	*L. coryniformis* ATCC 25600	Celluclast and Novozyme (28)	48	25	75	0.5	4
Defatted rice bran (100)	*L. delbrueckii* IFO 3202	Cellulase-Y-NC	36	28	28	0.77	8
Pretreated cardboard (41)	*L. coryniformis* ATCC 25600	Celluclast and Novozyme (22.8)	48	23	56	0.49	17
α-Cellulose (100)	*L. lactis* RM2-24	*P. janthinellum* EU1 (10)	48	73	73	1.52	This work

which enzymes are most active. No cellobiose accumulation was observed during the fermentation at any time. Cellobiose was either converted to glucose by β-glucosidase present in the cellulase preparations or utilized by the mutant strain to produce lactic acid. We obtained 73 g l^{-1} of lactic acid from 100 g l^{-1} of α-cellulose. The yield (g g^{-1}) and productivity (g l^{-1} h^{-1}) of lactic acid were 0.73 and 1.52, respectively (Table 2). This is, to our knowledge, the highest yield and productivity of D-lactic acid reported in the literature so far from cellulose (Table 3).

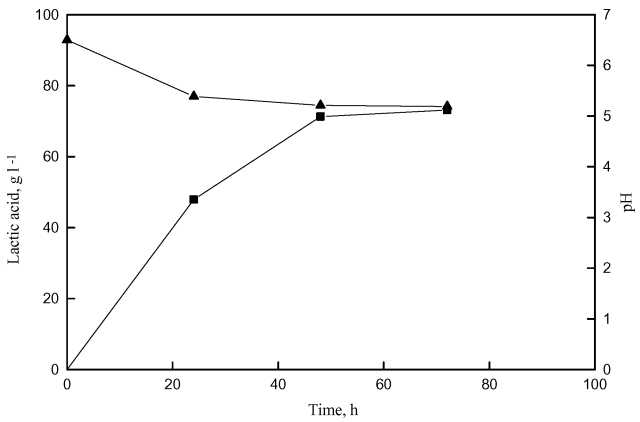

Fig. 3 Course of lactic acid production (■) and pH (▲) during SSF of α-cellulose (100 g l^{-1}) using mutant enzyme preparation (EU1, 10 FPU g^{-1}).

Conclusion

Lactobacillus lactis mutant RM2-24 is capable of utilizing high concentrations of cellobiose, producing lactic acid with high yield (0.8 g lactic acid per g cellobiose). Batch experiments were conducted for conversion of cellulose to lactic acid by simultaneous saccharification and fermentation (SSF) using cellulase preparations derived from mutants of *Penicillium janthinellum* (EU1) and *Lactobacillus lactis* (RM2-24). Batch SSF yielded 73 g l^{-1} of lactic acid from 100 g l^{-1} of α-cellulose with a yield of 0.73 g per g of cellulose substrate and a productivity of 1.52 g l^{-1} h^{-1}.

In conclusion, *Lactobacillus lactis* mutant RM2-24 is a promising strain for the production of D-lactic acid from cellulosic materials in SSF. Bottlenecks like feedback inhibition by glucose and cellobiose were removed by using this strain, leading to the complete conversion of cellulosic substrates to the value-added products like lactic acid. This strain utilized cellobiose effectively and produced lactic acid in a homo-fermentative manner. These studies show the potential of such a strain for producing value-added products from renewable biomass.

Acknowledgements

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