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Thermostable levansucrase from *Bacillus subtilis* BB04, an isolate of banana peel

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Abstract

Extensive screening resulted in the isolation of *Bacillus* sp. from Banana peel that produces considerable amount of thermostable levansucrase of molecular size 52kDa. 16S rRNA sequence analysis suggests that it belongs to *Bacillus subtilis* and was designated as strain BB04. Levansucrase was sucrose inducible, showed optimum activity at 50°C and pH 6.0. It was stable at pH range 6.0 - 7.0. Ca^{2+} at 1.0 $mmol^{-1}$ concentration enhanced levansucrase activity by 24%. However levan production was highest at 40°C and pH 6.0. Cane molasses and juice proved to be good sources of sucrose for levan production. *B. subtilis* BB04 produced relatively more levan using cane molasses (11.32 gl^{-1}) as sucrose source than in cane juice (4.81 gl^{-1}).

Keywords: *Bacillus subtilis*, thermostable levansucrase, levan, cane molasses and juice.

Introduction

Levansucrases (β-2, 6-fructan: D-glucose-1-fructosyltransferase, E.C.2.4.1.10) are involved in synthesis of fructan polymers known as levans. Levans are derived from sucrose, consists of linear or branched chains of fructose units attached to sucrose by β (2→6) glycosidic bond (Hernandez and Banguela 2006). Levans are used as viscosifier, stabilizer, emulsifier, gelling or water-binding agent in food, cosmetics and nutraceutical industries (Rairakhwada et al. 2010). Levansucrases catalyze two different reactions - hydrolysis of sucrose when water is used as the acceptor and transglycosylation to form fructose polymers releasing glucose (Ozimek et al. 2006). They are produced by several microorganisms (Bezzate et al. 2000; Morales-Arrieta et al. 2006; Hernalsteens and Maugeri 2008; Van Hijum et al. 2004).

Microbial levansucrases use different acceptors *in vitro*, such as water (in sucrose hydrolysis reactions), glucose (in interchange reactions) and sucrose or fructan (in polymerization reactions) (Vela´zquez-Herna´ndez et al. 2009). Levansucrases from *Zymomonas mobilis*, *Rahnella aquatilis* JCM-1683, *Pseudomonas syringae* and *Bacillus sp. TH4-2* are found to be thermostable (Sangiliyandi et al. 1999; Seo et al. 2000; Hettwer et al. 1995; Ammar et al. 2002). However, optimum temperature for levan production was not as high as their hydrolytic activity except for *Bacillus sp. TH4-2* (Ammar et al. 2002). The industrial use of levansucrases has long been hampered by costly production processes which rely on mesophilic bacteria and plants (Kucukasik et al. 2011). The present study illustrates the characterization of thermostable levansucrase *Bacillus subtilis* BB04 which efficiently converts sucrose in low cost raw material like cane juice and molasses to levan.

Material and methods

Isolation of levansucrase producing microbial system

Organisms isolated from different sources (sugarcane baggasse, ripened fruits, vegetables, organic wastes etc.) were screened for the levansucrase activity on media containing 10% (w/v) sucrose in agar plates. *Bacillus* sp isolated from Banana fruit peel was found to have maximum levansucrase activity. The bacterium was identified using 16S rRNA sequence analysis and maintained on LB medium at 4°C and designated as *Bacillus subtilis* BB04.

16SRNA sequence analysis

Bacillus genomic DNA (Ausubel et al. 1995) was subjected to PCR amplification using *Bacillus* 16S rRNA specific primers. The PCR mixture contained 15µg of template DNA, 2.5µl of 10X buffer with 1.5mmol l^{-1} $MgCl_2$, 2.5µl of 2.0mmol l^{-1} dNTPs, 2.5µl of 10.0µmole $ml^{1}$of oligonucleotide primers 16S_8F (5'AGAGTTTGATCCTGGCTCAG 3') and 16S_1391R (5'

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GACGGGCGGTGTGTRCA 3'), 3U Taq DNA polymerase in a total volume of 25µl. Reaction was initiated with denaturation at 94°C for 3min followed by 32 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 90s and final extension at 72°C for 10min. The PCR product was purified by PCR preps DNA purification system (Promega, India) and was sequenced using primers 704F (5' GTAGCGGTGAAATGCGTAGA 3') and 907R (5' CCGTCAATTCMTTTGAGTTT 3') and sequence was analyzed using Sequence Scanner (Applied Biosystems) software. The rDNA sequence contigs were generated using Chromas Pro software and analyzed using online NCBI BLAST database. Partial 16S rRNA sequence from *Bacillus subtilis* BB04 has been submitted to EMBL-EBI nucleotide sequence database (HE605037).

Levansucrase assay

B.subtilis BB04 was grown on LB medium supplemented with 5% (w/v) sucrose at 50°C overnight. The cell free culture supernatant was subjected to acetone precipitation at 60% saturation. Precipitate was collected by centrifugation 17,400 x g for 15 min at 4°C, dissolved in 50 mmol l^{-1} sodium phosphate buffer pH 6.0, and desalted using 10ml Zeba spin column (Thermo Scientific, India). Protein concentration was determined at 280nm using BSA as standard. Aliquots were stored at -20°C until further use. Enzyme activity was assayed by adding 50µg of enzyme to 100µl of 17% (w/v) sucrose and 1.0 mmol l^{-1} of $CaCl_2$ in 50 mmol l^{-1} sodium phosphate buffer pH 6.0 followed by incubation at 50°C for 60 min. The activity was measured as the amount of glucose released using glucose oxidase kit (Merck, India). One unit of levansucrase was defined as the amount of enzyme that produced 1.0 µmol of glucose per min under standard conditions. Levansucrase was analyzed on SDS and Native PAGE **(**Sambrook et al 1989). In Native PAGE, gel was cut into 1cm pieces and individual piece was incubated in 8.5 % (w/v) buffered sucrose at 50°C for 60 min. The levansucrase activity for each gel piece was measured as mentioned above.

Levan production

For levan production, enzyme assay mixture was incubated at 40°C for 12h. Levan was separated by adding 4 volume of chilled 70% (v/v) aqueous ethanol. Mixed by inversion and centrifuged at 17,400 x g at 5°C. The pellet was re-suspended in sterile distilled water and heated at 80°C for 10min. The suspension was hydrolyzed in 0.1N HCl at 80°C for 10min and assayed using 1% resorcinol reagent (Ashwell 1957). Levan content was expressed in terms of fructose as g l^{-1} (Viikari and Gisler 1986).

Effect of pH and temperature on Levansucrase activity and levan formation

Effect of pH on levansucrase activity and levan formation was determined by incubating 50µg of enzyme with buffered sucrose ranging from pH 5.0 to 9.0 (50 mmol l^{-1} of sodium acetate buffer, pH 5.0, sodium phosphate buffer , pH 6.0 and 7.0, TrisCl buffer, pH8.0 and 9.0) using the standard assay conditions. Optimum temperature for the activity and levan formation was determined by incubating reaction mixture at 4, 30, 37, 40, 50, 60, 70 and 80°C. pH stability of levansucrase was examined by pre-incubating the enzyme for 15min with buffered sucrose ranging from pH 5.0 to 9.0 (50 mmol l^{-1} of sodium acetate buffer, pH 5.0, sodium phosphate buffer , pH 6.0 and 7.0, TrisCl buffer, pH 8.0 and 9.0). Temperature stability of the enzyme was tested by pre-incubation for 15 min, pH 6.0 at different temperature up to 80°C and residual activity of levansucrase was measured. The levansucrase activity of the pre-incubated sample at 50°C was taken as 100%.

Effect of metal ions on enzyme activity

Enzyme (50µg) was incubated with 0.5 mmol l^{-1} EDTA for 10min at 50°C to titer the cations. One mmol l^{-1} solution of Na^{+}, K^{+} ,Ca^{2+}, Hg^{2+}, Fe^{3+}, Mn^{2+} and Mg^{2+} in 50 mmol l^{-1} sodium phosphate buffer pH 6.0 was pre-incubated with the enzyme (normal and pretreated with EDTA) for 10 min. Cations were applied as chloride salts. The reaction was started by addition of 8.5% (w/v) buffered sucrose to determine the residual activity**.** Controls were incubated with distilled water.

Effect of sucrose concentration and time on levansucrase activity and levan production

Enzyme (50ug) was incubated with 0- 13.5% (w/v) sucrose in 50 mmol l^{-1} sodium phosphate buffer pH 6.0 at 50°C for 60min. Controls were incubated with distilled water instead of sucrose. Further, the enzyme solution was incubated with 8.5% (w/v) sucrose in 50 mmol l^{-1} sodium phosphate buffer pH 6.0 at 50 and 40°C. Aliquots were drawn up to 24h at three hour interval. Glucose released and levan formed was quantified. Levansucrase (0-100µg) was spotted on 8.5% (w/v) sucrose (1.35%) agar plate and incubated overnight at 40°C and the whitish slimy spots formed were photographed under UV light for better visualization.

Levansucrase activity and levan production using cane molasses and cane juice

Cane molasses was clarified with 0.1N H_2SO_4. pH of the clear supernatant was adjusted to 6.0 using 0.1N NaOH. Cane juice from Co 86032 variety was heated at 80°C to get rid of innate invertase. The juice was filtered using Whattman Filter paper No 3. Clarified molasses and cane juice was analyzed by HPLC (Waters) for sucrose, glucose and fructose content, using Ultra Amino column, Restek (150mm length and 100A° pore size) with RI detector (Waters 410).

Enzyme assay mixture contained 50µg of enzyme and clarified cane molasses and cane juice diluted to contain 1, 3, 6, 9, 12, 15 and 18% of sucrose. In case of cane molasses 50 mmol l^{-1} sodium acetate buffer pH 6.0 was used as molasses formed insoluble precipitates in presence of phosphate. Fifty mmol l^{-1} sodium phosphate buffer pH 6.0 was used for cane juice. The reaction mixtures were incubated at 50°C for 60 min and assayed for the activity.

Levansucrase activity and levan production on cane molasses and cane juice using B. subtilis BB04 inoculum

For growth analysis, cane molasses and juice was diluted to 6% sucrose and fortified with 0.2% yeast extract and 0.2% $(NH_4)_2SO_4$ (Han and Watson 1992). Cell growth was monitored by measuring the OD at 600nm at regular intervals. Enzyme activity and levan was determined according to standard protocol.

Results

Identification of Bacillus subtilis levansucrase

16S rRNA sequence analysis confirmed the culture as *Bacillus subtilis* and designated as *Bacillus subtilis* BB04. It produced slimy white glistening colonies on 10% (w/v) sucrose agar after incubation at room temperature for 48h (Fig. 1). Phylogenetic analysis of 16SrRNA gene sequences suggest that it is related to *Bacillus subtilis ATCC 663* (Fig. 2). Molecular weight of the enzyme was estimated to be 52 kDa by SDS PAGE, and showed highest levansucrase activity in gel assay (Fig. 3).

Effect of pH and temperature on levansucrase activity and levan formation

Maximum levansucrase activity was observed at pH 6.0 and about 97% activity was retained at pH 7.0 in contrast to 71% at pH 5.0. Optimum yield of levan was in the pH range 6.0 to 8.0, (Fig. 4.a). At 50°C, levansucrase activity was highest; however levan production was higher at 40°C. Levan synthesis was reduced by 32% at 60°C (Fig. 4.b). Enzyme activity was stable in the pH range 6.0 to 7.0.

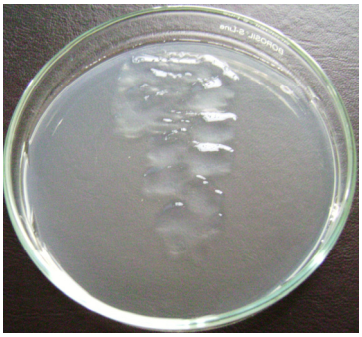

Figure 1: Levan formation by *B. subtilis* BB04 levansucrase. The bacterium was grown on 10% (w/v) sucrose agar. Whitish opalescent slimy colonies were observed after 48h incubation at room temperature.

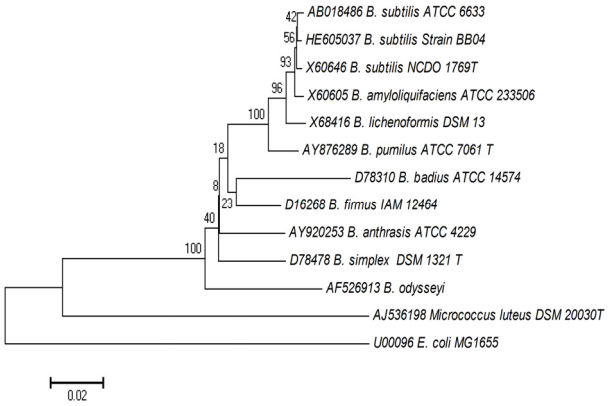

Figure 2: Neighbor-joining tree based on 16S rRNA gene sequences (1400 bases) showing the phylogenetic relationship between *B. subtilis* Strain BB04 and its closest relatives. *Micrococcus luteus* DSM 20030T and *Escherichia coli* Strain MG 1655 were used as out group in the tree. Bootstrap values >50% are given at nodes.

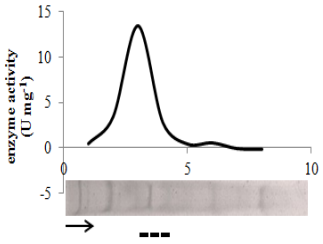

Figure 3: On gel activity assay of *B. subtilis* BB04 levansucrase on Native PAGE. Gel pieces of 1cm were assayed for levansucrase activity. The arrow indicates the direction of the run. The dotted line indicates the gel piece showing maximum activity.

Bacillus levansucrase retained less than 20% of its maximum activity at alkaline pH range 8.0-9.0. The enzyme was stable at 30-50°C and showed steep decrease above 50°C.

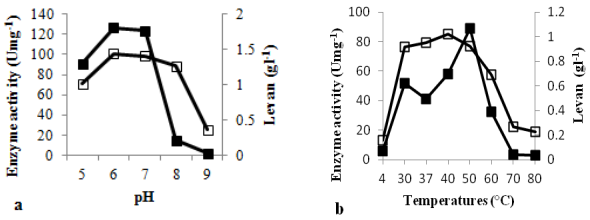

Figure 4: Effect of (a) pH and (b) temperature on levan production and activity of levansucrase from *B. subtilis* BB04. Levansucrase activity (■) and levan formation (□).

Effect of metal ions on enzyme activity

Ca^{2+} ions enhanced levansucrase activity by 24%. Na^{+} and K^{+} did not show any significant effect. Mg^{2+} reduced the enzyme activity by 23%. Enzyme activity was inhibited to more than 50% by Fe^{3+}, Mn^{2+} and Hg^{2+}. More than 50 % of the enzyme activity was blocked after EDTA treatment. However, in presence of Ca^{2+}, activity was restored to 55% of its initial activity. Levan production was increased by 42% in presence of Ca^{2+}; whereas with other metal ions, levan formation was negligible (Table 1).

Table 1: Effects of cations and EDTA on the overall activity of *B. subtilis* BB04 levansucrase and formation of levan.

Ion	% of residual activity[a]	% of residual activity after EDTA treatment[b]	% of levan formed[a]	% of levan after EDTA treatment[b]
None	100.0	43.0	100.0	--
Ca	124.0	55.0	142.3	29.3
Na	97.1	37.0	--	--
K	99.4	36.3	--	--
Mg	76.5	44.5	--	--
Fe	45.8	36.4	--	--
Mn	46.9	36.1	--	--
Hg	36.6	36.2	--	--

a: reaction mixtures were incubated with 1.0 mmol l^{-1} cation for 10min,

b: reaction mixtures were pre-incubated with 0.5 mmol l^{-1} EDTA for 10min followed by 1.0 mmol l^{-1} cation and assayed for residual levansucrase activity.

Effect of sucrose concentration on enzyme activity and levan formation

Levansucrase activity increased with sucrose concentration (up to 8.5% w/v). The activity was almost constant at higher sucrose concentrations. *K*m of levansucrase was at 0.57% (w/v) sucrose (16.8 mmol l^{-1}) and the *V*max was 127.2 Umg^{-1}. Steep increase in levan formation was noticed till 5% sucrose concentration and was found to be stabilized at higher concentrations (Fig. 5.a). Levansucrase attained maximum activity within 12h; however the activity slightly decreased after 15h. Levan content increased exponentially till 6h and almost stabilized (Fig. 5.b). Plate assay for levan production indicated that amount of the polymer produced is proportional to the enzyme concentration (Fig. 6).

Levansucrase activity in cane molasses and cane juice

Cane molasses and juice contained 23% and 18% sucrose respectively. HPLC analysis revealed about 2.8% fructose and 2.9% glucose in cane molasses. Increase in levansucrase activity was noticed up to 6% sucrose concentration in molasses and cane juice

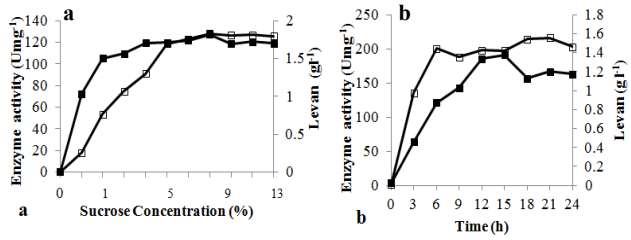

Figure 5: Effect of (a) sucrose concentration and (b) incubation time of the reaction mixture on *B. subtilis* BB04 levansucrase activity (■) and levan production (□).

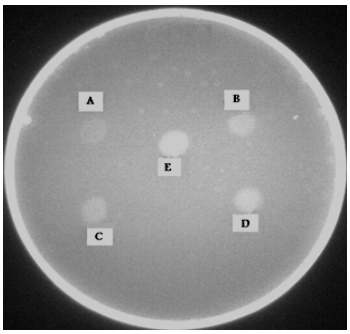

Figure 6: Effect of *B.subtilis* BB04 levansucrase concentration on levan production. A: 20 B: 40 C: 60 D: 80 and E: 100 µg of enzyme was spotted on 8.5 % (w/v) sucrose water agar plate. Levan formation was seen as whitish layer formed at the spotted area.

(Fig. 7). In cane molasses, the activity sharply decreased at higher sucrose levels. For cane juice, levansucrase activity was maximum at 6% and retained ~70% activity at higher sucrose concentration.

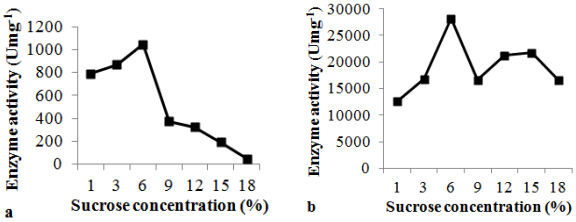

Figure 7: *B. subtilis* levansucrase activity with cane molasses (a) and cane juice (b) as source of sucrose.

Levansucrase synthesis and levan production by B. subtilis BB04 grown in cane molasses and juice

B. subtilis BB04 culture was grown at 37°C in fortified cane molasses and cane juice with final sucrose concentration of 6%. Culture attained stationary phase in 15h in both media. Cell dry weight was higher at late exponential phase. Gradual reduction in dry weight was observed after 12h in cane molasses whereas after 21h in case of cane juice. In cane molasses, levansucrase activity increased exponentially along the time and got stabilized. This was correlated with the levan production. However in case of cane juice, a rapid reduction in the levansucrase activity was observed after 9h with no significant change in levan production. The amount of levan formed using cane molasses was greater than cane juice (Fig. 8.a, b). The results suggest that sucrose in the form of cane molasses would be a good choice for production of levans.

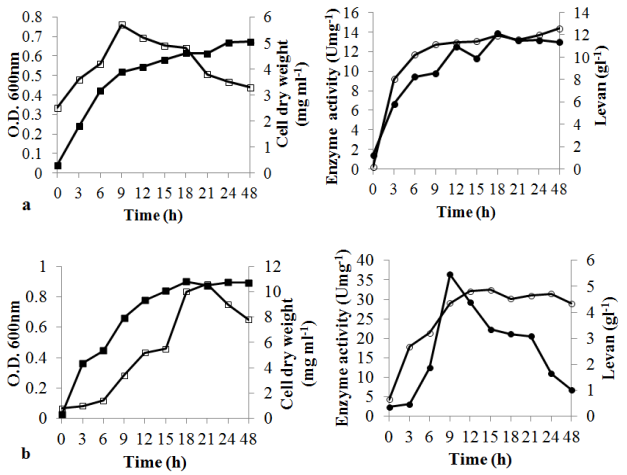

Figure 8: Growth analysis of *Bacillus subtilis* BB04. (a) Cane molasses and (b) Cane juice. OD $_{600nm}$ (■), cell dry weight (□); levansucarse activity (○) and levan formation (●) was determined during the growth.

Discussion

Levansucrases belong to glycoside hydrolases 68 (GH68) family catalyzing hydrolysis of sucrose and also transfer fructose residues to the suitable acceptors. Bacterial levansucrases are generally known as extracellular proteins, and produce high molecular weight fructans (Seo et al. 2000). In plants, fructans are used as reserve carbohydrates stored in cell vacuoles and their production is induced under abiotic stress viz. high light, cold and drought (Hernandez and Banguela 2006). Levan production is implicated in conferring resistance to environmental stress such as water deprivation, nutrient assimilation and biofilm formation in microbial systems (Vela´zquez-Herna´ndez et al. 2009). This study reports the characteristics of a thermostable levansucrase isolated from *Bacillus subtilis* BB04 isolate from Banana peel. *Bacillus subtilis* BB04 colonies produces levan, seen as typical whitish glistening phenotype on 10% (w/v) sucrose agar plates. *Bacillus licheniformis,* a levan producer, resulted in similar type of colonies on sucrose agar plates (Ghaly et al. 2007). Levansucrase from culture filtrate of *Bacillus subtilis* BB04 produced typical whitish glistening spots on sucrose agar plate and the results are in concurrence with levansucrase activity from *Pseudomonas syringae* isolate (Li and Ullrich 2001).

Levansucrase consists of hydrolytic and transfructosylation activity. Studies indicate that the optimum temperature for transferase activity is lower than the hydrolase activity (Oseguera et al. 1996; Ammar et al. 2002). *B. subtilis* BB04 levansucrase was found to be thermostable, with temperature optimum of 50°C. However, the optimum temperature for levan production was 40°C. Most of the levansucrases from *Bacillus sp.* produced levans at 30°C (Vela´zquez-Herna´ndez et al. 2009), with exception of levansucrase from *Bacillus sp.* TH4-2 isolate which showed highest levansucrase activity at 60°C and levan production at 50°C (Ammar et al. 2002). *B. subtilis* BB04 levansucrase activity was optimum at pH 6.0. *B. megaterium* levansucrase was found to be active between pH 6.0 and 7.0. The enzyme activity significantly fell at pH values below 5.6 and above 7.6 (Homann et al. 2007).

Incubation with EDTA blocked more than 50% of *B. subtilis* BB04 levansucrase activity. Ca^{2+} is essential for optimal enzyme activity. The activity was increased by 24% in presence of 1.0 mmol l^{-1} Ca^{2+}. 3D structural analysis of levansucrase suggest that amino acid residues associated with the putative Ca^{2+} ion-binding site are conserved in most of the fructosyltransferases characterized from

gram-positive bacteria (Meng and Futterer 2003). Ca^{2+} restored *Lactobacillus panis* levansucrase activity after EDTA treatment (Waldherr et al. 2008). Heavy metal ions like Fe^{3+}, Hg^{2+} and Mn^{2+} inhibited enzyme activity significantly.

*K*m value of the enzyme was similar to that of thermostable levansucrase from *Bacillus* sp. TH4-2 isolate (16.7 mmol l^{-1}) (Ammar et al. 2002). However, thermostable levansucrases from *R. aquatilis* JCM-1683 (Ohtsuku et al. 1992), *Lactobacillus reuteri* (Van Hijum et al. 2004) and *Zymomonas mobilis* (Yanase et al. 1992) were reported to have higher *K*m values; 50, 21 and 122 mmol l^{-1} respectively. Levan content was highest at 5% (w/v) sucrose and it remained constant at higher sucrose concentrations. Levan yield using *B. subtilis* (natto) Takahashi was seen to be decreased at the higher sucrose concentrations (Shih et al. 2005). In enzyme spot assay in the present study showed that polymer production is directly proportional to the enzyme concentration.

Molasses and cane juice are inexpensive and easily available low-cost raw material as source of sucrose for bulk production of levan polymer. Studies on levan production using *Halomonas sp*. indicate that levan yield (1.84 g l^{-1}) was lower with 50 g l^{-1} sucrose as substrate compared to sugar beet molasses (12.4 g l^{-1}) at 30 g l^{-1} sucrose concentration (Kucukasik et al. 2011). In our study, cane molasses as substrate gave higher yield of levan compared to cane juice. Levan yield after 12h was found to be 11.32 gl^{-1} in molasses and 4.81 gl^{-1} in cane juice, at 60 gl^{-1} of sucrose concentration. The increase in levan yield in molasses might be due to the presence of other carbon sources like glucose and fructose along with sucrose which enhances transfructosylation activity. *Halomonas* sp. AAD6 cells were found to utilize other monosaccharides like glucose, fructose and raffinose present in molasses besides sucrose for production of levan (Kucukasik et al. 2011). Levan production by *Paenibacillus polymyxa* NRRL B-18475 was higher using sugar beet molasses (38.0 gl^{-1}) than sugar cane syrup (19.6 gl^{-1}). Further, cane juice fortified with 0.2% peptone proved to be a better medium for levan production (Han and Watson 1992). Levan production was maximum at the early stationary growth phase of *B. subtilis* BB04 (Fig. 8. a, b). Studies with *Aspergillus japonicus*-FCL 119T and *Aspergillus niger* ATCC 20611 suggest that cane molasses is a good substitute for sucrose for the production of enzyme as well as fructooligosaccrides (Dorta et al. 2006).

Conclusion

In the present study, we have characterized a thermostable levansucrase with high transfructosylation activity, and analyzed its efficiency for levan production using low cost sucrose sources. Levansucrase from *B. subtilis* BB04 isolated from Banana peel was found to be most active at 50°C, with optimum levan production at 40°C and tolerant for wide range of pH. Cane molasses was found to be a better substrate for levan production than cane juice, possibly due to the presence of other carbon sources in molasses which are known to enhance transfructosylation activity. The results of this study suggest that the thermostable levansucrase from *B. subtilis* BB04 is quite efficient in converting sucrose to levan using cane molasses and cane juice. Both the enzyme and the sucrose sources can be effectively used for commercial production of levan.

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