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Statistical optimization of exopolysaccharide production by *Lactobacillus plantarum* NTMI05 and NTMI20

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ABSTRACT

In this study, 27 strains of Lactic acid bacteria (LAB) were isolated and identified from different milk sources. All the isolates were biochemically characterized and screened for their ability to produce exopolysaccharides (EPS), among which two isolates namely *Lactobacillus plantarum* NTMI05 (197 mg/L) and *Lactobacillus plantarum* NTMI20 (187 mg/L) showed higher EPS production. Both the isolates were molecular characterized and tested for their probiotic properties. The chemical composition of EPS from *L. plantarum* NTMI05 and NTMI20 revealed the presence of 95.45% and 92.35% carbohydrates, 14 ± 0.1and 11 ± 0.15 mg/L lactic acid, 10.5 ± 0.2 and 9 ± 0.1 mg/mL of reducing sugar, respectively. HPLC analysis showed galactose at the retention time of 2.29.The maximum EPS yield was optimized for the media components like glucose (20 g/L), yeast extract (25 g/L) and ammonium sulphate (2 g/L) using Central Composite Design and Response Surface Methodology (RSM). Under optimum conditions the predicted maximum EPS production was 0.891 g/L, 0.797 g/L, while the actual experimental value was 0.956 g/L and 0.827 g/L for *L. plantarum* NTMI05 and NTMI20, respectively. The antioxidant capacity was also evaluated by DPPH and reducing power assay proving the potentiality of these organisms in food and dairy industries.

1. Introduction

Lactic Acid Bacteria (LAB) are ubiquitous in nature. Milk from different mammals can be used as a source of LAB. In addition to the synthesis of lactic acid, there are several LAB traits that could confer desirable attributes to specific fermented products, as in the case of production of exopolysaccharides (EPS) in dairy fermentations. The EPS produced by LAB has received increasing attention mainly because these biopolymers improve the physical properties of fermented food products. Therefore, studying the probiotic traits of new or commercial strains of LAB before their application in the formulation of functional foods is of special relevance [1]. Probiotics are beneficial bacteria that favorably alter the balance of intestinal microflora, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection. Characterization of probiotic bacteria from goat milk [2], sheep milk [3] and breast milk [4] has already been studied. Bacterial growth is often accompanied by production of exopolysaccharides (EPS), which have relevant ecological and physiological functions. It has been shown that EPS of LAB contribute to the texture, mouth feel, taste perception and stability of fermented products. Furthermore, it has been reported that EPS of LAB have anti-tumor [5] anti-ulcer [6] and cholesterol-lowering activities [7]. Sometimes, EPS producing LAB is responsible for the undesirable ropiness of food products such as wine, cider and beer.

Sugars such as glucose, lactose, sucrose and mannose are utilized by LAB strains as the carbon source for EPS production [8]. The composition of the culture medium (carbon and nitrogen sources) influences the production and concentration of EPS [9]. Carbon sources such as oat-based non-dairy medium, whey permeate medium, sugar beet molasses and sago starch [10] are also found to be utilized by LAB to produce EPS. The other important parameters in EPS production include the pH of culture media and the incubation temperature.

The main strategy of bioprocess optimization is single factor optimization, i.e., changing one factor at a time keeping the other

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constant. One of the disadvantages of this method is that the effect of interaction of the factors cannot be assessed. This could be overcome with the help of statistical experimental designs that are being used for many decades and can be applied to search for optimal conditions of a targeted response [11]. The popular choices among statistical designs are central composite design (CCD) and response surface methodology (RSM) with various designs [12]. RSM is a collection of mathematical and statistical techniques for designing experiments, building models, searching optimum conditions of factors for desirable responses and evaluating the relative significance of several affecting factors even in the presence of complex interactions. RSM has been recently used for modeling and optimization of bioprocesses such as fermentation media, cultivation and process conditions [13,14], enzyme production [15,16] and extracellular polysaccharide production of *Oudemansiella radicata* [17].

This study has been focused on isolation and characterization of probiotic LAB strains from different sources of milk and screening efficient EPS producers with optimization of growth parameters for higher EPS production using RSM for the influence of different nutrients and cultivation conditions. The RSM with CCD was applied to determine the effects of significant parameters and their interactions to identify the optimum values and conditions that can be experimentally validated. Though various studies focused on EPS production by LAB isolated from different environmental sources through RSM optimization, our aim has been to evaluate the potentials of LAB isolated from different sources of milk and increase the EPS production further by optimizing growth kinetic parameters.

2. Materials and methods

2.1. Sampling and isolation of lactic acid bacteria

The LAB isolates were obtained from various sources like goat milk, cow milk, curd (cow milk), camel milk, sheep milk and buffalo milk. The samples were collected in sterile containers and immediately transported to laboratory at ambient temperature and stored at 4 °C until used in experiments. Decimal dilution of these samples were plated in MRS agar plates (HiMedia, Mumbai, India) and incubated at 37 °C for 48 h under static condition. Later, each colony was plated separately on MRS (de Man, Rogosa and Sharpe) agar plates [18] and subsequently subcultured for maintaining the pure cultures and kept frozen at −20 °C in MRS broth supplemented with 25% glycerol.

2.2. Phenotypic and biochemical characterization of selected isolates

The isolated colony formed on the MRS agar plates was identified using microscopy, gram staining, biochemical tests and carbohydrate fermentation tests [19]. The morphology was determined by microscopy of cells grown in MRS broth at 37 °C for 48 h.

2.3. Isolation and purification of EPS

The exopolysaccharide (EPS) was isolated with slight modifications of the method described earlier [20]. The cultures were heated at 100 °C for 10 min to inactivate enzymes potentially capable of polymer degradation and the cells were removed by centrifugation at 11,500 × *g* for 15 min at 4 °C. The supernatant was precipitated with double volume chilled ethanol and stored overnight at 4 °C. The resultant precipitate, after overnight storage was collected by centrifugation at 2,500 × *g* for 20 min. The pellet was dissolved in deionized water and dialyzed for 48 h against distilled water at 4 °C with replacement of water four times a day and then lyophilized. Freeze-dried EPS was used for further characterization.

2.4. Analysis of chemical composition of EPS

The EPS were analyzed for total carbohydrate content by phenol-sulphuric acid method [21] and protein content by Folin-Lowry method [22]. The lactic acid produced by the organisms was determined by Barker & Summerson assay [23] and the amount of reducing sugars present in the exopolysaccharide was estimated by DNS (Dinitro-salicylic acid) method [24].

2.5. Probiotic characterization studies

The probiotic properties of the selected isolates were determined by checking their tolerance to acid and bile salts [25], the degree of hydrophobicity [26] and mucin adhesion [27]. The phenotypic identification of exopolysaccharides produced by the selected strains was performed by alcian blue staining [28] and ruthenium agar method [29].

2.6. Molecular characterization of selected bacterial cultures

The genomic DNA from two selected probiotic bacterial isolates was extracted [30]. The extracted DNA was subjected to 16S rRNA gene amplification using the specific primers UB16S-F (5′AGAGTTTGATCCTGGCTCAG3′) and UB16S-R (5′ACGGCTACCTTGTTACGACT3′). All PCR reactions were carried out in a 30 μL volume containing 15 μL of Primer Taq premix (Taq polymerase, dNTPs, 2× Buffer with Mg+), 12 μL of Milli Q water, 1 μL (10 pmoles/μL) of each primer and 1 μL (50 ng) of bacterial DNA. The amplifications were performed with DNA thermal cycler (Veriti® –Thermal cycler, Applied Biosystems, California, USA). The reactions were carried out using the following conditions: 94 °C for 6 min (initial denaturation), 94 °C for 30 s (denaturation), 52 °C for 45 s (annealing), 72 °C for 45 s (elongation), 72 °C for 7 min (final elongation) with 35 cycles. The electrophoresis was carried out in 1.2% agarose (Sigma, Missouri, USA) and the bands were observed and photographed using the gel documentation system (Slite140, Avegene, New Taipei city, Taiwan).The sequences of the PCR products were determined by using a BigDye Terminator Cycle Sequencing v2.0 kit on an ABI 310 automatic DNA sequencer (Applied Biosystems, California, USA) according to the manufacturer's instructions. The 16S rRNA gene sequences determined for the probiotic strains were deposited in the GenBank database and the accession numbers were obtained.

2.7. Phylogenetic tree analysis *and* in silico *studies*

Based on the sequenced data, the phylogenetic tree was constructed using MEGA 5.05 [31], for aligning the sequences by Neighbor joining method [32]. Secondary structure prediction of 16S rRNA gene was analyzed using RNA*alifold* server version 2.5.8. (http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi).These secondary structures were used for the significance of observed differences in 16S rRNA gene sequence data. The theoretical restriction profiles of 16S rRNA gene sequence of each species, which had a high percentage of identity in the alignment of the BLAST algorithm, were compared with the restriction profiles of the isolates by Nebcutter software version 2.0 (http://tools.neb.com/NEBcutter2/index.php).

2.8. FT-IR characterization of EPS

FT-IR was performed using KBr method to determine the functional groups presented in EPS of the two strains tested with