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Isolation, optimization, and purification of extracellular levansucrase from nonpathogenic *Klebsiella* strain L1 isolated from waste sugarcane bagasse

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ABSTRACT

A newly isolated acidophilic bacterial strain from waste sugarcane bagasse was identified as *Klebsiella* species by biochemical and 16S rRNA sequencing methods. *Klebsiella* sp. strain L1 was selected for its ability to produce extracellular levansucrase. The optimization of carbon source, nitrogen source, sucrose concentration, NaCl concentration, initial pH and temperature of the growth medium in the flask fermentation medium. In the investigation, sucrose acts as a superior carbon source whereas yeast extract act as the best nitrogen source for *Klebsiella* sp. strain L1. 10% sucrose concentration and 2% sodium chloride (NaCl) concentration act as an inducer for levansucrase production. Isolate growing and produce optimum levansucrase under acidic condition (pH 5.0) with normal temperature range (40 °C). Synthesis of levan polymer by isolate was detected using thin layer chromatography (TLC) plate method. SDS PAGE result shown that the molecular weight of levansucrase from *Klebsiella* sp. strain L1 was 43 kDa. Moreover, isolate has a γ-hemolysis reaction on blood agar which indicated their non-pathogenic nature.

1. Introduction

Levansucrase (E.C.2.4.1.10), belonging to the glycoside hydrolase family 68 (GH68), is responsible for the levan production. Levan is an extracellular polysaccharide or natural fructan composed of D-fructose residues. D-fructose residues were linked predominantly by β-(2,6) linkage in the backbone with occasional β-(2,1) branch chains (Han et al., 2015). The levan has a vast range of application in food industries which include stabilizer, flavoring agent, color carrier and food thickening agent and also used in cosmetics industries (Belghith et al., 1996; Duboc and Mollet, 2001; Park et al., 2003). Moreover, levan has the biotechnological application, it acts as a sweetener for diabetics patients (Cha et al., 2001).

In nature, low molecular weight levansucrase produced by limited numbers of plant species but a large number of microbial species are able to produce high molecular weight levansucrase (Poli et al., 2009; Han et al., 2015). Several microorganisms, including bacteria and fungi are reported for levansucrase activity with levan production. Few bacterial genera such as *Bacillus*, *Pseudomonas*, *Streptococcus, Lactobacillus,* and *Leuconostoc* have the ability to synthesize levan (Han, 1990). However, no previous study on the synthesis of levan by *Klebsiella* species has been reported. To our knowledge, this is the first report describing the production of acidophilic levansucrase by genus *Klebsiella*.

In the present study, *Klebsiella* sp. strain L1 was isolated from the sugarcane bagasse and screened for levansucrase production. Production of levansucrase was improved by optimizing different growth parameters like carbon and nitrogen sources, sucrose and NaCl concentration, pH and temperatures. Moreover, the production of levan polymer was characterized via thin layer chromatography (TLC) method and molecular weight of levansucrase was detected via SDS PAGE.

2. Materials and methods

2.1. Sample collection and isolation

Waste sugarcane bagasse samples were collected from different areas of Surat, Gujarat, India sugarcane juice vendors (Vesu: 21°09′25″N, 72°46′43″E, Anuvrat Dwar: 21°09′36″N, 74°47′43″E). Samples were inoculated in enrichment medium (minimum salt medium (MSM) +5% sucrose). After overnight incubation, serial dilutions of samples were performed and 0.1 ml of each sample was spread on the nutrient agar plate. The plates were incubated at 35 °C for 24–48 h. Bacterial colonies grown on a plate were purified by repeating streaking.

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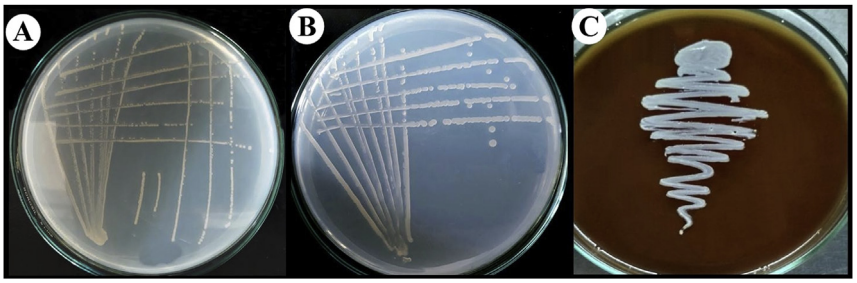

Fig. 1. (A) *Klebsiella* sp. strain L1 on nutrient agar plate, (B) Levansucrase production on sucrose agar plate, (C) Hemolytic activity test on blood agar plate.

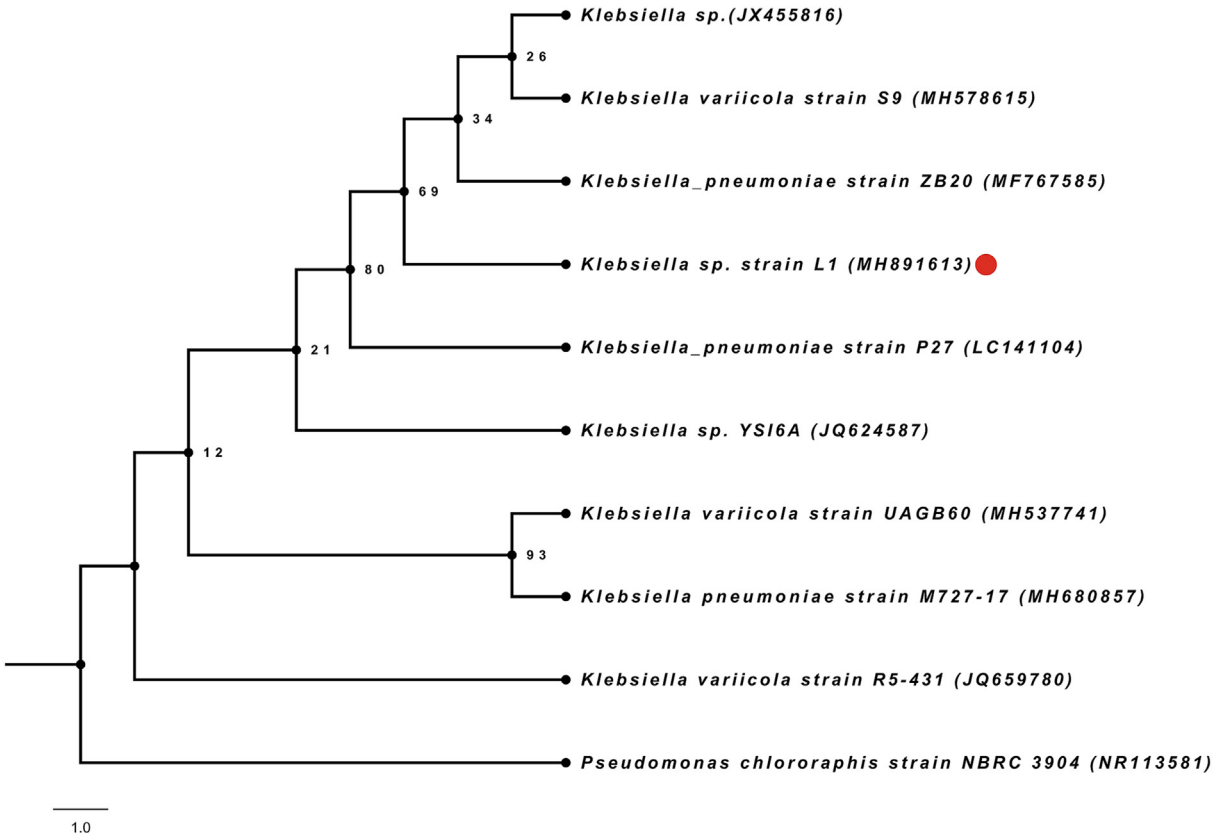

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences showing the position of *Klebsiella* sp. strain L1 (●). *Pseudomonas chlororaphis* strain NBRC 3904 is used as an out group.

2.2. Screening of levansucrase

Bacterial isolates were screening on sucrose agar plate (sucrose 2.5%, tryptone 1%, yeast extract 1%, NaCl 0.5%, K_2HPO4 0.025%, agar 3% and pH 5.0) for checking levansucrase production. The plates were incubated at 35 °C for 24–48 h. Production of mucoid bacterial colonies on a sucrose agar plate indicates positive levansucrase production. The positive isolate (L1) maintained on the sucrose agar medium slants at 4 °C for further study.

2.3. Identification of bacterial strain

Morphological and biochemical characterization of isolate L1 was done by Gram staining, spore staining, colony surface, pigmentation, motility test, oxidase, citrate utilization, indole production, nitrate reduction, sugar utilization (D-glucose, lactose, sucrose, D–sorbitol and D–xylose). Non-pathogenicity of the isolate was confirmed on blood agar according to Adnan et al. (2017).

Molecular identification of bacterial isolate was carried out by 16S rRNA gene sequencing method. Genomic DNA was isolated using NaCl-cTAB method (Wilson, 2001). Amplification of 16S rRNA gene was carried out by using a pair of universal primer 27f (5′AGAGTTTGATC MTGGCTCAG3′) and 1492r (5′CGGTTACCTTGTTACGACTT3′) with 1X final concentration of ReadyMix™ Taq PCR reaction mix (Sigma-Aldrich®, India) and, template DNA (50 ng/μl). The reaction was carried out in Thermal cycler (Applied Biosystems Veriti®). PCR reaction mixture contained 1X reaction mixture (10 μl), forward primer (1 μl), reverse primer (1 μl), genomic DNA template (2 μl), and nuclease-free water (6 μl). PCR program was adjusted as: initialization at 95 °C for 4 min, 35 cycles of denaturation 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min; followed by final elongation step at 72 °C for 5 min withhold at −4 °C for ∞ time. Amplified PCR products were detected on 1.2% agarose gel electrophoresis. Purification of amplified PCR product was done using GenElute™ PCR Clean-up kit (Sigma- Aldrich®, India). Purified PCR product was sequenced by Eurofins Genomics India Pvt Ltd., Bangalore. Sequence match analysis was carried out using the Basic Local Alignment Search Tool (BLAST) on NCBI and sequences were submitted to GenBank database of NCBI.

Table 1
Morphological and biochemical characteristics of *Klebsiella* sp. strain L1.

Characteristic	Result
Morphological characterization	
Gram reaction	–
Cell shape	Short rod
Spore formation	–
Colony shape	Round
Surface	Smooth
Pigmentation	–
Motility	–
Biochemical characterization	
Oxidase	+
Citrate utilization	+
Indole production	+
Nitrate reduction	+
D-glucose	+
D-sorbitol	+
D-xylose	+
Lactose	+
Sucrose	+

+, positive; -, negative; Colony characteristics on sucrose agar plate.

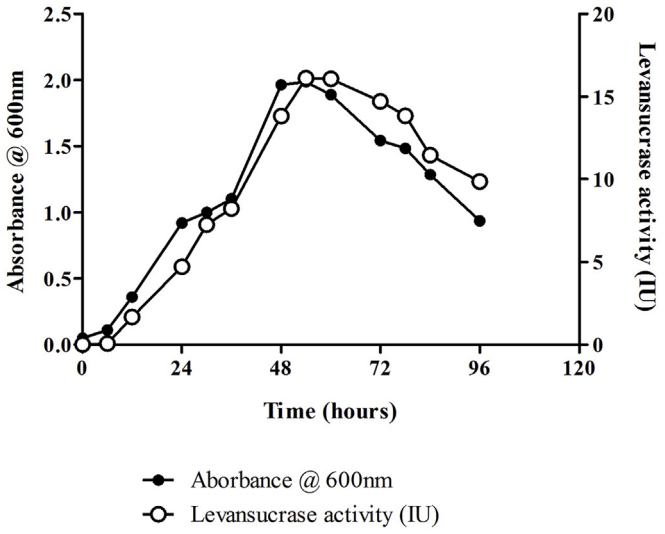

Fig. 3. Time course of growth and levansucrase production by *Klebsiella* sp. strain L1.

Phylogenetic analysis of the isolates sequences by BLAST search available in the NCBI, GenBank database. Sequences were aligned with the help of ClustalW and embedded in MEGA7.0. Aligned data was analyzed with PartitionFinder (Lanfear et al., 2012) for optimal partitioning strategy and evolutionary substitution model. Maximum Likelihood (ML) analyses were employed to infer phylogenetic relationships in RaxML (Silvestro and Michalak, 2012). 1000 bootstrap replicates were run under GTR + I model to assess clade support.

2.4. Fermentation conditions

The production of levansucrase enzyme and measurement of growth was performed in Erlenmeyer flasks containing: sucrose 10%, tryptone 1%, yeast extract 1%, NaCl 2.5%, K_2HPO_4 0.025%, and pH 5.0 (sucrose was separately autoclaved at 110 °C). Flask was inoculated with 5% inoculums size of log phase seed culture and incubation was carried out in a rotary shaker (150 rpm) at 40 °C. A small amount of sample was withdrawn and used to measure growth (600 nm) and levansucrase activity.

2.5. Enzyme assay

The enzyme reaction was initiated by adding 0.1 ml enzyme extract into 0.9 ml of 1 M sucrose in 0.1 M sodium acetate buffer (pH 5.0). The reaction was carried out at 40 °C for 10 min. All assays were run in triplicate. The concentration of released glucose was measured by the dinitrosalicylic acid method (Miller, 1959). One unit of levansucrase was defined as the amount of enzyme that produced 1 µmol of glucose per minute under standard conditions. (Expressed as IU = International Unit).

2.6. Protein estimation

The protein content was determined according to Lowry et al. (1951). Bovine serum albumin was used as a standard to determined protein concentration.

2.7. Optimization of different growth parameters

The effects of important growth parameters such as carbon, nitrogen source, sucrose concentration, NaCl concentration, pH, temperature were optimized for enzyme production. For the carbon source, 10% (glucose, galactose, lactose, maltose and sucrose); nitrogen source, fixed 1% (peptone, yeast extract, beef extract, tryptone and ammonium sulphate ($(NH_4)_2SO_4$)); sucrose concentration (2.5–15%), NaCl concentration (1–5%), medium pH, (4 − 10); temperature, (30–60 °C) were used to optimized for enhanced production of levansucrase.

2.8. Separation of levan polymer

The supernatant was obtained by centrifugation at 10,000 rpm for 15 min at 4 °C. The levan was precipitated by adding the double volume of chilled absolute ethanol and the mixture was kept overnight at 4 °C. The precipitate was collected by centrifugation at 10,000 rpm at 4 °C for 20 min and re-dissolved in de-ionized water. The aqueous solutions were dialyzed against distilled water at 4 °C for 24 h, and then levan was dried using lyophilizer.

2.9. Thin layer chromatography for levan

For the detection of levan, thin layer chromatography (TLC) was used. In which, the lyophilized sample was applied on silica gel plates saturated with solvent chloroform:acetic acid:water (6:7:1). Mixture of standard glucose, fructose, and sucrose was spotted along with samples. Plates were sprayed with 5% sulfuric acid (prepared in methanol), air dried and then heated at 100 °C until spots appeared (Dahech et al., 2013).

2.10. Purification of levansucrase

The cell-free crude enzymes were precipitated using ammonium sulphate saturation (40–80%). After desalted, the dialyzed enzyme was loaded into a Sephadex G-100 column (2.5 × 50 cm), previously equilibrated with 0.1 M sodium acetate buffer, (pH 5.0) and enzyme was eluted by using 50 M NaCl gradient. Two ml sample was collected with the flow rate of 15 ml/h. Each fraction was analyzed for protein and enzyme activity. The levansucrase activity fractions were pooled, desalted, concentrated and applied on DEAE-cellulose ion-exchange column (1.5 × 30 cm), previously equilibrated with the same buffer by using 0.0–0.5 M NaCl gradient with a flow rate of 30 ml/h. Again each fraction was analyzed for protein and enzyme activity. All the steps were carried out at 4 °C.

2.11. Molecular mass determination by SDS-PAGE

The SDS-PAGE analysis of purified levansucrase was performed in a

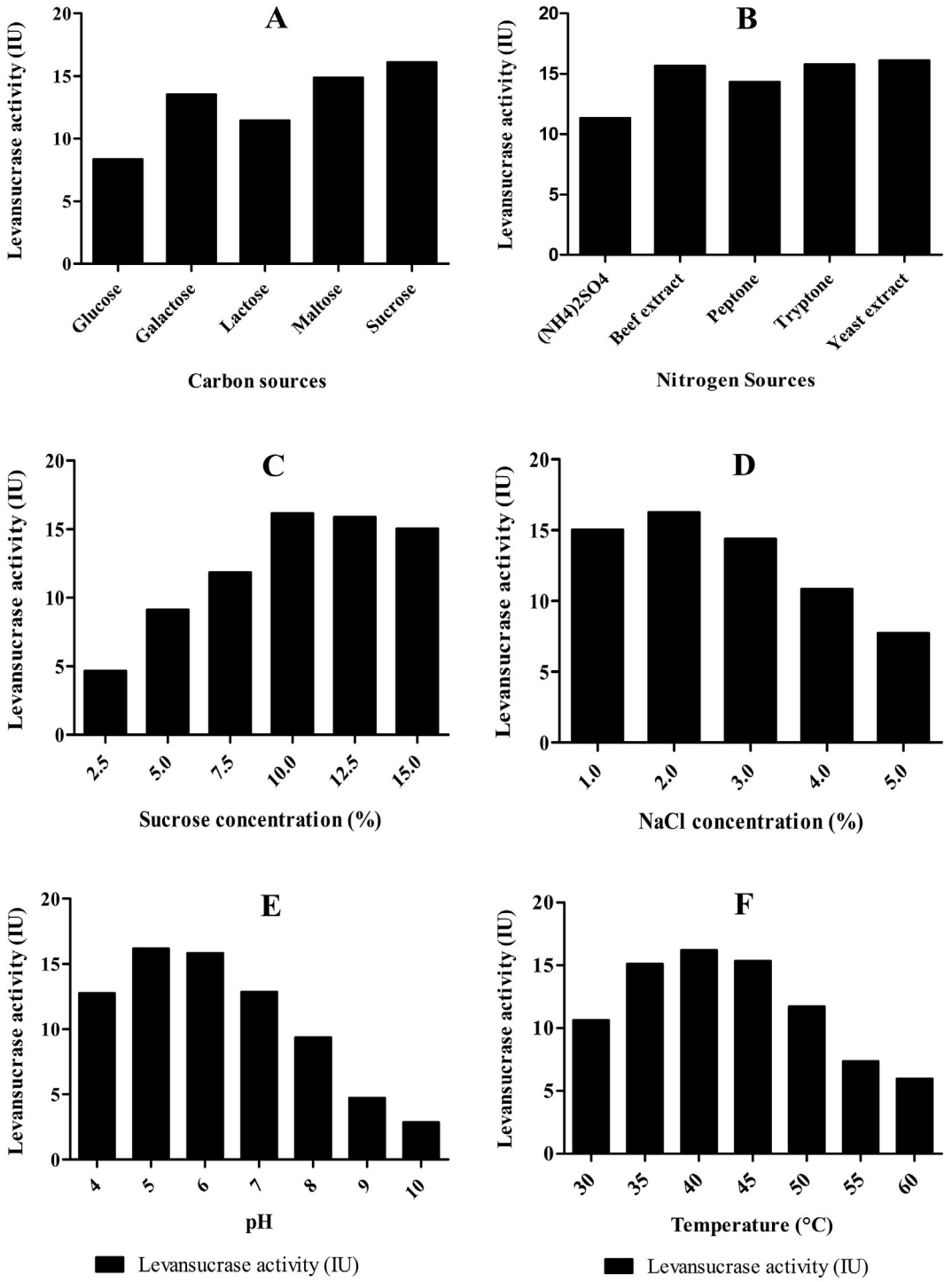

Fig. 4. Effect of different parameters for levansucrase production (A) carbon, (B) nitrogen, (C) sucrose concentration, (D) NaCl concentration, (E) pH, (F) temperature on *Klebsiella* sp. strain L1.

12.5% acrylamide gel (Laemmli, 1970) using Mini Gel Electrophoresis unit (Bio-Rad, Hyderabad, India) and visualized by Coomassie brilliant blue R-250. The protein ladder range from 14.4 kDa to 94.0 kDa molecular weight was used.

3. Results and discussion

3.1. Isolation of bacterial strains

In the present study, the aim was to isolates levansucrase enzyme producing bacteria from sugarcane bagasse. Based on the morphological characteristic including, shape, size, edge, pigmentation etc total 11 different bacterial strains were isolated on nutrient agar plate.

3.2. Screening of levansucrase producer

Out of 11 isolates, one bacterial isolate (L1) produced mucoid colonies on the sucrose agar plate, which indicated their ability to produce levansucrase enzyme (Fig. 1B). However, mucoid colonies were not obtained when streaked on nutrient agar plate (Fig. 1A). This result

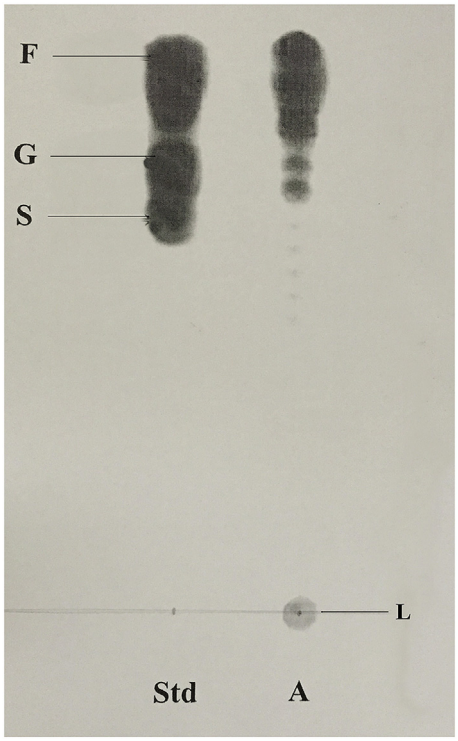

Fig. 5. TLC analysis of products produced by levansucrase reaction from (A) *Klebsiella* sp. strain L1. F, fructose; G, glucose; S, sucrose; L, levan.

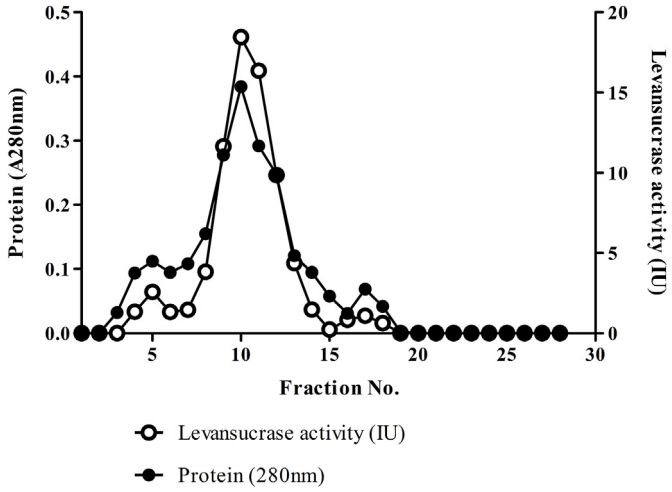

Fig. 6. Elution profile of levansucrase from DEAE-cellulose column chromatography.

was supported by Ghaly et al. (2007) and Vaidya and Prasad (2012), who previously reported that levan producers were mucoid colonies on sucrose containing agar plates.

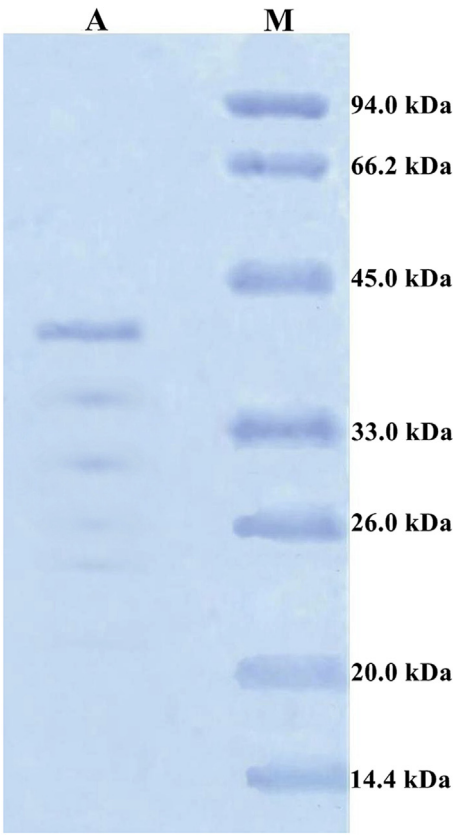

Fig. 7. SDS-PAGE analysis of levansucrase (M) Marker, (A) *Klebsiella* sp. strain L1 (43 kDa).

3.3. Identification of bacterial isolate

Based on morphological, biochemical and 16S rRNA sequencing method, bacterial strain L1 identified as *Klebsiella* sp. (Fig. 2). Strain L1 produce round shape, medium size, and flat, opaque, colorless with entire margin colonies. In microscopic observation, cells of L1 strain were gram-negative, short rod shape and non-spore forming. It was oxidase, citrate, indole, nitrate positive and able to ferment sugar like D-glucose, D-sorbitol, D-xylose, lactose, and sucrose. Strain L1 has luxurious growth at 5.0 pH and at 40 °C temperature (Table 1). Moreover, strain L1 has γ-hemolytic activity on a blood agar plate which indicates non pathogenic nature of isolates (Fig. 1C). After successful identification, nucleotide sequences were deposited in NCBI with accession number MH891613.

3.4. Growth curve study and levansucrase production

Fermentation study of bacterial isolate *Klebsiella* sp. strain L1 shown that the growth and levansucrase production was corresponding associated. Strain L1 was metabolically highly active and speedily growing. Maximum growth (biomass) was observed at 48 h and gradually decreased after 54 h. Levansucrase productions were maximized during stationary phase (54 h) and remain same up to 66 h (Fig. 3). In addition,

Table 2
Purification steps of levansucrase from *Klebsiella* sp. strain L1.

Purification step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Recovery (%)	Purification fold
Crude	812.5	92	8.83	100	1
40–80	519.3	34.89	14.88	63.91	1.68
Sephadex G-100	354.8	17.9	19.82	43.66	2.24
DEAE-cellulose	184.6	3.83	48.19	22.72	5.45

isolate L1 were considered as an acidophile, based on optimum enzyme production and cell growth at pH 5.0.

3.5. Effect of different carbon and nitrogen sources

To determine the effect of different carbon sources on the production of levansucrase, bacterial isolate L1 was inoculated into a separate flask containing 10% of glucose, galactose, lactose, maltose, and sucrose with pH 5.0. Highest levansucrase production was obtained by *Klebsiella* sp. strain L1 (16.11 IU) in the presence of sucrose as a carbon source (Fig. 4A). Enzyme activity was comparatively lower in glucose containing media as a carbon source. According to Wu et al. (2013), a medium containing sucrose as a carbon source was utilized by microbial strain to produced levan polymer via transfructosylation reaction of levansucrase. The effect of different nitrogen sources such as peptone, yeast extract, beef extract, tryptone, and $(NH_4)_2SO_4$, in which yeast extract act as the best nitrogen source for *Klebsiella* sp. strain L1 (16.13 IU) (Fig. 4B). The similar result was found in Wang and Rakshit (2000) and Belghith et al. (2012) study, when they used yeast extract as a nitrogen source. However, levansucrase production can vary according to the microorganism, carbon and nitrogen sources (Wang and Rakshit, 2000; Nguyen et al., 2005).

3.6. Effect of sucrose and NaCl concentration

Enhancement of levansucrase production by using a different concentration of sucrose and NaCl in the fermentation medium. Results showed that sucrose concentration at 10% was the excellent levansucrase inducer in *Klebsiella* sp. strain L1 (16.15 IU) (Fig. 4C). The concentration of sucrose was playing an important role in cell growth as well as in levansucrase production. According to Chen and Liu (1996), if the medium contains sucrose concentration less than 10%, then microbes utilized it's for cell metabolisms. The higher sucrose concentrations induce higher enzyme activity but cell growth inhibition occurred. Belghith et al. (2012) found higher levansucrase production by *Bacillus* sp. using 30% sucrose but cell growth was restricted. Varying NaCl concentrations also contribute to bacterial growth and levansucrase production. In this study, 2% NaCl concentration was optimal for isolate *Klebsiella* sp. strain L1 (16.26 IU) (Fig. 4D). Several bacterial strain, such as *Bacillus licheniformis* BK AG21 (Wahyuningrum and Hertadi, 2015), *Halomonas* and *Chromohalobacter* sp. (Nasir et al., 2015) reported that NaCl concentrations necessary for their optimum growth.

3.7. Effect of pH and temperature

Both parameters play an important role to achieved maximum levansucrase production. Isolate *Klebsiella* sp. strain L1 (16.17 IU) shown optimum levansucrase production at 5.0 pH. Enzyme production was decreased as increasing pH 8–10 (Fig. 4E). Maximum levansucrase production is reported in the range of pH 5.0 and 6.5 (L' Hocine et al., 2000; Sangeetha et al., 2002; Belghith et al., 2012). Besides pH, different temperature range (30, 35, 40, 45, 50, 55, 60 °C) was taken for the maximizing the production of levansucrase. Isolate *Klebsiella* sp. strain L1 shown highest production (16.21 IU) at 40 °C temperature optima (Fig. 4F). In the previous study, *Rhodotorula* sp. optimum temperature of 40 °C (Barbosa, 2007); *Bacillus macerans* EG-6 optimum temperature of 37 °C (Park et al., 2001) was reported.

3.8. Identification of levan polymer by TLC

Fermentation reaction product levan was analyzed using the TLC plate method. A mixture of sugars was identified by comparing with glucose, fructose, and sucrose as standards. Qualitatively observation shows that hydrolysis of sucrose by levansucrase was given the mixture of glucose, fructose, levan, sucrose and other fructooligosaccharides (Fig. 5). Every hydrolyzed product concentration depended on initial sucrose concentration (Viikari and Linko, 1986; Belghith et al., 2012).

3.9. Purification of levansucrase

The purification of levansucrase enzyme from *Klebsiella* sp. strain L1 was assessed with the help of ammonium sulphate precipitation, Sephadex G-100, and DEAE-cellulose column chromatography. First, the crude culture was precipitated by ammonium sulphate fractional (40–80%). Recovered fraction checked for the enzyme activity, protein estimation and used for further purification. The partially purified enzyme was further purified by Sephadex G-100 column with the help of 50 M NaCl gradient and further purification with DEAE-cellulose ion exchange chromatography column at a NaCl concentration of 0.2 M (Fig. 6). The fractions (no. 9–11) having maximum specific activity were concentrated. The final purification fold of *Klebsiella* sp. strain L1 levansucrase was 5.45 with a 22.72% recovery and which had a specific activity of 48.19 IU/mg protein. The summary of purification of levansucrase enzyme from *Klebsiella* sp. strain L1 has been shown in Table 2. The purified levansucrase enzyme bend sizes were found approximately 43 kDa (Fig. 7). Presence of some contaminating enzymes has also appeared in SDS PAGE gel, which was not removed at purification steps. Previously reported *Pseudomonas syringae* had 45 kDa molecular weight Hettwer et al. (1995) and *Bacillus* sp. TH4-2 contain 56 kDa (Ammar et al., 2002). According to Annamalai et al. (2009), the molecular weight of the enzymes is strain specific not species specific.

4. Conclusion

In the present work, newly isolated non-pathogenic acidophilic bacterial strain *Klebsiella* sp. strain L1 was acting as a potential levansucrase producer. Optimizing different growth parameters for isolate *Klebsiella* sp. strain L1 produced high levansucrase. Strain L1, potentially hydrolyzed sucrose with levansucrase enzyme to produce levan polymers which makes their potential applications like in food and pharmaceutical industries. Further studies will be needed for cloning and expression of levansucrase producing gene into a suitable host and optimization for the mass production.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101107.

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