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POTENTIAL MICROORGANISM FOR THE DIRECT PRODUCTION OF L-LACTIC ACID FROM CASSAVA STARCH WITHOUT CARBON DIOXIDE PRODUCTION

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The application of a starch-utilizing and homofermentative microorganism will be of great benefit to the lactate production industry as well as the protection of carbon dioxide production and accumulation in human surrounding environment. A homolactic bacterium isolated from the cassava starch waste sample in Nakhon Ratchasima Province, Thailand, could actively convert cassava polysaccharides to L-lactic acid. The isolate produced the maximum amount of lactic acid of about 9 g/l in the suitable medium containing 2% cassava starch in laboratory scale. The gene encoding lactate dehydrogenase, a key enzyme in lactic acid fermentation by most lactic acid bacteria, of the microorganism was detected. The nucleotide sequence of the gene was analysed. To enhance the lactic acid production from cassava starch, the starch-utilizing strain was improved by exposing to two mutagens: Ultraviolet (UV) light and N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG). A mutant obtained from UV-UV-MNNG treatments could produce lactic acid at approximate ten percents higher than its original strain. The strain was finally selected and maintained for using as the potential microorganism for the direct production of lactic acid from cassava starch without carbon dioxide production. After subculturing the culture for sixth times, the mutant could still produce the similar amount of lactic acid as before the first subculture.

1 Introduction

L-Lactic acid can be efficiently produced by the microbial fermentation of glucose. A potential growth market for the acid is its use as a raw material for the biodegradable polymer production. The application of a homofermentative microorganism in lactate industry will protect the production and accumulation of carbon dioxide in human surrounding environment. Based on a cheap and abundant starchy raw material such as cassava starch produced in Thailand, the homolactic starch-utilizing strain will be very useful for the lactate production. The production cost in the hydrolysis step, liquefaction and saccharification, of starch to glucose could be minimized. Some bacterial strains have been studied for their lactate production capabilities. Examples are *Lactobacillus amylovorus* [16, 17], *Lactobacillus plantarum* NCIM 2084 [9], metabolically engineered *Escherichia coli* RR1 [3], and *Lactococcus lactis* IO-1 [7, 8]. Linko and Javanainen [10] also reported the capability of homofermentative *Lactobacillus casei* NRRL B-441 strain to produce mainly L-lactic acid. However, potential microorganisms for the acid production are still desirable.

Lactate dehydrogenase is a key enzyme in lactic acid fermentation by most lactic acid bacteria. Most bacterial species possess only one lactate dehydrogenase gene [6]. The DNA sequence analysis of the gene encoding lactate dehydrogenase is an important prerequisite to the genetic manipulation and improvement of starch utilization and lactic acid production strains. The lactate dehydrogenase gene polymorphism is also useful for lactococcal classifications [15].

In this study, an attempt to obtain a potential microorganism for the direct production of L-lactic acid from cassava starch without carbon dioxide production was carried out. The DNA sequence analysis of the microbial gene encoding lactate dehydrogenase as

well as the strain improvement by mutagenesis to enhance the lactic acid production from cassava starch of the selected isolate was also investigated.

2 Methods

2.1 Starch-utilizing microorganism for L-lactic acid production

One hundred and thirty four bacterial strains isolated from starch waste samples in Nakhon Ratchasima Province, Thailand, were screened for their starch utilization and lactic acid production capabilities without gas, carbon dioxide, formation using the cassava starch medium modified from Glucose medium [7] and the medium for cultivating lactic acid bacteria [1]. *Lactococcus lactis* IO-1, a homolactic fermenting and non-starch utilization strain which is able to convert glucose to L-lactate with a conversion rate greater than 90%, was used as the reference strain. An efficient isolate compared to *Lactococcus lactis* IO-1, was selected. The suitable medium for growth and acid production of the selected microorganism was investigated.

Time-courses of lactic acid fermentation were studied using the optimized medium containing cassava starch for the starch-utilizing strain, and glucose for *Lactococcus lactis* IO-1. Fermentations were conducted in 125-ml Erlenmeyer flask with working volume of 50 ml. Seed cultures were prepared from late-exponential phase cells adjusting the concentration at approximately 10^6 CFU/ml, and used as 2% (v/v) inocula. The fermentation lasted for 72 hours. Cell growth was monitored spectrophotometrically at A_{562}. The remaining starch (substrate) in the fermentation medium was measured at A_{580} in the presence of iodine [12, 5]. Reducing sugar/residual glucose was determined in bacterial filtrate by the method of Bernfeld [2]. The pH was measured using pH meter. Lactic acid was determined by High Performance Liquid Chromatography (HPLC).

2.2 Investigation of the gene encoding L-lactate dehydrogenase

The gene encoding lactate dehydrogenase of the selected starch-utilizing and homofermentative strain as well as *Lactococcus lactis* IO-1 was detected by amplifying bacterial DNA in the polymerase chain reaction (PCR) using ten primer pairs [6, 11, 13, 15] designed from sequences encoding bacterial lactate dehydrogenase. Purified DNA was isolated from broth cultures using the Wizard® Genomic DNA Purification Kit (Promega, Promega Corporation, U.S.A.). PCR amplifications were performed as described by the sources of primers.

DNA sequencing and sequence analyses of the gene encoding L-lactate dehydrogenase of some of these amplified fragments were performed using the Applied Biosystems (ABI) PRISM Terminator cycle sequencing kit, and data obtained from ABI PRISM® 377 DNA Sequencer. Comparisons of these nucleotide sequences were then performed.

2.3 Strain improvement by mutagenesis

Both physical (Ultraviolet [UV] light) and chemical (N-Methyl-N′-Nitro-N-Nitrosoguanidine, MNNG) mutagens were applied for improving the selected lactate-producing isolate to maximize their lactic acid production from starchy material using procedures as described by Baltz [4].

Mutants were collected at the survival of about 10% of the treated cell population, then selected by direct screening of surface cultures growing on the suitable starch agar medium containing 1% of cassava starch with the incubation at 37°C under anaerobic conditions for 48 hours. Selected colonies in which they gave larger clear zones with the

iodine reaction than colonies from untreated cells, the organism started with, and from the majority of treated cells were further tested for their starch utilization and lactic acid production in cassava starch broth at the maximum production period obtained from time-course investigation results.

The genetic stability of the selected mutant strain was initially determined by subculturing the cultures for six times. Then the subcultured cells were tested for their lactate production in the optimized starch broth medium.

3 Results and Discussion

3.1 Starch-utilizing microorganism for L-lactic acid production

The homofermentative bacterium strain, isolate SUT-5, isolated from a starch waste sample collected from the Cassava Starch Production Plant, was selected among 134 bacterial isolates. The selected isolate was tentatively identified as belonging to the genus *Lactococcus* according to its morphological and some biochemical characteristics, and confirmed as the homofermentative microorganism.

When compared to their lactic acid production capabilities using the suitable medium composed of ingredients as follows (g/l): carbon source either glucose (for *Lactococcus lactis* IO-1) or cassava starch (for isolate SUT-5), 20; pancreatic digest of casein, 5; K_2HPO_4, 6; yeast extract, 3; tri-ammonium citrate, 1; $MgSO_4.7H_2O$, 0.57; $MnSO_4.7H_2O$, 0.12; and $FeSO_4.H_2O$, 0.03; at the initial pH 7.0; the isolate SUT-5 could produce lactic acid of 9.4 g/l, which was the maximum amount achieved after 24-hour incubation (Figures 1). Starch (substrate) was dramatically lost from 2- to 12-hour growth. While our reference strain, *Lactococcus lactis* IO-1, could produce lactic acid at the concentration about 10 g/l at 24-hour growth. The two strains also gave the maximum growth when cultured for 24 to 32 hours.

3.2 Investigation of the gene encoding L-lactate dehydrogenase

The most successful amplification of the gene encoding L-lactate dehydrogenase was obtained when using three sets of primers (LDHF1: 5′-ATGGCTGATAAACAACGTA-3′/LDHR1: 5′-TTAGTTTTTAACTGCAGAAG-3′, LDHF2: 5′-GTTGCTGCTAACCCAGTTGA-3′/LDHR1: 5′-TTAGTTTTTAACTGCAGAAG-3′, and LDHF1: 5′-ATGGCTGATAAACAACGTA-3′/LDHR2: 5′-GTCAAGATGTCAACTGGGTT-3′) designed from sequences complementary to conserved sequences at the extreme ends of the coding region of *Lactococcus lactis* L-lactate dehydrogenase gene [15]. Both isolate SUT-5 and *Lactococcus lactis* IO-1 produced results of approximate 1,000-bp, 670-bp, and 400-bp amplification products with the three specific primer pairs respectively (Figure 2). There was no consistent results obtained from the amplification reaction using primers designed from both the partial amino acid sequence of a lactococcal lactate dehydrogenase [13], and sequences encoded amino acid sequences of the bacterial lactate dehydrogenase according to Llanos *et al.* [11].

Sequence analyses and comparisons of about 670-bp and 400-bp amplification fragments were then performed. The comparisons of about 670-bp and 400-bp fragment sequences showed that isolate SUT-5 had 47% and 75% homology respectively with *Lactococcus lactis* IO-1 (Figure 3, for example).

3.3 Strain improvement by mutagenesis

Two mutagens (UV light and MNNG) were applied for the isolate SUT-5 mutagenesis. For UV exposure, it was found that approximate ten percents of survival cells were

obtained when the parent cells were irradiated for 10 minutes. And for the MNNG treatment, fifty percents of survival cells were obtained when the parent cells were exposed to the chemical at the concentration of 400 μg/ml. Nineteen mutants were selected from double and triple UV, UV-MNNG, UV-UV-MNNG, UV-MNNG-UV, and UV-MNNG-MNNG treatments, and then compared for their lactic acid production capabilities in the optimum medium containing 2% of cassava starch under anaerobic conditions at 37°C for 24 to 32 hours. Most of the mutants could produce lactic acid at about 3-10% higher than its original strain (Table 1). A mutant obtained from UV-UV-MNNG treatments could produce lactic acid at approximate 10% higher than its original strain, and was finally selected and maintained for using as the potential strain for the direct production of lactic acid from cassava starch.

For the study of genetic stability of the selected mutant strain for homolactic fermentation, the strain produced the consistent concentration of lactic acid over the experimental period (Table 1).

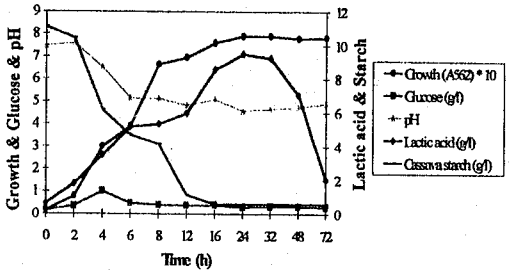

Figure 1. Lactic acid production from cassava starch by bacterial isolate SUT-5.

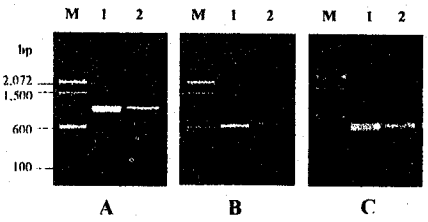

Figure 2. Agarose gel electrophoresis of PCR fragments generated with specific primers: (A) LDHF1/LDHR1, (B) LDHF2/LDHR1, and (C) LDHF1/LDHR2, of L-lactate dehydrogenase gene. Lanes: M, 100bp DNA ladder (GIBCOBRL) as a molecular weight marker; 1, Lactococcus lactis IO-1; 2, isolate SUT-5.

Table 1. Lactic acid production by starch-utilizing bacteria in the optimal medium containing 2% of cassava starch.

Bacterial strain	Lactic acid (g/l)	
	Before subculturing	After subculturing six times
SUT-5	9.48	9.61
SUT-5 mutant[1]	11.89	11.83

[1] Mutant obtained from UV-UV-MNNG treatments

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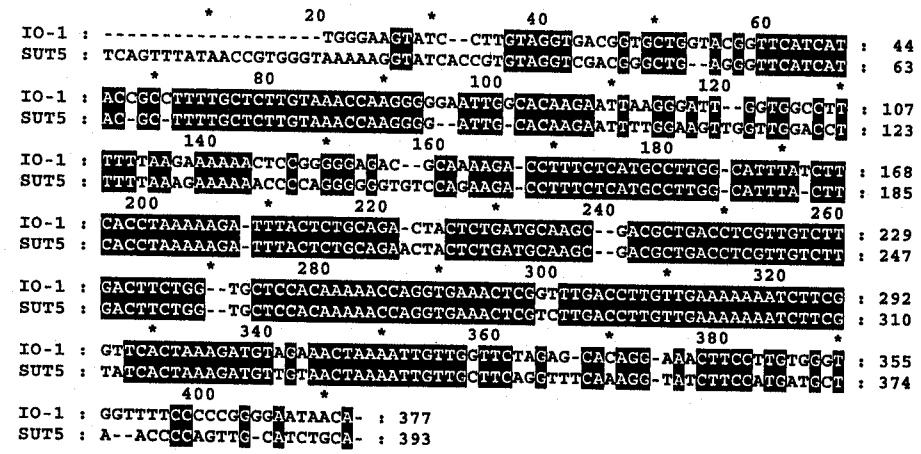

Figure 3. Alignment of nucleotide sequences (from the 5'terminus) of lactate dehydrogenase genes amplified using primers LDHF1/LDHR2 of *Lactococcus lactis* IO-1 (top) and isolate SUT-5 (bottom).

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