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RESEARCH ARTICLE

Isolation of a novel dextransucrase producer from the intestine of *Harpadon nehereus*

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Abstract

Lactobacilli are almost ubiquitous and are found in a variety of ecological niche including the gastrointestinal tract of humans and animals. The current study was aimed at understanding the diversity of lactobacilli in the intestinal microflora of Bombay duck (*Harpadon nehereus*). A total of 23 isolates were obtained from the 15 fish samples procured from Ferry Wharf (Bhaucha Dhakka). After confirming the purity of the isolates, they were subjected to microscopic and biochemical analysis and were characterized up to the species level. Out of the 23 isolates obtained, 6 of them were found to belong to *L.brevis*, 9 belonged to *L.plantarum*, 7 belonged to *L.fermentum* whereas 1 belonged to *L.confusus*. These isolates were further evaluated for their ability to produce dextransucrase. Out of the 23 isolates, only *L.confusus* demonstrated this ability and produced 4.98 DSU/ml of the enzyme. The identity of *L.confusus* was further confirmed by determining its 16S rRNA gene sequence. Owing to its ability to produce dextransucrase and being GRAS, this novel isolate is a potential candidate for industrial production of dextransucrase and thereby dextran.

INTRODUCTION

Lactic acid bacteria (LAB) is a heterogeneous group of industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits. The genera that comprise the LAB are at its core *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus,* and *Streptococcus,* as well as the more peripheral *Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Sporolactobacillus, Tetragenococcus, Vagococcus,* and *Weisella*. Out of these, the most important genus of the family is *Lactobacillus.* It is also the most numerous genus of the family comprising of 215 species and 19 subspecies. The genus *Lactobacillus* consists of a genetically and physiologically diverse group of rod-shaped, Gram-positive, non-spore forming, non-pigmented, catalase negative and microaerophilic to strictly anaerobic fastidious organisms. The members of the genus *Lactobacillus* possess two major advantages in that some of them are known to be probiotics and possess the GRAS status[1]. Secondly, they also produce many metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, diacetyl and bacteriocins which are believed to play an important role in preserving the nutritive values of the food.[2] Owing to these properties and the increasing awareness related to probiotics, isolation of these lactobacilli and investigation of their properties have become very imperative.

In general, Lactobacilli have complex nutritional requirements and occur in habitats with a rich nutrition supply.[3] They have limited biosynthetic ability, having evolved in environments that are rich in amino acids, vitamins, purines and pyrimidines. Most of them are free-living or live in beneficial or harmless associations with animals whereas some are opportunistic pathogens. They are found in a variety of milk and milk products, decomposing plant material and fruits, dairy products, fermented meat and fish, beets, potatoes, mash, sauerkraut, sourdough,

pickled vegetables, silage, beverages, plants, water, juices, sewage and in cavities (mouth, genital, intestinal and respiratory tract) of human and animals where they are reported to be the normal flora and play a beneficial role.[4]
The Gastrointestinal tract of fish consists of a very dynamic and complex microbial ecosystem which is important from the nutritional, physiological and pathological point of view. Although the muscle of living fish is sterile, the skin, mucus, gills and gut contain significant bacteria including LAB. The composition of these organism and their quantity vary according to the fish species, temperature, salinity and dissolved oxygen of the water, degree of pollution, feed and stress among others. [5] Recently, a considerably diversity of LAB have been reported to colonize the guts of live fish and have been confirmed as part of the natural microbiota of healthy marine and freshwater fish. Although a few LAB species have been described as fish pathogens, most indigenous LAB are harmless and are considered as potential functional cultures in the production of numerous fermented fish products or as probiotics that could improve fish health. In fish the presence of LAB is meanwhile well documented and researchers have described their presence in the intestines of various fish species at larval, fry and fingerling stages inhabiting ponds. However there is little information about the presence of *Lactobacillus* in the intestines of marine fish, whereas other groups of LAB have been studied in more detail.[5–7]

Furthermore many strains of lactobacilli are known to synthesize extracellular polysaccharides (EPS) which fulfil a variety of diverse functions including cell protection, adhesion of bacteria to solid surfaces and participate in cell-cell interactions. Incorporation of EPS or EPS-producing cultures in dairy foods can provide viscosifying, stabilizing and water-binding functions. They also contribute to the mouth-feel, texture and taste perception of fermented dairy products. EPS may even play a role in the probiotic activity of certain LAB.[8] One such EPS synthesized by LAB is dextran. Dextransucrase (E.C. 2.4.1.5) is responsible for the production of dextrans which are a class of large extracellular glucans produced by the organisms belonging to the genera *Lactobacillus*, *Leuconostoc* and *Streptococcus.* Extensive information is available for *L.mesenteroides*, however relatively less is known about the other dextran producers. Although each bacterial strain produces a unique glucan, a common structural feature of all dextrans is a high percentage (up to 95%) of α (1→6) linkages with a smaller proportion of either α (1→2), α (1→3), or α (1→4) linkages. This results in a highly branched dextran molecule.

Conventionally, milk, milk products and fermented foods have been documented as a well-known source for LAB, but isolates from the intestines of animals, humans and fish have become an increasingly important source for such strains due to an increased awareness of their importance as probiotics. The present study focuses on the screening, isolation and characterization of lactobacilli obtained from the guts of fish procured from **Ferry Wharf** (also called as Bhaucha Dhakka). It is a wharf along the coast of Dockyard Road on the Eastern seafront of Mumbai which serves as a port for numerous fishermen who bring in their daily catch. A total of 15 adult Bombay duck (*Harpadon nehereus*) were selected and sent to the lab for analysis. The samples were processed and colonies were obtained. After confirming the purity of the isolates and classifying them as lactobacilli, they were further subjected to a battery of biochemical tests in order to characterize them up to the species level. The isolates were further analyzed for their ability to produce dextransucrase and thereby dextran.

Material and Methods

Collection of Fish Samples:
A total of 15 freshly caught adult Bombay duck (*Harpadon nehereus*) were procured from Ferry Wharf and immediately transferred to the lab in temperature controlled boxes. Further analysis of the fish samples were carried out at the lab immediately.

Isolation of Lactobacilli from Bombay duck (*Harpadon nehereus)*
The fish samples were processed immediately after reaching the lab. The numbers of incidental organisms were reduced by washing the fish skin five times with 70% ethanol. Then, the ventral surface of the fish was opened with sterile scalpels in order to gain access to the digestive tract. A small portion of the digestive tract was cut and transferred into sterile saline. The intestinal contents were homogenized using a vortex mixer and diluted appropriately in normal saline.[9] The dilutions were pour plated on MRS agar and incubated at 37°C anaerobically for 24 to 48 hours. After incubation, the well isolated colonies were further picked and transferred to new MRS agar plates by streaking. Colonies exhibiting typical characteristics of lactobacilli on agar surface were picked up randomly and transferred into MRS broth for further enrichment. Consequently their purity was further assessed on MRS agar.

Identification of Lactobacilli

The pure isolates were subjected to biochemical characterization and identification as per the Bergey's Manual of Determinative bacteriology and open source softwares like PIBWin[10] and IDENTAX.[11] Macroscopic appearance, size, shape, colour and texture of the colonies were recorded along with microscopic characteristics to aid in identification.

The isolates were stained by Gram's Method and examined under microscope for purity. Those isolates readily identified as Gram positive rods and catalase negative were included for further characterization [12] which included cytochrome oxidase; growth at 15°C, 37°C and 45°C; acid production from carbohydrates (1 % w/v) - L-arabinose, D-fructose, cellobiose, esculin, lactose, maltose, sucrose, D-galactose, D-mannose, raffinose, salicin, melebiose, melezitose, mannitol, rhamnose, D-ribose, trehalose, sorbitol and D-xylose in MRS broth devoid of glucose and beef extract with phenol red as indicator; production of acid and gas from 1 % glucose (MRS broth without beef extract); methyl red and Voges-Proskauer test in MRVP medium; nitrate reduction in nitrate broth, indole production in tryptone broth, production of ammonia from arginine and growth on acetate agar.[9,13,14]

Screening for Dextransucrase activity of the isolates

A volume of 1% of culture (0.1 $Absorbance_{630nm}$) was inoculated into 10 ml MRS broth and incubated at 30°C under anaerobic conditions. The fermented broth (10 ml) was taken after 9 hours and centrifuged at 10,000 rpm for 20 mins at 4°C and the cell free supernatant was used for the estimation of enzyme activity. The assay of dextransucrase was carried out in 1 ml of a reaction mixture in 50 mM sodium acetate buffer,1 mM $CaCl_2$, pH 5.4, containing (5%) sucrose and using the cell free extract (100 µl) as the enzyme source.[15] The reaction mixture was incubated at 30°C for 30 mins and the reducing sugar was estimated using the method of Nelson [16] and Somogyi [17]. A standard curve was prepared with fructose. One unit (U) of dextransucrase 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.[15] It is expressed as DSU/min.

DNA Sequencing of the Dextransucrase Positive Isolate

The identification of the positive isolate was done by DNA sequencing of the 16S rRNA gene. DNA Sequencing of the isolates was done by Microbial Culture Collection, **National Centre for Cell Science** (**NCCS**), Pune, India.
Sequencing of pure isolate was done using multiple PCR primers in ABI 3730XL sequencing machine. DNA isolation (PCR Template preparation) was done by Phenol-Chloroform method and PCR purification by PEG-NaCl method.

Primer*	Sequence (5'-3')	Target Group	Reference
8F	AGAGTTTGATCCTGGCTCAG	Universal	Turner et al, 1999
907R	CCGTCAATTCMTTTRAGTTT	Universal	Lane et al, 1991

Table 1: Primers used for DNA sequencing

Result and Discussion

Identification of Lactobacilli

The preliminary investigations included macroscopic analysis, microscopic analysis, Gram-positive bacilli, lactic acid biosynthesis, endospore test, milk coagulation activities and the negative catalase reaction. Microscopically

they were Gram-positive rod shaped, non- motile, catalase negative and exhibited absence of endospore. The isolates were tolerant to a range of salt concentrations (1-9%) and also demonstrated the ability to coagulate milk. The isolates were also found to be negative for MR-VP tests, nitrate reduction, indole test, cytochrome oxidase and H_2S production. The results of these tests permitted the classification of the working bacterium into the genus *Lactobacillus*.

Biochemical Characterization and Assessment of Dextransucrase activity

Identification was carried out up to the species level with the help of biochemical tests given in Bergey's Manual of Determinative bacteriology and Bergey's Manual of Systematic bacteriology whereas open source softwares like IDENTAX and PIBWin were also used to aid the identification and help develop an identification scheme.[9,18,19] A total of 23 isolates were obtained from the fish samples. Amongst the others, Lab Isolate MS12 which was identified as *L.confusus* tested positive for dextransucrase activity.

Sr.No	Identified species	No. of Isolates	Dextransucrase Activity
1	*L.brevis*	6	Negative
2	*L.plantarum*	9	Negative
3	*L.confusus (MS12)*	1	Positive
4	*L.fermentum*	7	Negative
	Total	23	-

Table2: Isolates Obtained from Fish samples

Determination of Dextransucrase activity of MS12

The lab isolate MS12 which was identified as *L.confusus* by biochemical characterization was further subjected to the estimation of dextransucrase producing ability.

Sr.No	**Organism**	**DSU/ml**
1	*L.confusus (MS12)*	4.98

Table3: Dextransucrase activity of the isolate MS12

DNA Sequencing of MS12

Sequencing of the lab isolate MS12 corroborated with the microscopic and biochemical results and confirmed its identity as *L.confusus*. Nucleotide sequence similarities of observed DNA sequences as determined by BLAST, version 2.2.28 (National Centre for Biotechnology Information databases) further confirmed the isolate as *L.confusus* strain SK9-2. The results have been listed as follows:-

Table 4: Nucleotide Sequence of MS12	GCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAA TTGCTTTGGAAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGT AGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGG ACTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCT GGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGT GCCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAAC TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAG CAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAACTCCCAGAGATGGAG CGTTCCCCTTCGGGGACAAAGGTGA

Isolate	NCBI hit	Total score	Query coverage	E value	Max indent	Accession No
MS12	*L. confusus* (*Weissella confusa*) strain SK9- 16S ribosomal RNA gene, partial sequence	856	100%	0	99%	JQ801710.1

Table5: BLAST Hit for the Isolate MS12

Discussion

Lactobacilli are almost ubiquitous and are found in a variety of ecological niche including the gastrointestinal tract of humans and animals. They are known to colonize in the intestines of various organisms resulting in conferring their probiotic effect on the host. The lactobacilli act by producing varied products such as bacteriocins, organic acids etc. which are capable of interfering with the growth of other microbes. Hence these end products have been applied to food systems to prevent the growth of undesirable bacteria. Due to the diversified applications presented by lactobacilli, it is highly imperative to study these organisms and their properties in more depth.

Several reports have cited the presence of lactobacilli in the intestinal flora of fresh and sea water fishes however; there is no information about their diversity in the intestine of Bombay duck. A total of 23 isolates were obtained from 15 individual samples and were characterized upto the species level. Out of the 23 isolates obtained, 26% were identified as *L.brevis*, 39% as *L.plantarum*, 30% as L.fermentum and 4% as ***L.confusus.*** The predominance of *L.plantarum* followed by *L.fermentum* is in consonance to previous reports, where L.plantarum has been reported to be in large numbers in the intestinal isolates of other fish. [18]

The isolates obtained were further evaluated for their ability to produce dextransucrase and thereby dextran**.** Out of all the isolates screened, only ***L.confusus*** demonstrated dextransucrase production and exhibited a yield of 4.98 DSU/ml. This isolate was further confirmed as ***L.confusus*** **(**now known as *Weissella confusa*)[20–22] by subjecting it to DNA sequencing of its 16S rRNA gene. ***L.confusus'*** ability to produce dextran is of particular interest because of its use as blood-plasma volume expander in the pharmaceutical industry. Dextran also finds other applications in the chemical industry as an adjuvant, emulsifier, carrier and stabilizer. Dextran and its derivatives have immense applications in food industry based on their ability to stabilize syrups, confectionaries and the likes, against crystallization and impart viscosity to sugar syrups. Therefore, it finds use in various food products as a conditioner, stabilizer, bodying agent and other related use. Dextran also has high specific superiority for stabilizing texture of icecreams and sherbats.[23] Also, the range of glucans and oligosaccharides produced by GTF (glycosyltransferase) enzymes present in lactobacilli may potentially act as prebiotic by stimulating the growth of probiotic strains or of beneficial endogenic microorganisms of the gastrointestinal tract. Owing to its physiological properties and its ability to influence rheological property of a particular substance, dextran gives an incentive to extending its horizon of probable applications to the food and nutraceutical industry. Due to its ability to produce dextransucrase and dextran, this novel dextransucrase producer – *L.confusus* needs to be examined for its potential as an industrial producer of dextransucrase and thereby dextran.

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