



Original Article

Bioprospecting of indigenous resources for the exploration of exopolysaccharide producing lactic acid bacteria



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ABSTRACT

Exploration of biodiversity lead towards the discovery of novel exopolysaccharide (EPS) producing microbes that have multiple applications. The safety compatibility status of lactic acid bacteria (LAB) makes it an attractive candidate for the production of EPS in industries. Therefore, new bacterial isolates are continuously being identified from different habitats. Current research was conducted to explore indigenous biodiversity for the production of dextranucrase, which is involved in the synthesis of dextran. Dextran is an EPS which is used in different industries. In this study, thirty-nine LAB were isolated from different food samples. The isolates were identified as genus *Leuconostoc*, *Weissella* and *Streptococcus* based on genotypic and phenotypic characteristics. Screening revealed that only eight isolates can produce dextranucrase in high titres. Fermentation conditions of dextran producing LAB was optimized. The results indicated that *Weissella confusa* exhibited maximum specific activity (1.50 DSU mg^{-1}) in 8 h at 25°C with pH 7.5. Dextran produced from *Weissella* proved to be a useful alternative to commercially used dextran produced by *Leuconostoc mesenteroides* in industries for various applications.

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1. Introduction

The need to explore biodiversity for the production of novel metabolites is increasing progressively. Bioprospecting is the most frequently used term for screening of biological resources for the extraction of commercially important compounds. It generally relies on the provision of novel biodiversity [1]. Microbes are considered as powerful source for the development of new bio-products. A diversity of chemical structure and functionality has been observed in compounds obtained from microorganisms. Lactic acid bacteria (LAB) is one of the most explored microbial community among all identified genera with the potential to produce different metabolites. LAB are ubiquitous in nature and commonly found in various fruits, vegetables, meat, dairy products, cereals and seafoods. During the past few decades, exploration of natural resources resulted in identification of several new taxa of lactic acid bacteria [2–4]. LAB comprises of diverse genera including *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, *Weissella*,

Oenococcus, *Sporolactobacillus*, *Teragenococcus*, *Vagococcus*, *Lactobacillus*, *Aerococcus*, *Carnobacterium* and *Enterococcus* [5]. These genera have proven record in production of important commercial metabolites [6,7].

LAB have potential applications in food, pharmaceuticals and chemicals. They are also considered as microbial cell factories as they can produce different types of extracellular polysaccharides [8,9]. All exopolysaccharides have broad commercial applications due to the versatility in their structural and functional properties. A wide range of exopolysaccharides are produced from lactic acid bacteria including dextran, mutan, alternan, reuteran, inulin and levan [10]. Among them, dextran has various commercial applications since its identification in 1861 [11]. Several species of *Leuconostoc* have been reported to produce exopolysaccharides [12–14]. However, recent studies focused on its isolation from other LAB such as *Weissella* and *Pediococcus* [15,16]. The exploration of new isolates is important because the characteristics of dextran varies from species to species. The applicability of this exopolysaccharide highly depends on the molecular weight, type of linkage and degree of branching [17]. Dextran produced from *Leuconostoc mesenteroides*, *Leuconostoc citreum* and *Weissella confusa* have α -1 → 2 (4.1%), α -1 → 2 (3.1%) and α -1 → 3 (11%), α -1 → 3 linked

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branching (2.3%), respectively. Dextran from *Leuconostoc citreum* is preferred as a prebiotic glucooligosaccharide as compare to other lactic acid bacteria whereas, dextran from *Weissella* is more linear than commercially used *Leuconostoc mesenteroides* B512F [18]. Linear dextran is highly soluble and have high viscosity while, branching decreases the solubility of the polymer [19].

Considering the fact that biopolymers have applicability in diverse field depending upon their structural characteristics. The current study is aimed to explore biodiversity of lactic acid bacteria from indigenous sources for the production of dextransucrase and dextran. After identification of a potential isolate, the fermentation conditions were optimized to enhance the secretion of the enzyme.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents used in the current study are of analytical grade and purchased from different companies. MRS broth, yeast extract, bacteriological peptone, vancomycin disk were purchased from Oxoid (Basingstoke, Hampshire, England); sucrose, dipotassium hydrogen phosphate, calcium chloride, sodium chloride were products of Serva (Heidelberg, Germany); arginine and magnesium sulphate were purchased from Scharlau (Barcelona, Spain). The reagents were prepared in deionized water unless otherwise stated.

2.2. Sample collection and bacterial isolation

For the isolation of bacterial strains, twenty-five indigenous samples including decayed fruits (*Mangifera indica*, *Malus domestica*, *Vitis vinifera*, *Prunus domestica* and *Prunus persica*), vegetables (*Brassica oleraceae* var. *capitate*, *Lycopersicon esculentum* and *Brassica oleraceae* var. *botyris*) and a dairy product (raw milk) were collected from different areas of Karachi, Pakistan. After collection, samples were chopped and transferred into MRS (de Man, Rogosa and Sharpe Agar) broth medium and incubated at 25 °C for up to 24 h under agitation (100 rpm). Serial dilutions were carried out up to 10⁻⁶ diluents. From each dilution, 100 µl was spread on sucrose (20 g L⁻¹) agar plate and incubated at 25 °C up to 48 h. Isolated colonies with glutinous texture were selected and identified. The pure colonies were preserved on tomato juice agar slant and stored at 4 °C for further analysis [20].

2.3. Bacterial growth measurements

Bacterial growth was measured spectrophotometrically by transferring the culture in MRS broth medium and incubated for 24 h at 25 °C. After incubation, the samples were centrifuged at 4 0,000 × g for 10 min at 4 °C. The bacterial cell pellet was suspended in normal saline (1.5 ml) and optical density (OD) was measured at 600 nm using normal saline as a blank. Afterwards, for the calculation of the number of bacterial cells present in the sample, the respective OD of each sample was compared with that of McFarland turbidity index [21].

2.4. Qualitative screening for dextransucrase and dextran production

All the selected isolates were screened for biopolymer synthesis. Dextransucrase is an inducive enzyme and specifically require sucrose in the medium for the production of dextran. Therefore, for the screening of the dextransucrase and dextran production all the isolates were cultivated in MRS broth containing sucrose (10 g L⁻¹) and incubated at 25 °C for 24 h. After incubation, strains were

selected on the basis of polymer production. The fermented broth become turned viscous due to dextran production.

2.5. Identification of bacterial isolates by taxonomic characterization

Pure bacterial isolates were identified through different morphological analysis and by performing various biochemical test.

2.5.1. Taxonomic characterization

For morphological identification of bacterial strains, Bergey's manual of determinative bacteriology was used [22]. Colonial characteristics and cell morphology were observed for identification. The bacterial isolates were subjected to different biochemical tests for the biochemical characterization including sugar fermentation and arginine hydrolysis tests. For sugar fermentation, bacterial cultures were inoculated into the medium containing 10 g L⁻¹ of different sugars (Arabinose, Sucrose, Maltose, Dextrose, Lactose, Mannitol, Trehalose, Xylose, Fructose) for up to 48 h at 25 °C. Colour change and gas production was observed for fermentation. To test for arginine hydrolysis, samples were analysed as described by Tindall et al. [23]. Arginine dihydrolase activity was determined by inoculating the strain in nutrient broth medium containing arginine (5 g L⁻¹) and bromocresol purple as a pH indicator. The inoculated strains were incubated at 35 °C for 48 h.

2.5.2. Molecular characterization

DNA sequence analysis was performed for further identification of bacteria. Genomic DNA was extracted from overnight bacterial culture using DNA extraction kit (Promega, USA). For amplification, polymerase chain reaction (PCR) of 16S rDNA was performed using universal primer set as 16S forward 5'-GAGTTTGATCCTGGCTCAG-3' and 16S reverse 3'-AGAAAGGAGGTATCCAGCC-5'. The program parameters are as follows: initial denaturation at 95 °C for 5 min, 95 °C for 1 min for 35 cycles, 55 °C for 45 s, 72 °C for 1 min followed by final elongation of 72 °C for 10 min and a hold at 25 °C. The amplified DNA was analysed using agarose gel electrophoresis (1%) and purified through PCR purification kit (Promega, USA). The purified products were then sequenced and sequence similarity was searched through BLAST (GenBank, <http://www.ncbi.nlm.nih.gov/blast/>). Sequences were aligned and phylogenetic tree was constructed by neighbour joining method using Mega software (version 7.0.1).

2.6. Quantitative screening for dextransucrase and dextran production

The pure bacterial isolates were screened for maximum dextransucrase activity. The inoculum was transferred into sucrose (20 g L⁻¹) containing broth (10 ml) and incubated at 25 °C for 24 h. The seed culture was transferred into flask (90 ml) and incubated further for 24 h. After fermentation, bacterial cells were harvested for 10 min at 40,000 × g. The cell free filtrate (CFF) was used for further determination of enzyme activity and total protein. Sucrose containing medium constitutes of (g L⁻¹): 5.0, yeast extract; 5.0, peptone; 15.0, dipotassium hydrogen phosphate; 0.5, calcium chloride; 0.1, magnesium sulphate; 0.1, manganese chloride and 0.1, sodium chloride. The initial pH of the fermentation broth was adjusted at pH 7.5 before sterilization.

Dextransucrase activity was determined by measuring the amount of reducing sugar liberated from sucrose using glucose as standard through method as described by Kobayashi and Matsuda [24]. For enzyme activity, CFF (50 µl) was incubated with 1.0 ml of sucrose (125 mg ml⁻¹) at 35 °C for 15 min. After incubation, the reaction was stopped by incorporating 1 N solution of NaOH (50 µl). One unit of enzyme activity is measured as Dextransucrase Units (DSU) and is defined as:

'The amount of enzyme required to liberate 1.0 μmol of fructose from sucrose per minute under standard assay conditions'.

The protein content of the CFF was determined by Lowry's method using BSA as standard with concentration ranging from 2 to 5.0 $\mu\text{g ml}^{-1}$ to 250.0 $\mu\text{g ml}^{-1}$ [25].

2.7. Optimization of physical parameters of fermentation

To determine the effect of fermentation time on the production of enzyme, bacterial culture was incubated at different time periods ranging from 0 to 48 h. The temperature was also optimized by incubating the bacterial culture at different temperatures from 20 °C to 35 °C. To adjust the pH of the medium, bacteria was cultivated in medium under different pH values ranging from 6.0 to 8.0.

All the experiments were performed in triplicate ($n = 3$) and the results were calculated as mean of all the experimental runs.

2.8. Dextran production and precipitation

Dextran was produced using sucrose (100 g L^{-1}) as a substrate. Dextran precipitation and quantification was carried out as described earlier [26].

2.9. Statistical analysis

All the data is represented as a mean \pm standard deviation of three replicates. The analysis was done by employing one-way analysis of variance and Tukey's multiple range test using SPSS software (version 17.0). Differences were statistically significant at $p < .05$.

3. Results and discussion

3.1. Isolation of lactic acid bacteria from fermented food products

Isolation of new microbes from natural resources is a significant approach for the production of novel products. Lactic acid bacteria have gained considerable attention due to their unique metabolic activities, GRAS status and antimicrobial properties. They are most commonly isolated from fermented foods [27]. However, several studies on the isolation of lactic acid bacteria from soil and plant samples have also been reported [28,29]. In the present study, thirty-nine bacterial strains were isolated from various fermented fruits, vegetables and raw milk. Among these selected thirty-nine isolates, only eight isolates showed dextransucrase activity along with polymer production. The selected isolates were used for subsequent work.

3.2. Identification and screening of isolates for dextransucrase and dextran production

For the identification of bacterial strains, different phenotypic characters were considered. Phenotypically, bacterial colonies were characterized on the basis of colonial characteristics as well as microscopic analysis. The colonies were white or grey white, concave, pinpointed, mucoid with smooth edges. All the cells were identified as Gram's positive with cocci or coccobacilli in shape. Detailed taxonomic study is represented in Table 1. Antibiotic susceptibility test was also examined and results showed that all the isolates were vancomycin resistant except BZ-5 which was sensitive to vancomycin. Biochemical characters were evaluated

Table 1
Taxonomic characteristics of the isolated lactic acid bacterial species for the identification.

| Bacterial isolate | Source | Taxonomic characteristics | | | Biopolymer production ^b |
|-------------------|------------------------------------|--------------------------------------------------------|-----------------|-------------------------------------------|------------------------------------|
| | | Colony morphology ^a | Gram's reaction | Cell morphology ^a | |
| BZ-1 | <i>Prunus persica</i> (Peach) | White, Pinpointed, Concave, Mucoid, Smooth Margin | Gram Positive | Coccobacilli, Chains (3–5 cells) | Producer |
| BZ-2 | <i>Mangifera indica</i> (Mango) | White, Pinpointed, Concave, Mucoid, Smooth margin | Gram Positive | Cocci, Chains (5–6 cells) | Producer |
| BZ-3 | <i>Brassica oleracea</i> (Cabbage) | White, Pinpointed, Concave, Mucoid, Smooth margin | Gram Positive | Cocci, Chains (4–5 cells) | Producer |
| BZ-4 | <i>Mangifera indica</i> (Mango) | Grey white, Pinpointed, Concave, Mucoid, Smooth margin | Gram Positive | Coccobacilli, Diplo or Chains (2–3 cells) | Producer |
| BZ-5 | Raw milk | White, Small, Concave, Mucoid, Smooth margin | Gram Positive | Cocci, Chains (10–12 cells) | Producer |
| BZ-6 | <i>Prunus persica</i> (Peach) | Grey white, Small, Concave, Mucoid, Irregular edges | Gram Positive | Coccobacilli, Chains (5–7 cells) | Producer |
| BZ-7 | Raw milk | White, Medium, Concave, Mucoid, Smooth margin | Gram Positive | Coccobacilli, Chains (3–4 cells) | Producer |
| BZ-8 | <i>Brassica oleracea</i> (Cabbage) | Grey white, Medium, Concave, Mucoid, Smooth margin | Gram Positive | Coccobacilli, Chains (5–6 cells) | Producer |

^a Growth was observed on MRS medium. The characteristics of the isolates were observed up to 48 h at 25 °C.

^b Biopolymer production observed using sucrose (50.0 g L^{-1}) at 25 °C after 24 h. The medium in the tubes becomes viscous indicating biopolymer production.

Table 2
Biochemical characteristics of the lactic acid bacterial isolates.

| Bacterial isolate | Biochemical tests | | Sugar fermentation ^c | | | | | | | | |
|-------------------|----------------------------------------|----------------------------------|---------------------------------|---|---|---|---|---|---|---|---|
| | Vancomycin susceptibility ^a | Arginine hydrolysis ^b | A | B | C | D | E | F | G | H | I |
| BZ-1 | Resistant | Positive | – | + | + | + | + | – | + | + | + |
| BZ-2 | Resistant | Negative | + | + | + | + | + | – | + | + | + |
| BZ-3 | Resistant | Negative | + | + | + | + | + | – | + | + | + |
| BZ-4 | Resistant | Negative | + | + | + | + | + | – | + | + | + |
| BZ-5 | Susceptible | Negative | – | + | + | + | + | + | + | – | + |
| BZ-6 | Resistant | Positive | – | + | + | + | + | – | + | + | + |
| BZ-7 | Resistant | Negative | + | + | + | + | + | – | + | + | + |
| BZ-8 | Resistant | Negative | + | + | + | + | + | – | + | + | + |

Sugars used for fermentation: **A:** Arabinose; **B:** Sucrose; **C:** Maltose; **D:** Dextrose; **E:** Lactose; **F:** Mannitol; **G:** Trehalose; **H:** Xylose; **I:** Fructose.

^a Antibiotic test was performed on MRS medium using vancomycin disk (30.0 μg) at 25 °C for 24 h.

^b Arginine hydrolysis was observed at 25 °C up to 48 h.

^c Tubes were monitored up to 48 h for acid and gas production.

through carbohydrate fermentation and arginine hydrolysis test. All bacterial isolates ferment sugar resulting in acid production whereas, no gas production was noticed after fermentation (Table 2). Arginine dihydrolase test is used for the identification of different genera of lactic acid bacteria. In arginine dihydrolase test, the bacteria utilize glucose and produces acid which shift pH from neutral to acidic side. This change in pH, activates arginine dihydrolase which finally hydrolyse arginine. Arginine hydrolysis is examined by the change in colour from purple to yellow after 24 h or revert to purple colour within 48 h [30]. This test is essential for the identification of different genera of lactic acid bacteria. The results showed that among different isolates BZ-1 and BZ-6 were capable of hydrolysing arginine indicating that they belong to genus *Weissella*. While, other isolates were identified as belonging to the genus *Leuconostoc* and *Streptococcus*.

According to Fusco et al. [31] most of the dextran producing species of genus *Weissella* can ferment cellobiose, fructose, galactose, maltose, ribose and sucrose and at the same time, they are unable to ferment arabinose, melibiose and raffinose. Most of the species of genus *Leuconostoc* have the ability to specifically ferment melibiose and arabinose which cannot be fermented by genus *Weissella* [32]. Therefore, it is further confirmed that isolate BZ-1 and BZ-6 belongs to genus *Weissella*.

The purified isolates were screened based on enzyme activity (Table 3). *Leuconostoc* sp. (BZ-7) showed highest enzyme activity (67.13 DSU ml⁻¹ h⁻¹), followed by other *Leuconostoc* species accordingly BZ-4 < BZ-2 < BZ-3 < BZ-8. The two species of genus

Weissella, BZ-1 and BZ-6 showed enzyme activities of 4.41 and 5.91 DSU ml⁻¹ h⁻¹, respectively. Whereas, *Streptococcus* species showed enzyme activity approximately 2.13 DSU ml⁻¹ h⁻¹. BZ-6 was selected and its identification was confirmed through 16S rDNA analysis as *Weissella confusa* KIBGE-IB38 [KY411713]. Other indigenously isolated strains BZ-1 and BZ-7 were identified as *Weissella confusa* KIBGE-IB39 [KY411819] and *Leuconostoc mesenteroides* KIBGE-IB40 [KY938040], respectively. The phylogenetic tree (Fig. 1) showed that the *Weissella confusa* KIBGE-IB38 and KIBGE-IB39 falls into a distinct clade and share maximum identity of 100% with other *Weissella* strains.

3.3. Optimization of fermentation conditions

Industrially, optimization is an important criterion to obtain high titre of enzyme which helps in reducing the production cost by maintaining favourable environment for the bacterial growth and metabolic activities [33]. Optimization was carried out using one factor at a time approach under batch fermentation conditions. In this study, different physical parameters including fermentation temperature, time and pH of the medium were optimized for *Weissella confusa*. The production of dextransucrase and dextran was monitored. Several reports are available for the optimization of fermentation conditions for *Leuconostoc* species [34–36]. However, information is scarce for *Weissella* species. Although very few reports available for maximum dextransucrase activity for *Weissella* species using factorial and conventional approaches [37,38].

Table 3
Production of dextransucrase from different species of lactic acid bacteria.

| Bacterial isolate | Genus ^a | Enzyme activity ^b (DSU ml ⁻¹ h ⁻¹) | Dextran production ^c (g%) | Percent conversion ^d |
|-------------------|--------------------------|----------------------------------------------------------------------|--------------------------------------|---------------------------------|
| BZ-1 | <i>Weissella</i> sp. | 4.41 ± 0.08 | 0.80 ± 0.016 | 8.0 |
| BZ-2 | <i>Leuconostoc</i> sp. | 9.81 ± 0.19 | 0.60 ± 0.012 | 6.0 |
| BZ-3 | <i>Leuconostoc</i> sp. | 10.77 ± 0.21 | 0.58 ± 0.011 | 5.8 |
| BZ-4 | <i>Leuconostoc</i> sp. | 5.45 ± 0.10 | 0.54 ± 0.010 | 5.4 |
| BZ-5 | <i>Streptococcus</i> sp. | 2.13 ± 0.04 | 0.12 ± 0.002 | 1.2 |
| BZ-6 | <i>Weissella</i> sp. | 5.91 ± 0.11 | 1.2 ± 0.024 | 12.0 |
| BZ-7 | <i>Leuconostoc</i> sp. | 67.13 ± 1.34 | 1.82 ± 0.036 | 18.2 |
| BZ-8 | <i>Leuconostoc</i> sp. | 12.53 ± 0.25 | 0.62 ± 0.012 | 6.2 |

Standard deviation: ±2%, n = 3.

^a Taxonomic identification based on morphological and biochemical characteristics.

^b Enzyme activity determined at 35 °C using sucrose (20 g L⁻¹).

^c Sucrose (100 g L⁻¹) was used as a substrate.

^d Calculation is based on the conversion of sucrose into dextran.

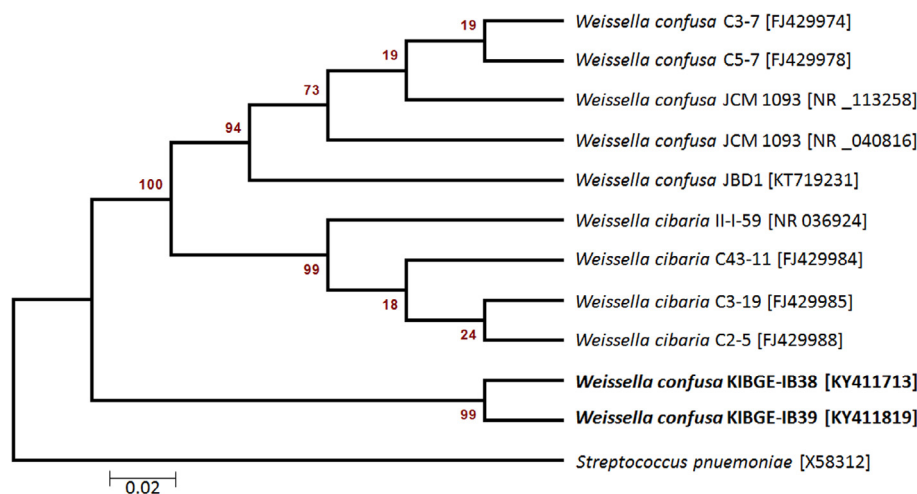


Fig. 1. Phylogenetic relationship of *Weissella confusa* KIBGE-IB38 and *Weissella confusa* KIBGE-IB39 with related species and genus of lactic acid bacteria.

3.3.1. Fermentation time

In the current study, the first parameter optimized was fermentation time, which has a significant effect on the cultivation of microbial cells and metabolites production. To determine the optimum time required for maximum bacterial growth and enzyme yield, *Weissella confusa* was incubated for different time intervals. The results indicate that the maximum enzyme yield depends on the bacterial cells growth. Maximum enzyme yield for *Weissella confusa* was 1.508 DSU mg⁻¹ in exponential growth phase with approximately 1.24 × 10⁸ cells ml⁻¹. Growth kinetics showed that after 06 h of fermentation the bacterial cells started to multiply rapidly and attained maximum activity in 08 h. Thereafter, microbial cells enter into the stationary phase which might be due to the depletion of nutrients in the medium (Fig. 2). According to another study, the maximum activity was also observed after 12 h [39].

3.3.2. Fermentation temperature

Temperature is also reported to have a direct effect on bacterial enzyme production as well as on its growth. It is reported that at low temperature, the fluidity of cytoplasmic membrane is reduced which ultimately effects the transport of metabolites across the membrane. While, at higher temperatures heat sensitive enzymes or proteins are inactivated due to the denaturation of structural components of bacterial cell [40]. In this study, the bacterial strain was cultivated at different temperatures ranging from 20 °C to 35 °C. The maximum activity was achieved at 25 °C for *Weissella con-*

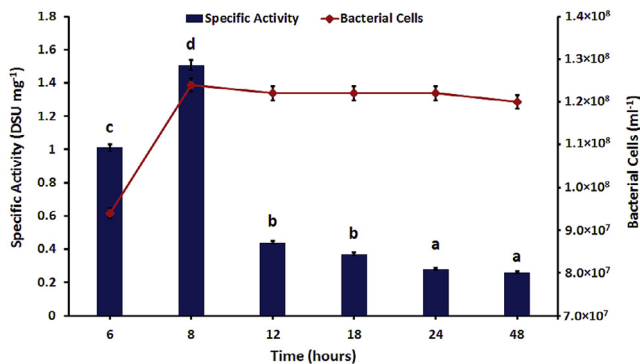


Fig. 2. Production of extracellular dextransucrase from *Weissella confusa* KIBGE-IB38 for different fermentation time periods. Symbols (means ± S.D., n = 3) having similar letters are not significantly different from each other (Tukey's test, $p < .05$).

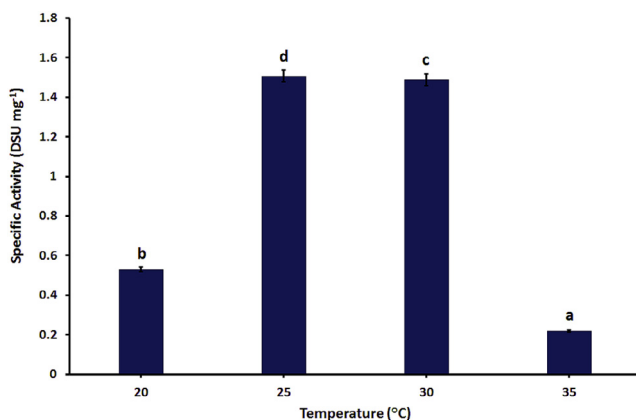


Fig. 3. Production of extracellular dextransucrase from *Weissella confusa* KIBGE-IB38 under various fermentation temperatures. Symbols (means ± S.D., n = 3) having similar letters are not significantly different from each other (Tukey's test, $p < .05$).

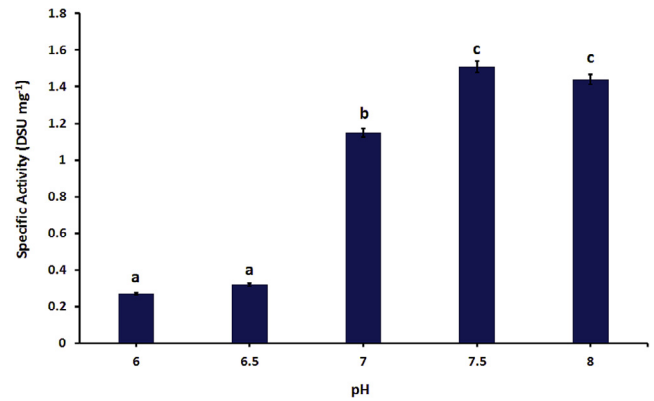


Fig. 4. Production of extracellular dextransucrase from *Weissella confusa* KIBGE-IB38 at different fermentation pH values. Symbols (means ± S.D., n = 3) having similar letters are not significantly different from each other (Tukey's test, $p < .05$).

fusa. The enzyme yield was not much effected by the change in temperature from 25 °C to 30 °C as shown in Fig. 3. Previously, the growth temperature reported for *Weissella* species was in between 15 °C and 45 °C [31]. It suggests that *Weissella* can survive and produce dextransucrase at a wider range of temperature. Similar results were reported and maximum enzyme activity was observed at 25 °C [41].

3.3.3. Fermentation pH

In the next step, fermentation pH was optimized as microbial cells are also pH sensitive. The pH of the medium affects the metabolic activity of microorganisms therefore, the pH of the medium was varied from 6.0 to 8.0. It was observed that with the increase in pH of the medium, the enzyme yield also increases and maximum activity was observed at pH 7.5 (Fig. 4). While, further increase in pH decreased the enzyme yield. At low pH (6.0 and 6.5) the isolates were unable to produce dextransucrase in higher titres. Several authors have also studied the effect of pH on dextransucrase activity from lactic acid bacteria [42–44].

4. Conclusion

Dextransucrase is an industrially important enzyme that produces dextran using sucrose. They are also able to produce oligosaccharides when acceptor molecules are present. Dextran and oligosaccharides both have been used for various applications in food industry. Therefore, different dextransucrase producing lactic acid bacteria were isolated. Among different isolates, the ones which showed polymer production were then selected and identified on the basis of taxonomical characteristics and molecular analysis. Moreover, optimization of fermentation parameters of *Weissella confusa* increase the dextransucrase activity. Further studies on the structural comparison of dextransucrase and dextran from *Weissella confusa* would be useful for their applications and is currently in progress.

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