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Production of Medically Important Lactic Acid by Lactobacillus Pentosus: A Biological Conversion Method

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Abstract

Objective: Lactic acid is one of the main components of lactated Ringer's solution and Hartmann's solution, which is mainly produced by the pharmaceutical industry for the medical purposes. But the production of lactic acid through chemical route has many disadvantages. Therefore, production of lactic acid through biological route has more advantages. Hence the present study aimed to produce lactic acid from the xylose sugar through the biological route by using novel Lactobacillus strain. **Methods:** Hyperactive Lactobacillus strains were isolated from the coir industry and identified using routine biochemical and molecular biology tools. Ethyl Methane Sulfonate (EMS) were used for the creation of mutation in the selected strains and cultivated under various cultivation medium. High Performance Liquid Chromatography (HPLC) was used for the quantification of individual sugars, lactic acid and acetic acid respectively. **Results:** Primary screening revealed that the strain L-3 showed significant amount of lactic acid under aerobic and microaerobic condition. 16S rRNA gene amplification and sequence analysis confirmed that the strain had close similarity with the Lactobacillus Pentosus. Shake flask cultivation revealed that the strain could able to produce maximum amount of lactic acid from xylose and exhibited higher cell density under control growth condition, cell growth (1.7 OD 600_{nm}) and lactic acid (15 mM) and acetic acid (11 mm). Interestingly, the EMS treated cells of the strain produced promising amount of lactic acid (18 g/L). **Conclusion:** The novel strain could able to produce comparatively significant quantity of lactic acid by green synthesis method. The lactic acid produced by this method could be useful to the pharmaceutical industry for the bulk level production of medically important drugs.

Keywords: EMS, Lactic Acid, Lactobacillus, Medically Important Drugs

1. Introduction

Lactic acid (2-hydroxypropanoic acid, CH_3–CH (OH)–COOH) is widely used as a preservative chemical in food industry, acidulant in pharmaceutical, cosmetic and chemical industry[1]. Currently, there is renewed demand for lactic acid as a feedstock for the production of biopolymer Poly-Lactic Acid (PLA), which is a promising biodegradable, biocompatible and environmentally friendly alternative to plastics derived from petrochemicals. It is a nonvolatile, odorless organic acid and is classified as GRAS (Generally Recognized as Safe) for use as a general-purpose food additive by FDA in the USA and other regulatory agencies[2]. It is mainly produced by chemical synthesis or by microbial fermentation. Chemical synthesis from petrochemical resources always results in racemic mixture of DL-lactic acid, which is a major disadvantage of this approach[3]. Conversely, microbial lactic acid fermentation by selecting an appropriate strain offers an advantage in terms of the utilization of renewable carbohydrate biomass, low production temperature, low energy consumption and the production of optically high pure lactic acid. Refined disaccharides or monosaccharides have been more frequently used as the carbon source for lactic acid production, but this is economically

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unfavorable. Therefore, conversion of biomass into lactic acid is highly attractive, because of their complex nature and non decomposing nature using microorganisms. The biochemical conversion of lignocellulosic biomass requires several processing steps designed to convert structural carbohydrates to monomeric sugars such as glucose, xylose, arabinose and mannose. Among the monomeric sugars, xylose is the major component of lignocellulose sugars cannot be efficiently metabolized by most of the microorganisms. For xylose fermentation, bacteria activate xylose isomerase enzyme to convert xylose to xylulose directly while most fungi convert xylose to xylulose through the intermediate of xylitol. Among the lactic acid bacteria, Lactobacillus delbrueckii, L. rhamnosus, L. helveticus, L. pentosus, L. bifermentans, L. acidophilus, L. bifermentans, L. brevis, L. plantarum, Leuconostoc lactis and L. lactis were widely studied for the production of lactic acid from lignocellulosic biomass[5–8]. These strains ferment xylose to lactic acid via a phosphoketolase pathway[9]. In metabolic pathway, one mole of xylose yields 1 mole of acetic acid and 1 mole of lactic acid. Hence the theoretical yield of lactic acid from xylose is about 60%. However, achieving the complete conversion of yield of lactic acid from xylose is difficult. Reports claimed that metabolic engineering of the phosphorketolase pathway to pentose phosphate pathway yield high lactic acid. Whereas, xylose consumption is less. The secretion of lactic acid is also enhanced by the use of yeast extract, meat extract, peptone in the culture medium, by stimulating the growth of lactic bacteria. However, the use of these substances in large quantities is very expensive and contributes 30% of the total cost of lactic acid production[10]. Therefore, it becomes necessary to screen and characterize numerous strains to obtain ideal lactic acid producing strains using xylose. The number of xylose degrading strains from the environment has steadily decreased. To cope with the demand of lactic acids and its derived products, researchers have been forced to look for novel microorganisms in unusual environment. Relatively, the microorganisms from coir industry samples have rarely been explored for microbial diversity and lactic acid production from xylose. Hence, there is an immense possibility to identify novel strains with high conversion efficiency of xylose to lactic acid from the coir industry area. The purpose of this study is to isolate and characterize active microorganisms with a higher cell density in xylose containing cultivation medium. Several Lactobacillus strains were screened by growing on xylose as a sole carbon energy source and L-3 strain had a higher cell density and produced considerable lactic acid was treated with mutagenic agents for selection of better lactic acid producers.

2. Materials and Methods

2.1 Isolation and Identification of Organisms

For isolation cultures, the enrichment cultivation medium containing xylose 10 g/L, yeast extract 3 g/L, beef extract 3 g/L, peptone 5 g/L, $NaCl_2$ g/L, K_2HPO_4 2 g/L, $KHPO_4$ 2 g/L was used[11]. Xylose in the medium was used as a carbon source to favor the growth of xylose utilizing microorganisms. The pH of the medium was adjusted to 6.0 using 1N NaOH or HCl. The medium was autoclaved at 121°C for 20 min before use. Throughout the experiment was autoclaved without xylose followed by addition of filter-sterilized xylose solution to avoid the loss of xylose. One gram of coir industry sample was suspended in sterile water and centrifuged at 8,000 rpm for 20 min to remove the heavy particulates. After proper dilution, the supernatant was serially diluted with 0.85% saline solution and transferred into the 250 ml flask and incubated at 30°C. After 3 d of incubation with three cycles of enrichment culture; 1 mL of the aliquot was first suspended in a series of 10-fold dilutions of sterile saline water (0.85% NaCl) from 10^{-1} to 10^{-6}. An aliquot of 0.1 mL of each dilution was taken and spread evenly over the surface of the basal agar medium containing 25 g/L xylose. The bacterial colonies formed on the plate were transferred to new plates and incubated for further purification. The purified strains were numbered as strain (L-1 to L-21). One strain having better growth and lactic acid production was selected and named as strain (L-3); for further characterization the strain was preserved in 20% glycerol (v/v) at -80°C

2.2 Molecular Identification

Strain L-3 DNA was extracted manually by boiling a loop full of culture in sterilized distilled water for ten minutes and centrifuged at 13,000 rpm for 10 min. The supernatant containing the DNA was used to amplify 16S ribosomal DNA fragments by PCR (Bio-Rad I cycler) using 27F (5' AGA GTT TGA TCG TGG CTC AG 3') and 1492R (3' GGT TAC CTT GTT ACG ACT T 5') primers.

The amplified PCR product 1500 bp was subjected to gene sequencing and the similarity of the sequences were checked to BLAST at http://www.ncbinlm-nih.gov/ search in for determining the phylogenetic relationship with the identified strains[12].

2.3 Selection of Better Strain by Mutagenesis

For selecting the better growing and tolerant in the presence of xylose, the strain was selected by treating Ethyl Methane Sulphonate (EMS). Briefly, 0.6 OD of 1 mL of freshly prepared L-3 isolate cells (10^9 CFU/mL), were washed twice with 1.5 ml of 0.1M KPO_4 buffer, pH 7.0 and mixed with 20 µL EMS solutions and incubated at varying time period. After incubation (15, 30, 45 and 60 min) the cells at each time point were mixed with 1 mL of 5% sodium thiosulfate. Further, the cells were washed twice with sterile distilled water and putative mutants were selected in fermentation medium containing 100 g/L of xylose. The survival was less than 2%. The better mutants were selected and once again treated by EMS for the identification of best strain for maximum cell growth and production of lactic acid.

2.4 Shake Flask Cultivation of Lactobacillus sp. L-3

Lactobacillus sp. KCC-10 was grown in 250 ml Erlenmeyer flasks containing 50 mL production medium contained 5 g/L yeast extract, g/L Nacl, 0.2 g/L $MgSO_4$. $7H_2O$, 0.05 g/L $MnSO_4 7H_2O$, 100 mM phosphate buffer and 100 g/L xylose and incubated at 30°C on an orbital incubator shaker. The culture flasks were inoculated to 0.1 OD_{600} with freshly prepared cells grown in production medium under the same culture conditions. The flasks were plugged with oxygen-permeable cotton plugs and incubated at 50 rpm for the micro-aerobic condition. The saline bottles were plugged with oxygen-impermeable rubber stops and sealed with aluminum caps and incubated at 200 rpm for the anaerobic condition. The samples were withdrawn periodically and analyzed for cell density (OD_{600}) and metabolites (xylose, lactic acid and acetic acid)[13].

2.5 Analytical Methods

The cell concentration was determined by measuring the OD at 600_{nm} in an ELISA reader (Bio-Rad). The amount of xylose, lactic acid and acetic acid present in the fermentation medium was quantified by High Performance Liquid Chromatography (Agilent Technologies, USA). The cell free supernatant was collected and separated through a SB-C_{18} column (4.6 mm × 150 mm, 5 µm) at 65°C using 5 mM H_2SO_4 as the mobile phase at 0.5 mL/min. Standard organic acids were used as the control for the calculation of the individual concentration in the fermented broth.

3. Results

3.1 Identification and Morphological Characteristics of Xylose utilizing Bacteria

The present study aimed to investigate the selection of microbial strains from the coir industrial effluent samples to convert xylose into lactic acid. Different colonies were selected based on the cell growth of xylose containing agar medium and designated as L-1 to L-21. Initially all the isolated strains were cultivated in 50 mL shake flask containing production medium with 100 mM xylose were screened for its high cell growth and lactic acid production. Among the selected strains, L-3 found to best over other strains in terms of growth (1.7 OD 600_{nm}) and lactic acid (15 mM) and acetic acid (11 mm) production under shake flask level. Biochemical characteristic and physiological characteristic revealed that the strain L-3 belongs to Gram-positive, light creamy in color, the live cells were short with cell size of approximately 1.3–0.9 µm diameter and can grow under micro-aerobic and anaerobic conditions. Utilization of various carbon sources by L-3 strain indicated a wide pattern of carbon source assimilation (Table 1). Based on the microscopic analysis and biochemical characteristics, the strain was identified as Lactobacillus and designated as L-3. The cell wall polyunsaturated fatty acid profile indicated that the strain L-3 had a match with Lactobacillus sp. 16 s rRNA sequence analysis strongly suggested that this strain belonged to the genus Lactobacillus Pentosus (Figure-1). 16S rRNA gene sequence has been deposited in the NCBI nucleotide sequence databases.

Table 1. Carbohydrate fermentation test of strain L-3

No.s	Tests	Result	No.s	Test	Result
1	Control	-	26	Esculin ferric citrate	+
2	Glycerol	-	27	Salicin	+
3	Erythritol	-	28	D-Celiobiose	+
4	D-Arabinose	+	29	D-Maltose	+
5	L-Arabinose	-	30	D-Lactose	+
6	D-Ribose	-	31	D-Meliobiose	-
7	D-Xylose	+	32	D-Saccharose	+
8	L-Xylose	-	33	D-Trechalose	+
9	D-Adonitol	+	34	Inulin	+
10	Methyl-D-Xylopyraniside	+	35	D-Melezitose	+
11	D-Galactose	+	36	D-Raffinose	-
12	D-Glucose	+	37	Amidon	-
13	D-Fructose	+	38	Glycogen	-
14	D-Mannose	-	39	Xylitol	-
15	L-Sorbose	-	40	Gentiobiose	+
16	L-Rhamnose	+	41	D-Turanose	+
17	Dulcitol	+	42	D-Lyxose	-
18	Inositol	-	43	D-Tagatose	+
19	D-Mannitol	+	44	D-Fucose	-
20	D-Sorbitol	+	45	L-Fucose	-
21	Methyl-D-Mannopyraniside	-	46	D-Arabitol	-
22	Methyl-D-Glucopyraniside	+	47	L-Arabitol	-
23	N-Acetyl-Glucosamine	+	48	Potassium Gluconate	+
24	Amygdalin	-	49	Potassium Gluconate 2-KetoGluconate	-
25	Arbutin	-	50	Potassium Gluconate 5-KetoGluconate	-

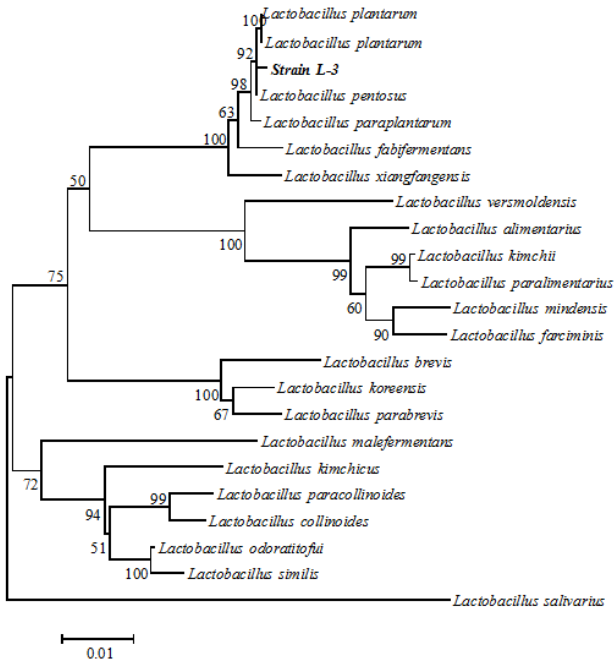

Figure 1. Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences of strain L-3.

3.2 Mutagenesis of the Lactobacillus Strains

Initial screening confirmed that strain L-3 showed better cell growth and lactic acid production profile. Therefore, strain L-3 was subjected to mutagenesis by EMS for the selection of better strain. The strains were allowed to kill by keeping them in the EMS for maximum time. Finally 98% of the cells were killed and remaining 2% were selected for further study. The survived cells were selected and further cultivated in higher xylose concentration and checked for their cell growth and organic acid production profile. Seven strains namely, M-1 to M-7 was selected and studied for the production of lactic acid and other organic acids. The production profile of the mutated strains was displayed in Table 2. Results indicated that among the seven strains, strain M-6 exhibited hyper lactic acid production followed by strain M-1. Besides the lactic acid production, the strain also produced significant level of acetic acids and succinic acid. The final growth profile of strain M-7 was its advantage. Further, the strains production profile was enhanced by optimizing the cultivation condition and growth profile under various cultivation medium.

Table 2. Primary screening of different mutant strains of L-3 for organic acid production

Strains	Microaerobic condition			Anaaerobic condition		
	Final cell growth	Lactic acid (g/L)	Acetic acid (g/L)	Final cell growth	Lactic acid (g/L)	Acetic acid (g/L)
M-1	2.1	7.24	3.5	1.58	12.5	6.9
M-2	1.15	3.45	1.05	1	5.9	2
M-3	0.91	4.5	2.5	0.98	6.49	1.6
M-4	1.25	4.45	1.05	1.02	4.68	2.9
M-5	0.91	2	0.5	1	4.15	2.9
M-6	1.45	10.5	4.6	1.29	11.5	7.5
M-7	3.1	18	16	2.75	21	15

3.3 Optimization of Lactic Acid Production

Different parameters such as pH, temperature and agitation speed were evaluated for better lactic acid production. Initially, the strain was cultivated under pH, 5, 6, 7 and 8. After incubation, the production profile was determined by HPLC and the cell growth was determined by spectrophotometer. Results indicated that the strain exhibited better production and growth under neutral pH. Under pH 7, the strain consumed 94 g/L of xylose and produced 40.74 g/L of lactic acid and 31.05 g/L of acetic acid. The final cell density at pH 7 was 3.41 (Table 3), whereas, at pH 7 and 8, the consumption of sugar and organic acid production profile was comparatively lesser. Cultivation under varying temperature indicated that the stain could grow well at 30 and 35°C. Under 30 and 35°C the strain consumed 94.95 and 96.8 g/L of sugar and produced 38.15 and 42.4 g/L of lactic acid. Therefore, it is concluded that the 35°C was the optimum for better lactic acid production. Cultivation under different rpm revealed that 100 rpm was better for the cell growth and lactic acid production.

Table 3. Optimizing the lactic acid production conditions for the mutant strain-M6

Different conditions		Final cell growth	Consumed Xylose (g/L)	Produced lactic acid (g/L)	Produced acetic acid (g/L)
pH	5	1.7	21.05	4.87	1.15
	6	3.1	67	21.74	12.05
	7	3.41	94	40.74	31.05
	8	2.71	48.35	23.13	14.5
Temperature(°C)	30	3.5	94.95	38.15	28.5
	35	3.53	96.8	42.4	30
	40	2.77	43.05	18.36	11
	45	1.1	8.1	4.07	2.17
Agitation (RPM)	50	3.5	93.4	38.15	30
	75	3.43	94.8	40.25	33.1
	100	3.53	96.46	43	31.7
	150	3.4	94.65	39.2	32

4. Discussion

The present study aimed to convert the xylose into lactic acid in the biological method using the novel Lactobacillus strains. Conversion of lignocelluloses biomass into useful product by chemical and biological route is the attractive method. Among the conversion method biological method had more importance because of its low input cost for the production. The mixed sugars consists of glucose, xylose and arabinose is converted into lactic acid by lactobacillus strains had some difficulties. The difficulties are the conversion of xylose into lactic

acid and other methodlites such as acetic acid. This is occurred because of the heterofermentation of xylose via the phosphoketolase pathways[14]. In the present study, the isolated xylose fermenting strains were identified as Lactobacillus among that the promising strain L-3 shared higher similarities with Lactobacillus Pentosus. Those novel strain able to ferment the xylose into lactic acid and acetic acids which clearly indicated the activation of metabolic enzyme (phosphoketolase) responsible for hetero-lactic acid fermentation as reported by the other strains such as E. mundtii QU 25[15,16].

Results indicated that the concentration of lactic acid and acetic acid was increased rapidly after 24 h of incubation under shake flask condition, indicating that when the identified strain was presumably adapted to the inhibitory compounds in the xylose mixture especially the degradation compounds such as furfural and other inhibitory compounds, it could efficiently utilize the fermentable sugars in the mixture. The fermentation experiments revealed high optical purity of lactic acid using these substrates as source of carbon[17]. Smith et al. (2014) claimed that two key strain development techniques, namely random mutagenesis and evolutionary engineering were sequentially used to strengthen the microbial strains for the lactic acid production against xylose as the subtract[18]. Simultaneously, selective pressure through xylose as carbon source was imposed the strain to enhance the metabolism via the xylose isomerase pathway[19,20]. The selected strain would be useful for further preparation of the useful commodity chemicals. Also, reports claimed that the Lactobacilus strains were useful for the food industry and also useful for the treatment of diseases[21].

5. Conclusion

The novel Lactobacillus strain useful to convert xylose into lactic acid and acetic acid were isolated from environmental sample and was identified both biochemical and molecular methods. 16S rRNA amplification and sequencing analysis confirmed that the strain belonged to Lactobacillus Pentosus. Shake flask cultivation method confirmed that the strain was able to convert xylose to lactic acid under aerobic and microaerbic condition. Fermentation parameters were optimized and the productions of lactic acid were enhanced in the standardized condition. Additionally, EMS treatment could generate new strains which can withstand higher xylose level and produce significant amount of lactic acid. In conclusion, the lactic acid produced by using this strain could be useful for the preparation of various medically important drugs and other routine medical agents.

6. Conflict of Interests

Declared none

7. References

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