ELSEVIER

Process Biochemistry 41 (2006) 120–126

PROCESS BIOCHEMISTRY

www.elsevier.com/locate/procbio

Strain improvement of *Lactobacillus delbrueckii* NCIM 2365 for lactic acid production

Sachin R. Kadam, Sudarshan S. Patil, Kulbhushan B. Bastawde, Jayant M. Khire, Digambar V. Gokhale *

NCIM Resource Center, National Chemical Laboratory, Pune 411 008, Maharashtra, India

Received 28 February 2005; received in revised form 24 May 2005; accepted 1 June 2005

Abstract

Various nitrogen sources were compared with yeast extract for efficient lactic acid production by *Lactobacillus delbrueckii* (NCIM 2365). None of the nitrogen source gave lactic acid concentration as high as that for yeast extract. The effect of yeast extract could have been due to its B vitamin content. Acclimatization and ultraviolet mutagenesis were used to develop strains of *L. delbrueckii* (NCIM 2365) that produced increased lactic acid concentrations. Four mutants (Ac-1, Ac-2, Uc-1 and Uc-3) were compared with the wild type *L. delbrueckii* with 100 g/l cane sugar concentration in a fermentation medium. All the four mutants produced higher levels of lactic acid with enhanced productivity than the wild type. Lactic acid fermentation from various carbohydrates by both wild strain and mutant Uc-3 was investigated. Sucrose, xylose and maltose were not utilized by both the wild and mutant strains. When the cultures were grown in a fermentation medium containing glucose, fructose, lactose or galactose as a carbon source, the average volumetric productivities exhibited by mutant Uc-3 were higher than those of wild strain. Mutant Uc-3 was compared with wild type, *L. delbrueckii* NCIM 2365 by fermentation with different concentrations of hydrolyzed cane sugar. The highest lactic acid concentration (135 g/l) in batch fermentation was obtained with 150 g/l of cane sugar with 90% lactic acid yield. Overall, mutants exhibited faster growth rates, shorter lag phases, higher productivity and greater lactic acid yield.

Keywords: Lactic acid fermentation; *Lactobacillus delbrueckii* mutants; Productivity

1. Introduction

Lactic acid and its derivatives have been widely used in food, pharmaceutical, cosmetic and industrial applications [1]. It has also been receiving great attention as a feedstock for manufacture of polylactic acid (PLA), a biodegradable polymer used as environmental friendly biodegradable plastic. PLA could be a good substitute for synthetic plastic derived from petroleum feedstock. Being highly reactive due to the presence of carboxylic and hydroxyl groups, lactic acid can undergo a variety of chemical conversions into potentially useful chemicals such as propylene oxide, propylene glycol, acrylic acid, 2,3 pentanedione and lactate esters [2,3].

Lactic acid is produced commercially either by chemical synthesis or by microbial fermentation. Approximately, 90% of the total lactic acid produced worldwide is made by bacterial fermentation and rest is produced synthetically by the hydrolysis of lactonitrile. The chemical synthesis of lactic acid always results in racemic mixture of lactic acid, which is a major disadvantage. Fermentative production of lactic acid offers the advantages in both utilization of renewable carbohydrates and production of optically pure L- or D-lactic acid depending on the strain selected. The physical properties of PLA depend on the isomeric composition of lactic acid [4,5]. Therefore, pure L- or D-lactic acid is polymerized to a high crystal polymer suitable for fiber and oriented film production and is expected to be useful in the production of ligand crystal as well [6].

Only a few lactic acid bacteria such as *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* could produce optically pure lactic acid [7]. Most of the

* Corresponding author. Tel.: +91 20 25882702; fax: +91 20 25882647.
E-mail address: dv.gokhale@ncl.res.in (D.V. Gokhale).

1359-5113/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2005.06.007

lactic acid bacteria generally have complex nutrient requirements and very low growth rates, which are the main drawbacks of industrial lactic acid fermentation processes. Therefore, there is a need for developing lactic acid bacteria, which require minimal amount of nutrients in a medium and convert carbohydrates in to optically pure lactic acid through homo-fermentative pathway.

L. delbrueckii NCIM 2365 is a homo-fermentative L(+) lactic acid producing strain selected through screening protocol. Our objective of the present work was to develop mutants of *L. delbrueckii* NCIM 2365 capable of producing enhanced levels of L(+) lactic acid with higher productivity. Two mutants were selected through UV-mutagenesis showing bigger acid formation zones on the selected medium. Two mutants were selected by acclimatization technique, which also exhibited bigger acid production zones on the selected medium. We examined the mutants for their growth and lactic acid production.

India is one of the largest countries producing 20 million tonnes of cane sugar (sucrose). Sucrose has been used commercially for the production of high fructose syrup using yeast invertase enzyme. We used synthetic medium with acid hydrolyzed sucrose and compared the growth and lactic acid production by mutant and parent strains.

2. Materials and methods

2.1. Materials

Galactose, lactose, fructose, maltose, yeast extract and MRS liquid medium were obtained from Hi-Media (India). Glucose was obtained from E-Merck, India. D-xylose was from s.d. fine-chemicals Ltd., India. Corn Steep Liquor was procured from Anil Starch Industries Ltd., Ahmedabad, India. Cane sugar was purchased from local market. Other nitrogen sources were available locally.

2.2. Methods

2.2.1. Micro-organisms and growth media

L. delbrueckii NCIM 2365 and four mutants were used throughout the studies. The cultures were maintained in liquid MRS medium supplemented with 0.1% $CaCO_3$. The cultures were transferred to fresh liquid MRS medium every 4 weeks and were used as stock cultures for preparation of inoculum. The growth medium consisted of (w/v) hydrolyzed cane sugar 10%, yeast extract 1%, and $CaCO_3$ 5%. The basic fermentation medium was the same as growth medium. The cane sugar was hydrolyzed by adding 1 ml of 20% H_2SO_4 in 100 ml of sugar solution. The acidified sugar solution was heated in boiling water bath for 20 min. Cane sugar and yeast extract in the fermentation medium were replaced with other sugars and nitrogen sources to evaluate their effects on lactic acid production. The ingredients of the media were separately added after sterilization at 121 °C for 20 min. The pH of both growth and fermentation media was adjusted to 7.0 prior to sterilization.

2.2.2. Inoculum preparation

Cells from stock cultures were transferred to 30 ml sterile growth medium in 50 ml screw cap tubes and incubated at 42 °C for 24 h under stationary conditions. This culture (2 ml) was then transferred to 100 ml of the growth medium in 250 ml screw cap conical flask. The flask was incubated at 42 °C with shaking (150 rpm) for 24 or 48 h till growth (OD) reached approximately 10. This culture was used as an inoculum to be transferred to fermentation medium.

2.2.3. Fermentation conditions

Fermentation experiments were carried out in 250 ml screw cap conical flasks containing 100 ml of fermentation medium. Flasks were inoculated with 5% inoculum culture and incubated in shaker incubator at 42 °C with shaking at 150 rpm. The culture samples harvested at various time intervals were centrifuged at 5000 rpm for 20 min to separate the cells. The supernatant was analyzed for sugars and lactic acid and for determining the pH of the fermentation broth. The supernatant was acidified by adding equal volume of 1N HCl where free acid is liberated.

2.2.4. Analytical methods

Cell growth was measured by spectrophotometer (UV–vis Spectrometer –117, Systronics, Mumbai, India) at a wavelength of 660 nm. Reducing sugar concentrations were determined by DNS method [8]. The amount of lactic acid was determined using a high performance liquid chromatography (HPLC) system (Dionex India Limited) equipped with UV- or RI-detectors. An ion exclusion column (Aminex HPX-87H, Biorad, Hercules, CA) was used at a temperature of 38 °C with 0.008 N H_2SO_4 as a mobile phase at a flow rate of 0.6 ml/min. An injection volume of the sample was 50 μl.

2.2.5. Mutagenesis and mutant selection

L. delbrueckii NCIM 2365 was grown in 30 ml MRS liquid medium with 0.1% $CaCO_3$ at 42 °C under stationary condition for 24 h. This culture was used to inoculate 30 ml MRS medium with pH 6.0 containing 0.1% $CaCO_3$. The culture was incubated at 42 °C up to 48 h where OD was found to be 6.0. These transfers were repeated till the growth (OD) in MRS medium at pH 6.0 reaches 9.0 within 24 h. The above-mentioned culture was further transferred to MRS liquid medium having pH 5.5 with $CaCO_3$ (0.1%). Such transfers were repeated several times in a medium having lower pH till we got sufficient growth (OD equivalent to 9.0 within 24 h.). The culture was finally acclimatized to grow in a medium with no $CaCO_3$ at pH 4.0 where we found good growth (OD 7.5 within 24 h of incubation). The final pH of the medium was found to be 3.5. The above-mentioned acclimatized culture after suitable dilution was plated on a solid medium containing hydrolyzed cane sugar 10%, yeast