Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria

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Citation

Abstract
Bacterial exopolysaccharides have wide applications in various industries. In this perspective, it is essential to explore the natural biodiversity for novel strains of exopolysaccharide synthesizing lactic acid bacteria (LAB). Two novel isolates of lactic acid bacteria with higher enzyme activity were screened and characterized based on a battery of microscopic, staining, metabolic, physiological and antibiotic sensitivity tests. The two isolates of LAB named SPO and SPA were cocci shaped, Gram Positive, catalase negative, heterofermentative, vancomycin resistant, broad spectrum carbohydrate fermentating with exopolysaccharide synthesizing activity. EPS synthesizing activity was confirmed by activity staining of EPS using sucrose as substrate. This confirmed that the EPS produced was dextran and the enzyme responsible for its synthesis is dextransucrase. The enzyme activity of SPO was 3.8 U/ml and that of SPA was 3.4 U/ml. For strain improvement, the isolates were subjected to UV radiation. The isolate SPO did not give promising results. However, SPA after UV-mutagenesis, generated two novel mutants, SPAm1 and SPAm2. The enzyme activity of SPAm1 was 4.9 U/ml and that of SPAm2 was 4.7 U/ml. The mutants possessed about 40% enhanced enzyme activity over the wild type strain.

INTRODUCTION
Since time immemorial, lactic acid bacteria are regarded as food grade micro-organisms. LAB have found wide applications as food preservatives, flavouring and texturizing agents for centuries [1] and are now used as starter culture in dairy industry, meat and vegetable fermentation. LAB have received great attention as the major group of probiotic bacteria promoting the growth of gut micro flora [2]. LAB are also reported to cure diarrhoea, irritable bowel disorder, allergies, lactose intolerance, urinary tract infections and to stimulate immunity (3,4,5). LAB capable of secreting antimicrobial peptides are used as food preservatives as well as health-promoting agents for humans [6]. Lactic acid bacteria have attracted immense commercial interests, for their capacity to secrete a host of exopolysaccharides having industrially useful physico-chemical properties [7,8]. Dextrans are a class of exopolysaccharides synthesised by Lactobacillus, Leuconostoc and Streptococcus belonging to LAB family. Sucrose is hydrolysed by the enzyme dextransucrase and the resultant D-glucosyl moieties are polymerised to produce dextran. Dextrans are employed as blood plasma substitutes, plasminogen activators, antithrombogenic agents and in treatment of iron deficiency anaemia [9,10]. Dextran are also tremendous usage in matrix preparation of chromatography columns [10]. Dextran have major use in food formulations as stabilizing, emulsifying, texturizing and gelling agent. Dextran is reported to enhance biocompatibility of biomaterials [11]. Considering the grand commercial usage of the dextrans, it is essential to discover novel isolates of LAB synthesizing bioactive exopolysaccharides. It is important to garner sufficient information about the characteristics of the EPS producing strains, as the optimal growth conditions, carbohydrate fermentation ability, antibiotic sensitivity studies have practical implications in maximizing the dextran production on large scale. The development of novel approaches in food and in pharmaceutoclinical therapies is broadening the potential for using lactic acid bacteria. [12]. Apart from screening the biodiversity for selection of new isolates, UV induced mutagenesis of the existing strains for improvement is a promising strategy. UV mutagenesis was carried out on the strains of L. delbrueckii (NCIM 2365) and screened four novel mutants exhibiting higher lactic acid productivity and yield with faster growth rates and shorter lag phases were reported [13]. The present study reports the isolation of two new strains of lactic acid bacteria from the
soil samples, their characterization. UV mutagenesis as a tool for strain improvement was explored for enhancing enzyme production.

MATERIALS AND METHODS
ORIGIN, CULTURE AND STORAGE CONDITIONS OF BACTERIAL ISOLATES

The soil samples were collected from the sugarcane fields of Assam (near Guwahati) and Orissa (Sundargarh). 1 g soil from the samples were suspended in 10 ml 0.85% sterile saline solution. The soil suspensions were subjected to serial dilution. 100 μl of the sample from 10^{4}, 10^{5}, 10^{6} and 10^{7} dilutions of soil samples were spread plated on sugarcane medium composed of filtered sugarcane juice containing sucrose (2%, w/v), peptone (1%, w/v), NaCl (0.1%, w/v), pH 7 [14]. The culture plates were prepared in duplicates. The plates were then incubated for 48h at 28°C. Appearance of colonies were checked and counted on a colony counter. The large size colonies were selected and picked by inoculation loop and streaked on modified MRS agar plates containing 2% sucrose [15]. The streaked plates were incubated for 12h at 28°C and stored at 4°C. Loopful of cultures were inoculated in 5 ml enzyme production medium described by Tsuchiya et al. (1952) [16] and grown for 12h at 25°C and 180 rpm. 1% of this 12h grown culture was again inoculated in 5 ml sterile medium of Tsuchiya et al. (1952) [16] in tubes and grown for 16h at 25°C and 180 rpm for further inoculations and enzyme assays.

SELECTION OF THE ISOLATES BASED ON ENZYME ACTIVITY

Enzyme activities of the two natural isolates were determined by the estimation of reducing sugars by the method of Nelson [17] and Somogyi [18]. The enzyme assay was carried out in 1 ml reaction mixture containing 5% sucrose, 20 mM sodium acetate buffer (pH 5.4) and 10-20 μl cell free supernatant. The enzymatic reaction was performed at 30°C for 15 min. 100 μl aliquot from the reaction mixture was taken for reducing sugar estimation. Fructose was used to plot the standard graph. One unit (U) of dextranucrase activity is defined as the amount of enzyme that liberates 1 μmole of reducing sugar (fructose) in 1 min at 30°C and pH 5.4.

The selected isolates were named as SPO and SPA after the author’s and place’s name. The isolates were grown in modified MRS-agar stabs at 28°C for 20h and maintained at 4°C. The isolates were propagated by sub-culturing every 2 weeks. The cultures were also preserved as glycerol stock frozen at -80°C in 22% (v/v) final sterile glycerol concentration for long term storage.

MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF ISOLATES

The phenotypic characterization of the isolates was followed as reported earlier [19, 20, 21]. The characteristics of these strains, such as Gram staining, morphology, catalase activity test were studied. The Gram staining of the isolates was performed. Bacterial smear was air dried and flooded with crystal violet solution for 30s, the stain was decanted by rinsing with running water, the slide was flooded with iodine solution for 30s, excess of this mordant was washed off with water and flooded with counter stain safranin for 30s, washed with water and the smear was observed under compound microscope.

The cell shape, size and their arrangement were studied by Scanning Electron Microscopy. The samples were prepared by centrifuging 1 ml of 12h grown culture at 5,000 rpm for 10 min. The cell pellets were dissolved in 1 ml of saline solution (0.85%, w/v). The sample was fixed with equal volume of glutaraldehyde (2.5%, v/v) for 2-4h. One drop of this bacterial smear was dehydrated using different percent of alcohol and finally dried in a vacuum desiccator. This dried sample was attached to the SEM stub with double-sided tape and coated with 10 nm Au in a sputter coater (SCH 620, Leo). The surface of the sample was viewed at various magnifications in Scanning Electron Microscope (Leo1330 VP) operated at 10.0 kV.

The catalase activity of the isolates was deduced by adding few drops of 3% H₂O₂ on 5 ml of 12h grown culture [22]. The E. coli culture was treated under identical conditions with H₂O₂ as positive control for catalase activity.

The hot loop test devised by Sperber and Swan (1976) [23] was followed to find the nature and type of fermentation. To the culture of isolates grown in MRS medium at 28°C for 24h, a red hot inoculation loop was plunged immediately to detect the evolution of CO₂.

The morphology of colony of isolates was examined on MRS agar plate after 24h of growth. Growth of isolates at initial pH of medium 3.3 and 9.6 were tested to determine their pH tolerance [24].
**GROWTH PROFILE STUDY**

Various growth parameters such as change in pH, cell optical density and enzyme activity [17,18] were studied at every 4h interval. Growth of culture was determined by measuring the cell optical density at 600 nm using the sterile medium as blank in UV-Vis spectrophotometer (Labomed, model UVD-300). To determine the effect of shaking on the growth of the isolates, loopful of the isolate cultures from the modified MRS stabs were grown in 5 ml enzyme production medium [16] and kept in static and orbital shaking incubator at 25°C±0.2°C. After 16h, 1 ml from the samples were withdrawn, centrifuged and cell free supernatant was analyzed for enzyme activity.

To determine the optimum incubation temperature for growth of the isolates, loopful of the isolate cultures were grown in 5 ml enzyme production medium and incubated at four different temperatures 22°C, 25°C, 28°C and 30°C in incubator shaker. After 16h, 1 ml from the samples were withdrawn, centrifuged and cell free supernatant was analyzed for enzyme activity as described earlier.

**ANTIBIOTIC SENSITIVITY**

The isolates were tested for their susceptibility to thirty antibiotics using agar disc diffusion test [25]. The antibiotic tests were performed using commercially available antibiotic octodiscs impregnated with Amoxyclav, Cephalexin, Ciprofloxacin, Clindamycin, Claxacillin, Erythromycin, Tetracyclin, Ampicillin, Carbenicilllin, Cephatoxamine, Chloramphenicol, Co-Trimazine, Gentamicin, Norfloxacxin, Oxacillin, Amikacin, Amoxyclllin, Bacitracin, Cephalothxin, Novobiocin, Oxytetracyclin, Vancomycin, Penicillin-G, Tobramycin, Cephaloridine, Kanamycin, Linomycin, Methicillin, Norfloxacxin and Oleandomycin purchased from Hi-media Pvt. Ltd. India. The isolates grown in MRS liquid medium were mixed in MRS-soft agar (0.8%, w/v agar) and poured over the MRS medium containing 1.8%, (w/v) agar. After 2 min, the antibiotic octodiscs were gently placed at the centre over the surface of the agar plates. The Petri plates were incubated in inverted position overnight at 28°C and were observed next day for zone of inhibition around the discs. The results were compared with that of Leuconostoc mesenteroides NRRL B-640 [26].

**CARBOHYDRATE FERMENTATION**

The isolates were tested for their carbohydrate fermentation ability [27]. From the overnight grown MRS broth containing 2% glucose as carbohydrate source, 50 µl was inoculated in 5 ml liquid MRS medium lacking glucose but containing Phenol red (0.04 g/L) as pH indicator and other test carbohydrates [26]. The test media were incubated at static condition, for 48h at 28°C. The acid production was observed between 24-48h. The acid production as a result of carbohydrate fermentation was indicated by a change in colour of phenol red to yellow. The results were compared with the standard strain Leuconostoc mesenteroides NRRL B-640 [26].

**DETECTION OF DEXTRAN SYNTHESIZING ACTIVITY**

Dextran synthesizing activities of the isolates were tested by loading purified enzyme from the isolates on a 7.5% acrylamide gel and conducting non-denaturing SDS-PAGE [28]. After the run, SDS was removed by incubating the gel in sodium acetate buffer (20 mM sodium acetate, pH 5.4, 0.3 mM CaCl₂ and 0.1%, v/v Tween 80) at 4°C for 30 min. The gel was incubated in sodium acetate buffer (20 mM sodium acetate, pH 5.4, 0.3 mM CaCl₂) supplemented with 5% sucrose for 48 h at 30°C. Following incubation, the gel was washed once with a solution of methanol:acetic acid (50:10, v/v) in water for 30 min, then with water for 30 min, and incubated in a periodic acid solution (1%, w/v periodic acid and 3%, v/v acetic acid) for 45 min at 25°C. After the periodic acid treatment, the gel was washed with water for 2h with several changes. The gel was then stained with 15 ml Schiff reagent (0.5%, w/v Fuchsin basic, 1% sodium bisulphite and 0.1 N HCl) until the magenta colour bands appeared.

**UV-MUTAGENESIS OF THE ISOLATES AND SELECTION OF MUTANTS**

A loopful from the culture of the natural isolate SPO and SPA, maintained in modified MRS agar as stabs were inoculated in 10 ml modified MRS medium [15] and grown at 25°C and 180 rpm for 12h. 1 ml from these cultures were serially diluted in 9 ml of 0.85% saline solution until the required dilutions were obtained. 100 µl of the cultures from the dilution factors 10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷ were spread-plated on modified MRS agar (1.8%, w/v) Petri-plates. Each dilution factor was taken in duplicates for accuracy of results. The Petri-plates were exposed to UV radiations for 15s, 30s, 60s and 90s [13]. Immediately after the UV irradiation, the plates were kept in the dark to avoid photo-reactivation. The control and UV treated Petri-plates were
incubated at 28°C for 36h. 35 larger colonies were selected from the culture plates for each isolate that showed about 1% survival rate. These colonies were analyzed for their enzyme assay. One loopful from each colony was grown in enzyme production medium [16]. The cell free supernatants obtained by centrifugation at 10,000 rpm and 4°C for 10 min, were analyzed for enzyme activity using reducing sugar estimation [17,18]. The protein concentration of the cell free supernatant was estimated by Lowry’s method [29].

RESULTS AND DISCUSSION
MORPHOLOGICAL, PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION

The appearance of purple colour of cells after Gram staining, confirmed the Gram positive nature of the isolates, a characteristics of lactic acid bacteria (Fig. 1, A and B). The morphology of cells observed by scanning electron microscopy revealed cocci shape and random arrangement in groups or chains (Fig. 2, A and B). The catalase activity test proved the catalase negative nature of the isolates as they could not hydrolyse H_2O_2, whereas, E. coli hydrolysed it, as vigorous bubbling was observed (Fig. 3, A, B and C). The hot loop test confirmed the hetero-fermentation nature of the isolates as the insertion of inoculation loop into the culture broth created a stream of bubbles symbolising the presence of CO_2. The initial medium pH of 3.3 and 9.6 did not support the growth of the isolates. The colonies measured were 1-2 mm in diameter, white, smooth, circular and slimy when grown on modified MRS agar at 28°C for 24h. If the strains are catalase negative, gram-positive and cocci shaped they can be identified as lactic acid bacteria.

Figure 1
Gram staining of the isolates showing purple colour, indicating their Gram positive nature (A) SPO and (B) SPA

Figure 2
Scanning Electron Microscopy of the isolates showing the cocci shape and random arrangement of the isolates (A) SPO and (B) SPA

Figure 3
Catalase activity test of the isolates showing their catalase negative nature (A) (B) SPO (C) SPA

Growth profile study

The cell density of the two isolates at 600 nm measured by a spectrophotometer showed the peak at 16h. The pH of the growth medium fell from 6.9 to 4.2, after 32h of incubation. Dextranucrase activity of both the isolates was highest at 16h. From these results, it was inferred that the enzyme activity is growth associated. Excess lactic acid production led to pH fall, that inactivated the enzyme after 16h (Fig. 4). The enzyme production was favoured by shaking over static conditions. This explains that the isolates are microaerophilic in nature. Both the isolates gave maximum enzyme activity at incubation temperature of 25°C.
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Figure 4
The cell growth, pH and enzyme activity profile of the isolates at 25°C under shaking condition (A) SPO (B) SPA

Figure 5
Antibiogram of the isolates and the standard NRRL B 640 using antibiotic octodiscs on MRS agar.

ANTIBIOTIC SUSCEPTIBILITY OF THE ISOLATES

In order to elucidate the antibiotic susceptibility of the isolates, a standardized filter-paper disc-agar diffusion assay was used. This method determines the efficacy of the drug by measuring the diameter of the zone of inhibition which results from diffusion of the antibiotic from the disc into the medium. In this procedure, the filter-paper discs of uniform size are impregnated with specified concentrations of different antibiotics and then placed centrally, on the surface of an agar plate seeded with the organism to be tested. Following incubation, the plates are examined for the zone of inhibition, surrounding the discs. The susceptibility of microorganism to a drug is determined by the size of this zone. A measurement of the length of the zone of inhibition is made. Based on this comparison, the test organism is classified to be resistant, moderate or susceptible to the antibiotic. The two isolates along with standard strain Leuconostoc mesenteroides NRRL B-640 were tested for susceptibility to twenty-nine antibiotics belonging to different classes. All the three strains were resistant to the antibiotics norflaxacin and vancomycin (Table 1). Resistance to vancomycin is characteristic feature of Leuconostoc genus [30]. All the three strains were sensitive to amoxycillin, carbenicillin, chloramphenicol, linomycin, oxytetracyclin, penicillin-G and tetracycline [30,26]. From Table 1, it can be observed that the antibiotic susceptibility pattern of the isolate SPO is 66% similar and that of SPA is 55% similar to Leuconostoc mesenteroides NRRL B-640.

CARBOHYDRATE FERMENTATION BY THE ISOLATES

The ability of the two isolates to degrade and ferment carbohydrates with the production of acid was tested. Both the isolates utilized cellobiose, dextrose, fructose, galactose, maltose, melibiose, sucrose, trehalose as colour changed to yellow (Fig. 5). The isolates showed weak fermentation towards lactose, rhamnose, xylose and were unable to ferment mannitol, raffinose and glycerol (Fig. 5). The extent of fermentation of carbohydrates were categorized as shown in Table 2. The carbohydrate fermentation profile of both the isolate SPO and SPA was 92% similar to B-640. These results are in agreement with those of Holt et al. (2001) [28] and Purama et al. (2008) [26] with few exceptions.

Figure 6
The carbohydrate fermentation pattern of the isolates using 14 sugars. The change of red to yellow colour indicates sugar fermentation.
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**Figure 7**
Carbohydrate fermentation of the natural isolates and NRRL B 640 after 24h.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Carbohydrates</th>
<th>B-640</th>
<th>SPO</th>
<th>SPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cellobiose</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>2</td>
<td>Dextrose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>3</td>
<td>Fructose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>Galactose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>Lactose</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>Maltose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>7</td>
<td>Mannitol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Melibiose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>9</td>
<td>Raffinose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Rhamnose</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Trehalose</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>13</td>
<td>Xylose</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

(+ + +) strongly positive; (+ +) fairly positive; (+) weakly positive; (-) negative

**DEXTAN SYNTHESIZING ACTIVITY OF THE ISOLATES**

Both the natural isolates SPO and SPA showed single magenta colour band on the gel after non-denaturing SDS-PAGE, confirming in situ dextranucrase activity (Fig. 6, A and B). The results are similar to those obtained with Leuconostoc mesenterides NRRL B-640 [31] and Leuconostoc dextranicum B-1146 [32] which also showed single band.

**Figure 8**
Dextran synthesizing patterns of the isolates (A) SPO and (B) SPA as observed by periodic acid Schiff protocol of activity staining.

**UV-MUTAGENESIS OF THE ISOLATE SPA AND SELECTION OF MUTANTS**

The colonies generated after UV irradiation of isolates SPO
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and SPA were enumerated by colony counter. It was observed that 30s and 60s exposure to UV radiation on the culture plates of $10^{-4}$, $10^{-5}$ and $10^{-6}$ dilution, generated mutants with approximately 1% survival rate. The colonies of isolate SPA appeared at $10^{-4}$ dilution without exposure and reduced survival with UV exposure are shown (Fig. 7, A and B). The survival rate for isolate SPA is shown in Table 3. The plates having approximately, 1% survival rate were selected for screening of mutants. From the selected plates of SPO having a total of 52 colonies and that of SPA having 41 colonies, 35 from each were picked for enzyme assay. Out of the 35 mutants of SPO, 8 showed higher enzyme activity than the wild-type SPO, but the increase was not significant and further studies on the mutants of SPO were not undertaken.

Out of the 35 mutants of SPA, 15 showed higher enzyme activity than the wild-type isolate SPA. The percent increase in the enzyme activity of the mutants is shown in Table 4. Out of 15, two mutants showed significant increase in enzyme activity and selected for propagation and for further studies. The wild-type isolate SPA showed enzyme activity of 3.4 U/ml, whereas the mutant SPAm1 showed 4.9 U/ml and mutant SPAm2 showed 4.7 U/ml, exhibiting enhanced enzyme activity by 44% and 38%, respectively (Table 4). The specific activity of the mutant SPAm1 was 1 U/mg and that of SPAm2 was 0.94 U/mg, whereas the specific activity of the wild type SPA was 0.58 U/mg, exhibiting enhanced specific activity by 72% and 62%, respectively as compared to the wild type SPA (Table 5).

CONCLUSIONS

In light of the documented biochemical and physiological characterization of the isolates their suitability for exploitation can be charted. The antibiotic resistance, carbohydrate fermentation profiles, sucrose hydrolyzing activity or polysaccharide synthesis activity determination are important for understanding of these industrially potential strains. The isolates SPO and SPA were Gram positive, heterofermentative, cocci and were lacking catalase activity. Both the isolates were resistant to the antibiotics viz. norflaxacin, ampicillin, amikcin, vancomycin, kanamycin, tobramycin and cephalaridine. Both the isolates were found sensitive to amoxyccillin, carbenicillin, cephalothin, cephotaxamine, chloramphenicol, clindamycin, linomycin, oxytetacyclin, methicillin, oleandomycin and
tetracyclin. Both the isolates utilized cellobiose, dextrose, fructose, galactose, maltose, mellibiose, sucrose and trehalose. Mannitol, raffinose and glycerol could not be fermented by any of the isolates. Both the isolates confirmed sucrose hydrolyzing activity by displaying the polysaccharide formation patterns in non-denaturing SDS-polyacrylamide gels.

The meteoric rise in the usage of exopolysaccharides in food, pharmaceutical and cosmetics industries emphasises the importance of exploration of the new species and characterization of their traits. Modification of the isolate SPA by UV-mutagenesis generated mutants showing enhanced enzyme activity, thus may find commercial applications.

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