Original Research Paper

Heterologous Expression and Characterization of Thermostable Levansucrase (*BsSacB*) from *Bacillus subtilis* BB03

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Abstract: Thermostable levansucrase encoding gene (*sacB*) with its promoter was isolated from *Bacillus subtilis* BB03. *BsSacB* is composed of 1419 bp, encoding 473 amino acid residues and with promoter regions TTGCAA (-35) and TAGAAT (-10). The amino acid sequence analysis of *BsSacB* showed conserved motifs of microbial fructosyltransferase belonging to Glycoside Hydrolase family 68 (GH68). Comparative analysis of the protein structure was carried out with 3D model of *BsSacB* constructed using PyMol software. *BsSacB* gene was expressed in *Escherichia coli* and the purified enzyme demonstrated thermo stability up to 50°C with a high hydrolytic activity at pH 6.0 and presence of Ca^{2+} promoted the activity by 15%. An attempt was made to further characterize the thermostable recombinant levansucrase from *B.subtilis* BB03 using crude sucrose rich substrates. Optimal levan production was seen with molasses (1.94±0.04 g/L) at 15 h as compared to cane juice (1.0±0.01 g/L) at 12 h. An increase in levan formation with gradual decrease in hydrolytic activity was distinctly evident in both the molasses and juice media.

Keywords: *B.subtilis*, Cane Juice, Cane Molasses, *E.coli*, Levan and Levansucrase

Introduction

Levans are prebiotic agents with potential health benefits, selectively support intestinal health and act as low calorie sweeteners (Byun *et al*., 2014; Kang *et al*., 2009). They are used as blending component in cosmetics to provide cell-proliferating and skin moisturizing effects (Abdel-Fattah *et al*., 2012). Due to their physical and biological functions as anti-tumor, anti-inflammatory agent, they have potential application in food and pharmaceutical industry (Yoon *et al*., 2004; Kim *et al*., 2005). Levans are water soluble fructo-oligosaccharides which form viscous solutions, hence used as emulsifier or encapsulating agent in cosmetics, biodegradable plastics, textile coatings and detergents (Kang *et al*., 2009).

Levans are synthesized by bacterial levansucrases which belong to GH68 family. They synthesize fructan oligosaccharides and levan by transferring fructosyl group of non-activated sucrose into fructan polymers with β (2→6) linkage (Seibel *et al*., 2006). Levansucrases catalyze two different reactions-hydrolysis of sucrose and transfructosylation to form fructose polymers by releasing glucose (Ozimek *et al*., 2006; Goldman *et al*., 2008). *Bacillus* species like *B. methylotrophicus*, *B.subtilis, B. amyloliquefaciens and B. megaterium,* are reported to be good producers of levansucrases (Zhang *et al*., 2014; Seibel *et al*., 2006; Homann *et al*., 2007; Rairakhwada *et al*., 2010; Vaidya and Prasad, 2012). However, poor availability and low stability of characterized microbial enzymes generates the need for investigation on levansucrases with improved physico-chemical properties (Maiorano *et al*., 2008).

For industrial exploitation of the potential of these enzymes, it is essential to gain knowledge about their

molecular and structural characteristics. Characterization, cloning and expression of levansucrase (SacB) from *Bacillus* sp. in a heterogeneous system like *E.coli* have been attempted. Studies on recombinant expression of levansucrase in gram positive bacterium like *B. megaterium* are in developing phase (Rairakhwada *et al.*, 2010; Korneli *et al.*, 2013). Several expression studies have been performed using *E.coli* system (Rairakhwada *et al.*, 2010; Kang *et al.*, 2005; Nakapong *et al.*, 2013); however they require addition of IPTG to induce levansucrase production. Further, the availability of low cost media and sucrose substrate is a major constraint in levan production. The present study report the isolation of *BsSacB* from *B.subtilis* with its own promoter inducible in presence of sucrose that can effectively be expressed in *E.coli*. The results of the studies on effect of temperature, pH, substrate concentration and utilization of alternate sucrose rich substrate on hydrolysis and trans fructoslysation activity using the recombinant *BsSacB* expressed in *E.coli* has been presented.

Materials and Methods

Genomic DNA Isolation and Cloning of BsSacB Gene

Genomic DNA of *B.subtilis* BB03, an isolate from the Banana peel, which produces thermostable levansucrase was isolated (Sambrook *et al.*, 1989) and used as template for amplifying *BsSacB*. Initially, the primers for *sacB* (LevF-5'GCACTGTCGCAAACTATCAC-3' and LevR-5'ACGTGATGCCGTCAATCG3') were designed based on the reported *sacB* gene sequences of bacterial levansucrases (Velázquez-Hernández *et al.*, 2009). PCR reaction was performed in a 25.0 µL reaction volume containing 2.5 µL of 10X Taq buffer B, 1.5 µL of 25.0 mM MgCl$_2$, 1.0 µL of 10 mM dNTPs (2.5 mM each), 1.0 µL of each primer, 1.0 µL of Taq DNA polymerase (3.0 U/µL; Bangalore Genei, India), 0.5 µL of isolated genomic DNA (0.3 µg/µL) and reaction conditions of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 66°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 7 min. The sequence of the amplicon obtained was analyzed on NCBI BLASTn, which showed homology with *sacB* of *B.subtilis* (X02730.1). Based on this, primers Lev 16F (5'GTTCTTTAGGCCCGTAGTCTG3') and Lev 19R (5'AATACGGTTAGCCATTTGCCTGC3') were designed and full length *sacB* along with the promoter was obtained. PCR amplification was performed and the products were sequenced. Amplicons were analyzed on 1% agarose/EtBr gel electrophoresis, purified using

QIAEX II gel extraction kit (QIAGEN, India) and ligated into pTZ57R/T vector (Fermentas, India) as per the manufacturer's instructions. Recombinant *pTZ:BsSacB* was transformed into *E.coli* DH5α. Positive colonies were analyzed by colony PCR using Lev16F and Lev19R primers as well as internal primers (Lev46F-5'CCGATGAACATCAAAAAGTTTGC3'; Lev74R-5'ATCTCTCAGCGTATGGTTATCC3'). Recombinant plasmid was isolated using QIAprep mini spin kit (QIAGEN) and sequenced using automated DNA sequencer (Mega BASE 500, Applied Biosystems) and verified with BLASTn analysis.

Bioinformatic Analysis of BsSacB

BLAST search was performed in NCBI (http://www.ncbi.nlm.nih.org) and protein prediction was performed in CBI (http://www.cbi.pku.edu.cn). Alignment of the *BsSacB* nucleotide and protein sequence with other structurally related levansucrase genes were performed using the ClustalW program. Phylogenetic tree was drawn with the Mega 4.0 program (Tamura *et al.*, 2007). Coding region of *BsSacB* (Acc.no.CBI68350) was analyzed by PSI-BLAST program (Position specific interactive BLAST) to identify structure of related protein. Atomic coordinates of the 1OYG structure, solved at 1.5A° resolution, was used as a template for comparative modeling. Sequence and structural alignment of *BsSacB* with 1OYG Chain A was performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Aligning was carried out without the insertion of gaps in the conserved secondary structural regions. Spatial restraints were derived and used in the three dimensional structure determination using PyMol molecular graphics software (www.pymol.org). The stereochemical quality of model was assessed by PROCHECK (Laskowski *et al.*, 1993; www.ebi.ac.uk/thornton-srv/software/PROCHECK/) at the same resolution as in 1OYG structure. The overall stereo chemical quality of the model was assessed by Ramachandran plot analysis.

Expression of Recombinant Levansucrase

E.coli harboring pTZ:*BsSacB* was grown in LB medium at 37°C for 12 h and used for levansucrase expression analysis. After the 12 h growth, the cells were lyzed by freezing at -80°C followed by thawing in 37°C water bath and vortexed for 5 sec., the freeze-thaw cycle was repeated 4 times respectively (modified with reference to Gay *et al.*, 1983). This was followed by centrifugation at 12000 × g for 10 min at 4°C. The freeze thaw method is found to separate the highly expressed recombinant protein from the bulk of endogenous *E.coli* proteins (Sangiliyandi and Gunasekaran, 2000). The

supernatant obtained was brought to 60% (v/v) with respect to acetone. Protein precipitated was collected by centrifugation 17,000 × g for 15 min at 4°C, dissolved in 0.05M sodium phosphate buffer, pH 6.0. The extract was further purified using Zeba spin columns (Thermo scientific), centrifuged at 3,040 rpm for 2 min. Protein concentration was determined at 280 nm using BSA as standard and analyzed by Native PAGE (Sambrook *et al.*, 1989). The unstained gel was sliced into 5 mm bits and extracted in 100 µL 50 mM sodium phosphate buffer, pH6.0 and assayed for levansucrase activity by standard procedure described.

Assay for Levansucrase and Levan Production

Levansucrase activity was assayed in a reaction mixture containing 6.5 µL of protein sample (100 µg/mL) and 50 mM sodium phosphate buffer, pH6.0, containing 8.5% (w/v) sucrose and 1.0 mM $CaCl_2$ followed by incubation at 50°C for 60 min and the amount of glucose released was measured using glucose oxidase kit (Merck, India; Vaidya and Prasad, 2012). For levan production, assay mixture was incubated at 40°C for 12 h. Levan produced was precipitated with 4 volume of ice-cold 70% aqueous ethanol and centrifuged at 17,400 × g at 5°C. The resulting precipitate was re-suspended in sterile distilled water and heated at 80°C for 10 min followed by hydrolysis in 0.1N HCl at 80°C for 10 min. After the hydrolysis, the levan content was assayed using 1% resorcinol reagent and expressed as fructose units (Viikari and Gisler, 1986).

Levansucrase Activity and Stability

Levansucrase activity and levan production profile was analyzed over a range of pH 5.0-9.0 (50 mM sodium acetate buffer of pH 5.0, 50 mM sodium phosphate buffer pH 6.0-7.0 and Tris-Cl buffer pH 8.0-9.0). Levansucrase was also analyzed for pH stability, (pH 5.0-9.0) by pre-incubating the enzyme sample at appropriate pH for 15 min prior to the assay. Optimum temperature for the activity and levan formation was determined by incubating reaction mixture at 4-80°C under standard assay conditions. Temperature stability of the enzyme was tested by pre-incubation at above mentioned temperatures for 15 min and residual activity was measured.

To examine the effect of metal ions, levansucrase was pre-incubated for 60 min at 4°C with 1.0 mM concentration of cations (Mg^{2+}, Cu^{2+}, Ca^{2+}, K^{1+}, Mn^{2+}; Fe^{3+} and Hg^{2+}) prepared in 50 mM sodium phosphate buffer, pH 6.0. Levansucrase activity and levan production profile was analyzed at different sucrose concentrations (1.0-13.0% w/v) in 50 mM sodium phosphate buffer pH 6.0 and temperature (50°C for sucrose hydrolase activity and 40°C for levan production). Michaelis constant (Km) and velocity maximum (Vmax) was calculated. To determine the effect of enzyme concentration, enzyme solution (0-100 µg/10 µL) was spotted on agar plate (1.35%) containing 8.5% (w/v) sucrose and incubated overnight at 40°C. Whitish slimy spots formed were photographed under UV light for better visualization. Effect of incubation time on levansucrase activity and levan produced was measured at every 3 h intervals for 24 h.

Cane Juice and Molasses as Substrate

Cane molasses or cane juice as source of sucrose was used to assess levansucrase activity and levan production. Prior to analysis, cane molasses was clarified using 0.1N H_2SO_4 and pH was adjusted to 6.0 using 0.1N NaOH. Cane juice was heated at 80°C for inactivating the innate invertase activity and filtered through Whatmann No. 3 filter paper. Clarified molasses or cane juice were analyzed for sucrose, glucose and fructose concentration by high performance liquid chromatography (Waters) using Ultra Amino column, Restek (150 mm length and 100A° pore size) with RI detector (Waters 410). *E.coli* -pTZ:*BsSacB* was grown in cane molasses and juice diluted to contain 1.0% sucrose, fortified with 0.2% yeast extract and 0.2% $(NH_4)_2SO_4$ (Han and Watson, 1992) in 500 mL Erlenmeyer flasks at 37°C under shaking conditions at 150 rpm for overnight. Gay *et al.* (1985) reported use of sacB as a counter selectable marker for *E.coli* and other gram negative bacteria. It was seen that the production of levansucrase in those microbial systems was lethal in presence of 5.0% sucrose in the medium. Similarly, higher sucrose concentration did not resulted in efficient cell growth in this system thus limiting our study for using sucrose concentration compatible with the growth. Further, growth was monitored by recording the OD at 600 nm; enzyme from the harvested cells at regular intervals was assayed for levansucrase activity and levan production.

Statistical Analysis

All the parameter analyses were repeated with three independent biological samples. The data were analyzed by one-way Analysis of Variance (ANOVA) and the treatment means were compared by using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$. Data were expressed as Mean ± Standard Error (SE).

Results and Discussion

Amplification and Sequence Analysis of BsSacb

Many bacterial levansucrases have been expressed in heterologous system and assayed for their ability to produce levans (Caputi *et al.*, 2013; van Hijum *et al.*, 2004; Homann *et al.*, 2007; Rairakhwada *et al.*, 2010). Partial conserved domain of levansucrase encoding gene, *SacB* was amplified using the primers LevF and LevR from *B.subtilis* BB03 genomic DNA. BLASTn analysis of the amplicon (~800bp; Fig. 1) revealed sequence similarity with levansucrase encoded by *sacN* of *B.subtilis* (X02730.1; 98%), *sacB* of *B.subtilis* (AY150365.1; 97%), *B.stearothermophilus* (U34874.1; 95%) and *B.amyloliquefaciens* (EU668142.1; 83%). Further, primers Lev16F and Lev19R were designed based on the levansucrase gene sequences of *B.subtilis* (X02730.1) and PCR amplification resulted in a ~1.8 kb fragment (Fig. 1a). The fragment was cloned into pTZ57R/T, giving pTZ:*BsSacB*. *BsSacB* gene (FN599519.1) contained promoter sequence, Ribosomal Binding Site (RBS) and protein coding region (Fig. 1b). *BsSacB* encodes for a putative protein having 473 amino acids with a predicted molecular weight of 52.9 kD. Based on the phylogenetic analysis, microbial fructosyltransferases have been classified into three groups-levansucrases from gram-positive, gram-negative microbes and fungi (Velázquez-Hernández *et al.*, 2009). Bacterial levansucrases belongs to GH68 family with typical conserved motifs-VWDSW motif functioning as a catalytic nucleophile, EWSGS (sucrose box I), YLVFE for sucrose hydrolysis and transfructosylation and DEIER (catalytic center) (Fig. 2). Phylogenetic analysis of levansucrases show significant homology in nucleotide sequences from *B.subtilis* strains and *Zymomonas mobilis* (Fig. 3).

Three dimensional structure of *BsSacB* protein was compared with crystal structure of *B.subtilis* levansucrase (CBI68350.1, 1OYG_A chain) using PyMol Molecular Graphics Software and PROCHECK to quantify the residues in available zones of the Ramachandran plot. It was found that 87.8% of residues were located in the most favored zones, 10.7% in allowed regions, 1.0% in generously allowed regions and 0.5% (two residues) in disallowed region. Ramachandran plot characteristic and the goodness factors (quality of covalent and overall bond-angle distances) confirm this to be the best fit for the predicted model proposed for *BsSacB* (Fig. 4).

Expression of BsSacB in E.coli

Proteins from the recombinant *E.coli* having pTZ:*BsSacB* were analyzed by native-PAGE and *in-gel* levansucrase activity. The protein band with levansucrase activity (3.9U/mg) from the *in-gel* assay (Fig. 5) corresponded to the molecular weight of 52 kDa by SDS-PAGE which is in accordance with the predicted molecular weight. *BsSacB* encoded protein is typical to monomeric enzymes with the molecular weight ranging from of 46-73 kDa. However studies report that levansucrases from *Rahnella aquatilis* JMC-1683, *Leuconostoc mesenteroides* B-512 FMC and *Actinomyces viscosus* T-14V exist as dimmers (Ohtsuku *et al.*, 1992; Kang *et al.*, 2005; Pabst, 1977)

Effect of pH and Temperature on Levansucrase Activity and Levan Synthesis

Levansucrase activity of *BsSacB* protein was maximum (6.5 U/mg) at pH 6.0 and the activity significantly reduced above pH 7.0. Eighty-seven percent of the activity was retained at pH 5.0. Optimum yield of levan was at pH 6.0 which was observed to be 14.3 g/L and reduced to 6.7 g/L at pH 7.0 (Fig. 6a). Levansucrase activity was maximum at 50°C (10.6 U/mg) and showed a steep decrease with increase in temperature above 50°C. However, levan production was optimum at 40°C (10.8 g/L) and reduced drastically at 60°C (1.03 g/L; Fig. 6b). Similar difference was observed in optimum temperature for sucrose hydrolysis activity and levan formation with other recombinant levansucrase clones expressed in *E.coli*. Studies on *R. aquatilis* levansucrase showed hydrolase activity at 40°C and levan formation at 30°C (Kim *et al.*, 1998). Levansucrase from *B.amyloliquefaciens* showed optimal activity at 30°C and levan production at 0-4°C (Rairakhwada *et al.*, 2010). Studies by Hettwer *et al.* (1995) revealed that *P.syringae* levansucrase activity and levan formation was maximum at 60°C and 18°C respectively. Sangiliyandi and Gunasekaran (2000) reported the levansucrase activity at 60°C and levan synthesis at 30°C by *Z.mobilis*. However, *BsSacB* levansucrase was found to be tolerant over a wide pH and temperature range, stable over a pH range from 5.0 to 7.0 (Fig.7a) and temperature range from 4 to 50°C (Fig.7b). These results put together suggest that *BsSacB* levansucrase shows higher thermal stability in hydrolytic activity as compared to that in transfructosylation reaction. However, it has been observed that at saturated levels, levan formed was found to rapidly hydrolyze by levansucrase itself at higher temperatures (Song *et al.*, 1998).
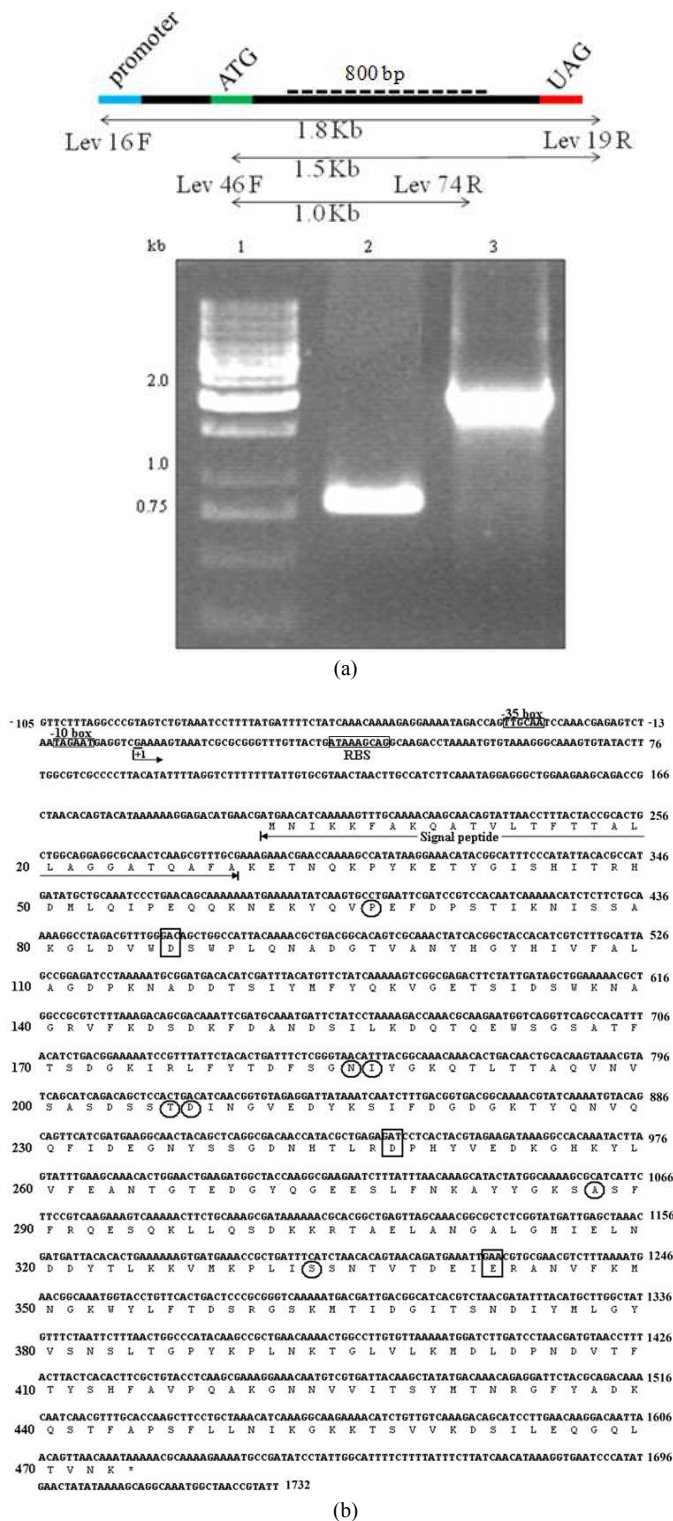
(a)

(b)

Fig. 1. (a) PCR amplification using *B.subtilis* BB03 genomic DNA as template, including schematic representation of designed primers. Lane 1: 1 kb DNA Ladder, Lane 2: PCR with primers Lev F and Lev R and Lane 3: PCR with primers Lev 16 F and Lev 19 R. (b) Nucleotide sequence and predicted amino acid sequence of the *BsSacB* gene from *B.subtilis* BB03. Nucleotides and amino acid residues are numbered on the left and right, respectively. The Ribosome Binding Site (RBS), -10 box, -35 box (in box), the active site residues (Asp86, Asp247, Glu342; in box), the amino acid changes (in circle), the stop codon (*) and the signal peptide sequences (horizontal arrows) are indicated
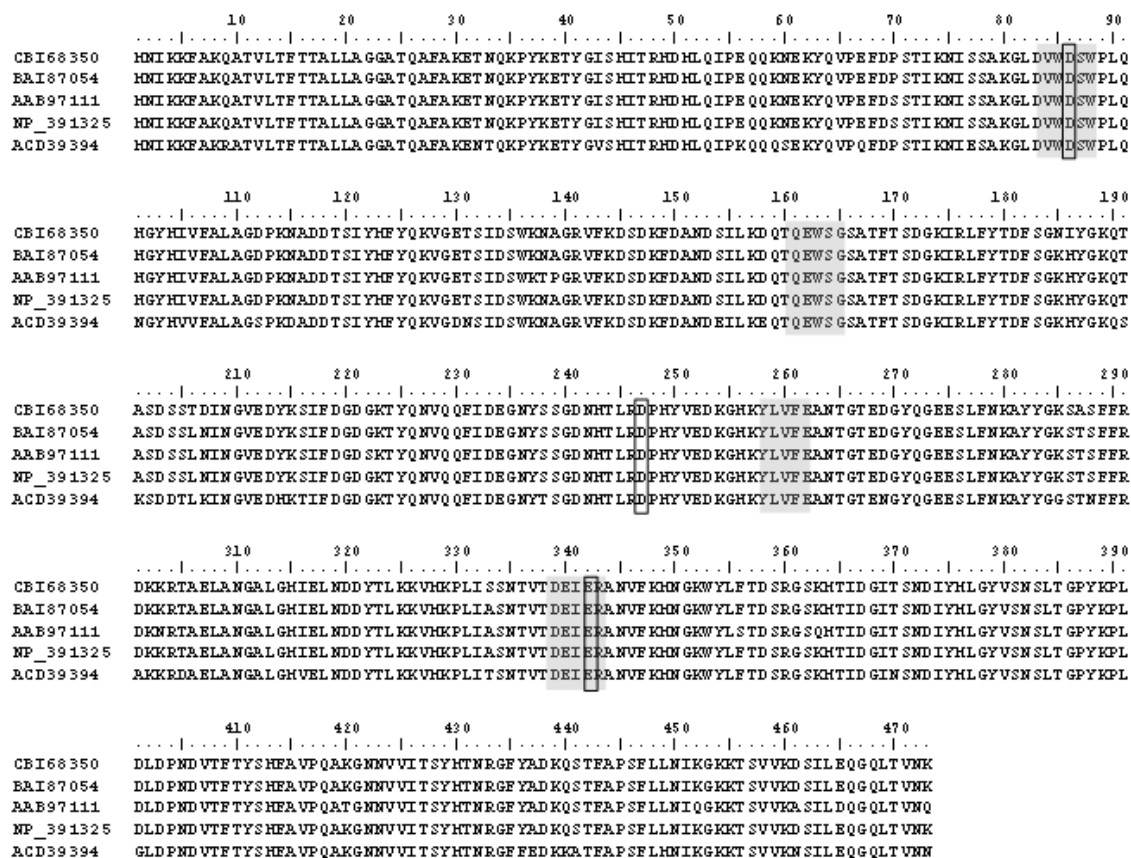
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              10        20        30        40        50        60        70        80        90
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
CBI68350  HNIKKFAKQATVLTFTTALLAGGATQAFAKETNQKPYKETYGISHITRHDHLQIPEQQKNEKYQVPEFDPSTIKNISSAKGLDVWDSWPLQ
BAI87054  HNIKKFAKQATVLTFTTALLAGGATQAFAKETNQKPYKETYGISHITRHDHLQIPEQQKNEKYQVPEFDPSTIKNISSAKGLDVWDSWPLQ
AAB97111  HNIKKFAKQATVLTFTTALLAGGATQAFAKETNQKPYKETYGISHITRHDHLQIPEQQKNEKYQVPEFDSSTIKNISSAKGLDVWDSWPLQ
NP_391325 HNIKKFAKQATVLTFTTALLAGGATQAFAKETNQKPYKETYGISHITRHDHLQIPEQQKNEKYQVPEFDSSTIKNISSAKGLDVWDSWPLQ
ACD39394  HNIKKFAKRATVLTFTTALLAGGATQAFAKENTQKPYKETYGVSHITRHDHLQIPKQQQSEKYQVPQFDPSTIKNIESAKGLDVWDSWPLQ

              110       120       130       140       150       160       170       180       190
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
CBI68350  HGYHIVFALAGDPKNADDTSIYHFYQKVGETSIDSWKNAGRVFKDSDKFDANDSILKDQTQEWSGSATFTSDGKIRLFYTDFSGNIYGKQT
BAI87054  HGYHIVFALAGDPKNADDTSIYHFYQKVGETSIDSWKNAGRVFKDSDKFDANDSILKDQTQEWSGSATFTSDGKIRLFYTDFSGKHYGKQT
AAB97111  HGYHIVFALAGDPKNADDTSIYHFYQKVGETSIDSWKTPGRVFKDSDKFDANDSILKDQTQEWSGSATFTSDGKIRLFYTDFSGKHYGKQT
NP_391325 HGYHIVFALAGDPKNADDTSIYHFYQKVGETSIDSWKNAGRVFKDSDKFDANDSILKDQTQEWSGSATFTSDGKIRLFYTDFSGKHYGKQT
ACD39394  NGYHVVFALAGSPKDADDTSIYHFYQKVGDNSIDSWKNAGRVFKDSDKFDANDEILKEQTQEWSGSATFTSDGKIRLFYTDFSGKHYGKQS

              210       220       230       240       250       260       270       280       290
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
CBI68350  ASDSSTDINGVEDYKSIFDGDGKTYQNVQQFIDEGNYSSGDNHTLRDPHYVEDKGHKYLVFEANTGTEDGYQGEESLFNKAYYGKSASFFR
BAI87054  ASDSSLNINGVEDYKSIFDGDGKTYQNVQQFIDEGNYSSGDNHTLRDPHYVEDKGHKYLVFEANTGTEDGYQGEESLFNKAYYGKSTSFFR
AAB97111  ASDSSLNINGVEDYKSIFDGDGKTYQNVQQFIDEGNYSSGDNHTLRDPHYVEDKGHKYLVFEANTGTEDGYQGEESLFNKAYYGKSTSFFR
NP_391325 ASDSSLNINGVEDYKSIFDGDGKTYQNVQQFIDEGNYSSGDNHTLRDPHYVEDKGHKYLVFEANTGTEDGYQGEESLFNKAYYGKSTSFFR
ACD39394  KSDDTLKINGVEDHKTIFDGDGKTYQNVQQFIDEGNYTSGDNHTLRDPHYVEDKGHKYLVFEANTGTENGYQGEESLFNKAYYGGSTNFFR

              310       320       330       340       350       360       370       380       390
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
CBI68350  DKKRTAELANGALGHIELNDDYTLKKVHKPLISSNTVTDEIERANVFKHNGKWYLFTDSRGSKHTIDGITSNDIYHLGYVSNSLTGPYKPL
BAI87054  DKKRTAELANGALGHIELNDDYTLKKVHKPLIASNTVTDEIERANVFKHNGKWYLFTDSRGSKHTIDGITSNDIYHLGYVSNSLTGPYKPL
AAB97111  DKNRTAELANGALGHIELNDDYTLKKVHKPLIASNTVTDEIERANVFKHNGKWYLSTDSRGSQHTIDGITSNDIYHLGYVSNSLTGPYKPL
NP_391325 DKKRTAELANGALGHIELNDDYTLKKVHKPLIASNTVTDEIERANVFKHNGKWYLFTDSRGSKHTIDGITSNDIYHLGYVSNSLTGPYKPL
ACD39394  AKKRDAELANGALGHVELNDDYTLKKVHKPLITSNTVTDEIERANVFKHNGKWYLFTDSRGSKHTIDGINSNDIYHLGYVSNSLTGPYKPL

              410       420       430       440       450       460       470
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
CBI68350  DLDPNDVTFTYSHEAVPQAKGNNVVITSYHTNRGFYADKQSTEAPSFLLNIKGKKTSVVKDSILEQGQLTVWK
BAI87054  DLDPNDVTFTYSHEAVPQAKGNNVVITSYHTNRGFYADKQSTEAPSFLLNIKGKKTSVVKDSILEQGQLTVWK
AAB97111  DLDPNDVTFTYSHEAVPQATGNNVVITSYHTNRGFYADKQSTEAPSFLLNIQGKKTSVVKASILDQGQLTVWQ
NP_391325 DLDPNDVTFTYSHEAVPQAKGNNVVITSYHTNRGFYADKQSTEAPSFLLNIKGKKTSVVKDSILEQGQLTVWK
ACD39394  GLDPNDVTFTYSHEAVPQAKGNNVVITSYHTNRGFFEDKKATEAPSFLHNIKGKKTSVVKNSILEQGQLTVWN
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Fig. 2. Amino acid sequence alignment of *Bacillus subtilis* BB04 (*CBI68350.1*) with levansucrase from *B.subtilis* subsp. natto (BAI87054), *B. stearothermophilus* (*AAB97111*), *B. subtilis* str. 168 (NP_391325.1) and *B. amyloliquefaciens* (*ACD39394*) respectively. The amino acid residues are numbered on the left and ruled on top. The residues essential for the catalytic activity Asp86, Asp247 and Glu342 are boxed. Region from 37-470 is designated as Glyco_hydro_68 family domain in *Bacillus sp*. The conserved motifs of microbial FTFs are indicated in shaded boxes
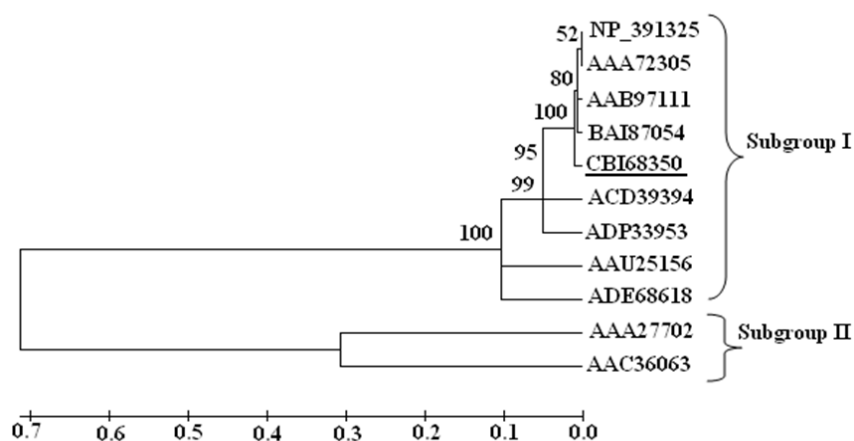

Fig. 3. Comparison of the deduced amino acid sequence of *B. subtilis* BB03 *sacB* with other levansucrases from gram-negative and gram-positive bacteria. Un-rooted dendrogram derived from the amino acid sequences. The tree was generated from a consensus of 1000 bootstrap replicates using neighbor joining method in MEGA 5 program. The scale bar indicates the relative amount of change along branches. The accession nos. of the sequences used in the comparison are indicated in the right margin, *B.subtilis* (BAI87054), *B.subtilis* (AAA72305), *B.subtilis* (NP_391325), *B. atrophaeus* (ADP33953), *B. amyloliquefaciens* (ACD39394), *Geobacillus stearothermophilus* (AAB97111), *B. megaterium* (ADE68618), *B. licheniformis* (AAU25156), *Zymomonas mobilis* (AAA27702) and *Pseudomonas syringae* (AAC36063) respectively
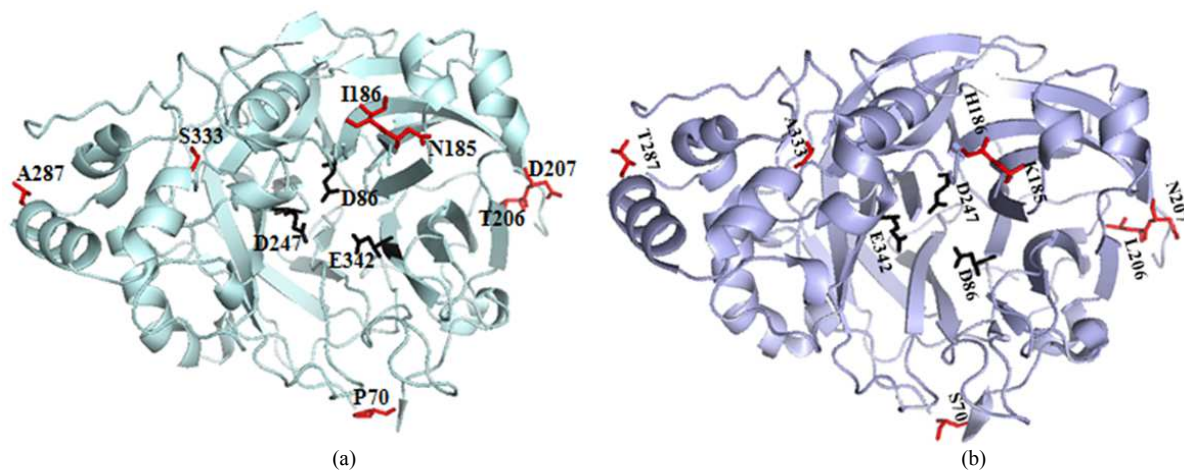
Fig. 4. The 3D structure of (a) thermostable *B.subtilis* BB03 levansucrase (CBI68350.1) was compared with (b) *B.subtilis* levansucrase (1OYG_A) using PyMol Molecular Graphics System. Essential active site residues (in black) were found to be conserved in both the structures and changes in amino acid residue were indicated in red
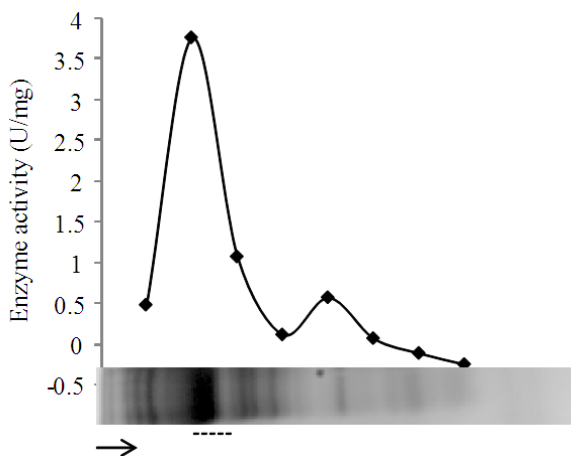

Fig. 5. *In gel* activity assay of *E.coli -pTZ:BsSacB* enzyme extract on native PAGE. Gel pieces were assayed for levansucrase activity. The arrow indicates the direction of the run. The dotted line indicates the gel piece showing maximum activity
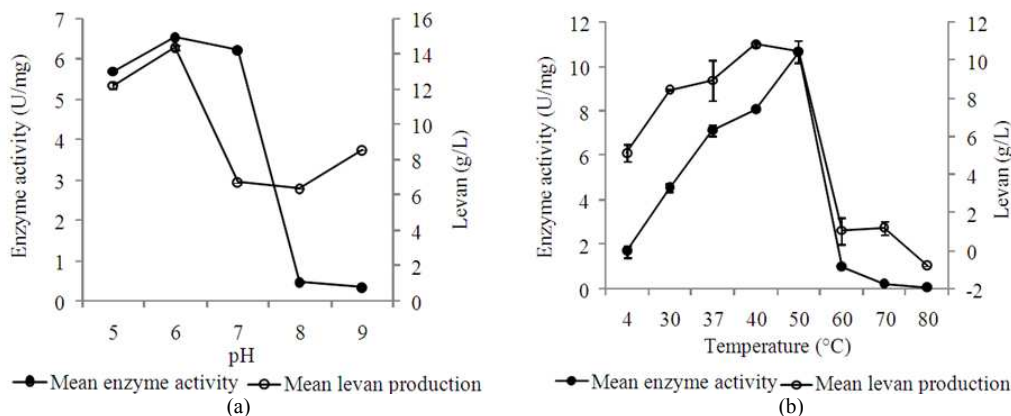
Fig. 6. Effect of pH and temperature on *E.coli* expressed recombinant FTF on levansucrase activity and levan formation Optimum (a) pH and (b) temperature was determined for levansucrase activity and levan formation. The enzyme was assayed at different pH (5.0 to 9.0) and temperatures (4°C to 80°C) under standard conditions
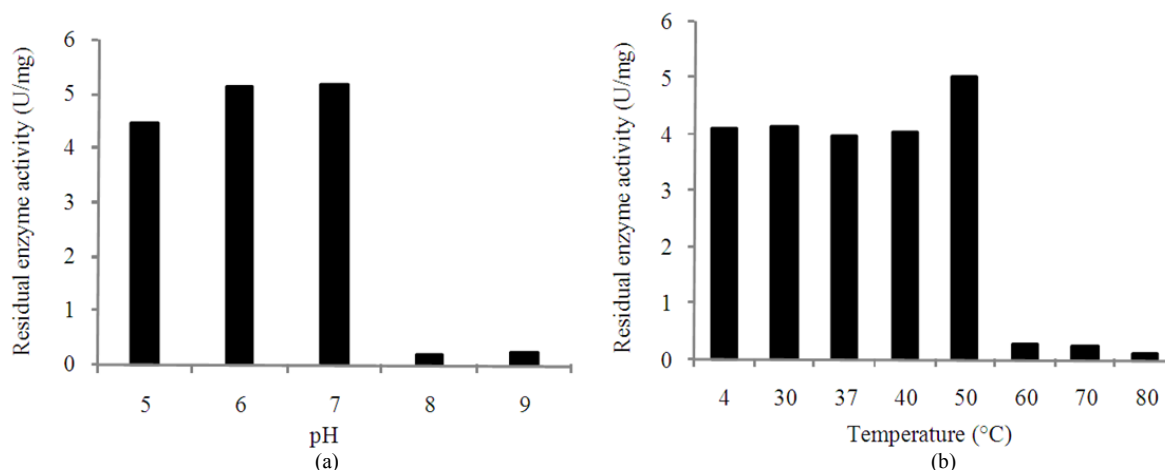
Fig. 7. pH stability (a) and thermal stability (b) of the enzyme was examined by pre-incubating the enzyme for 15 min at respective pH and temperatures followed by standard assay conditions

Table 1. Effect of cations and EDTA on *E.coli* expressed recombinant FTF on levansucrase activity and levan production

Ion	Relative activity[a] (%)	Relative activity after EDTA treatment[b] (%)	Levan[a] (%)	Levan after EDTA treatment[b] (%)
None	100.0	27.1	100.0	--
Ca	115.0	44.6	129.1	12.2
Na	100.3	27.3	--	--
K	94.4	29.0	--	--
Mg	60.8	28.8	--	--
Fe	40.3	26.5	--	--
Mn	36.7	27.3	--	--
Hg	32.3	26.7	--	--

*reaction mixtures were incubated with 1.0 mM cation for 10 min
**reaction mixtures were pre-incubated with 0.5 mmol/L EDTA for 10 min followed by 1.0 mmol/L cations and assayed for residual levansucrase activity

Effect of Metal Ions on Enzyme Activity and Levan Formation

Most of the levansucrases require metal ions as cofactors. *Lactobacillus reuteri*, *Leu. mesenteroides* and *B.subtilis* levansucrases require Ca^{2+} (van Hijum *et al.*, 2004; Ozimek *et al.*, 2006; Morales-Arrieta *et al.*, 2006; Velázquez-Hernández *et al.*, 2009) whereas Fe^{3+} can also act as an substitute cofactor for levansucrase by certain *B.subtilis* strains (Chambert *et al.*, 1990). Table 1 shows the effects of metals ions on levansucrase activity and respective levan yield. Ca^{2+} enhanced levansucrase activity by 15%. Na^{+} and K^{+} did not show any significant effect. Mg^{2+}, Fe^{3+}, Mn^{2+} and Hg^{2+} ions inhibited levansucrase activity more than 50%. The competition and replacement of a majority of smaller bivalent cations with Ca^{2+} may result in a deformed tertiary structure and may be the reason for an inhibitory effect (Waldherr *et al.*, 2008). EDTA blocked more than 70% of the enzyme activity. However, in presence of Ca^{2+}, activity was restored to 44% of its initial activity. Levan production was 29%

higher in presence of Ca^{2+} compared to other metal ions. Sequence alignments of family GH68 proteins showed the presence of calcium ion-binding site and Asp339 was identified as key amino acid residue coordinating Ca^{2+} binding (Meng and Futterer, 2003) in majority of levansucrases from Gram-positive bacteria (Ozimek *et al.*, 2006) including *BsSacB* (Fig. 2).

Effect of Sucrose Concentration on Enzyme Activity and Levan Formation

Maximum levansucrase activity and levan formation was observed at 8.5% sucrose (Fig. 8). Activity was slightly reduced at higher sucrose concentration (9.0-13%). Studies have shown that at higher sucrose concentration, the transfructosylation is a preferred reaction with noticeable decrease in hydrolase activity (Ammar *et al.*, 2002). *L. sanfranciscensis* TMW levansucrase expressed in *E.coli*, showed maximum levansucrase activity at 6.8% sucrose, however increased levan production was observed with increase in sucrose concentration above 7.0% (Tieking *et al.*, 2005). *K*m of the *BsSacB* levansucrase was 42.5 mmol/L of sucrose,
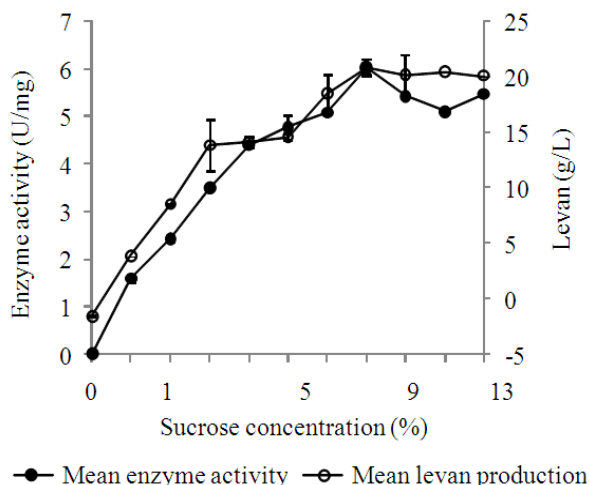
Fig. 8. Effect of sucrose concentration on *E.coli* expressed recombinant FTF on levansucrase activity and levan formation
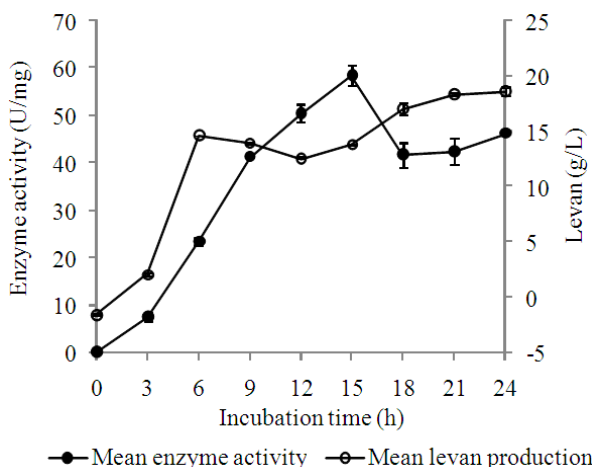

Fig. 9. Effect of different incubation time on sucrose hydrolysis activity and levan formation by *E.coli*-pTZ: *BsSacB* levansucrase

Vmax of 5.909 U/mg and exhibited sigmoidal curve with substrate concentration. Km value reported for levansucrase was on par with enzymes from *B. amyloliquefaciens*, 47 mmol/L (Rairakhwada *et al.*, 2010) and *R. aquatilis* JCM-1683_50 mmol/L (Ohtsuku *et al.*, 1992) but differed with levansucrases from *Leu. mesenteroides* B-512 FMC _26 mmol/L (Kang *et al.*, 2005), *Z. mobilis* _160 mmol/L (Yanase *et al.*, 1992) and *Pseudomonas syringae* _122 mmol/L (Hettwer *et al.*, 1995).

Effect of Incubation Time

Maximum levansucrase activity was at 15 h of incubation time and decreased. However a steep increase in levan production was observed up to 6 h followed by gradual increase till 24 h with 8.5% (w/v) sucrose (Fig. 9).
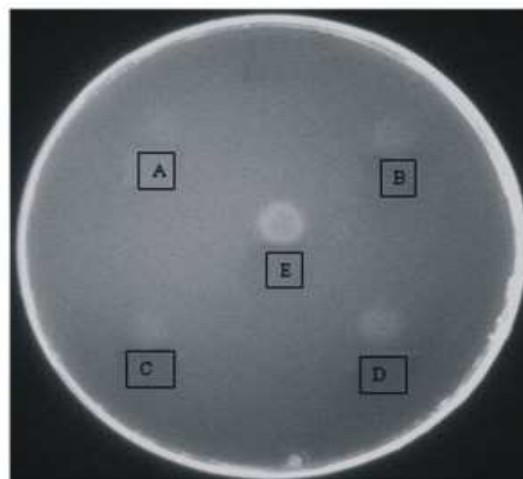
Fig. 10. Effect of concentration of *E.coli* expressed recombinant FTF on levan production. A: 20 B: 40 C: 60 D: 80 and E: 100 μg of enzyme was spotted on 8.5% (w/v) sucrose agar plate. Levan formation was seen as whitish layer formed at the spotted area

Similar study reports that recombinant levansucrase from *B. amyloliquefaciens* synthesized levan till 24 h (Rairakhwada *et al.*, 2010). However, wild type *sacB* from *Z. mobilis* B14023 was expressed in *E.coli* produced levan from the initial period of fermentation and reached a maximum concentration at 16 h (6.8 g/L and 10.7 g/L respectively) (Ananthalakshmy and Gunasekaran, 1999). The production capacity of recombinant *R. aquatilis* levansucrase was found to reduce with prolonged incubation time at saturated levels of levan (Kim *et al.*, 1998). Since levansucrase has both transfructosylation and hydrolase activity, hydrolysis of fructans can be expected at higher concentration of levans (Song *et al.*, 1998). Sucrose agar plate assay for levan production showed whitish slimy layer and was directly proportional with the enzyme concentration (Fig. 10) which is on par with our earlier observations (Vaidya and Prasad, 2012). Similarly, levan production from *B.subtilis* NRC 33a levansucrase was also related to effective concentration of enzyme. The conversion of fructose to levan was 84% and the sucrose concentration was critical to levan synthesis (Abdel-Fattah *et al.*, 2005).

Enzyme Synthesis and Levan Production by Levansucrase from E.coli -pTZ:BsSacB Grown in Cane Molasses and Cane Juice

Attempts have been made to commercially produce fructooligosaccharides utilizing agro-industrial byproducts rich in sucrose (Sangeetha *et al.*, 2005). Levan are useful prebiotic compounds with enormous application in food and pharma industry and reduction in its production cost has high impact on its industrial production and usage (Kucukasik *et al.*, 2011). Cane molasses and juice contained 23.0 and 18.0% sucrose respectively. HPLC

analysis revealed about 2.8% fructose and 2.9% glucose in cane molasses. *E. coli* harboring *BsSacB* showed comparatively slow growth in fortified cane molasses and cane juice formulated to 1.0% sucrose (attained stationary phase in 9 and 15 h, respectively; Fig. 11a and b) as compared to wild type. In previous study, *B.subtilis* strain BB04 was grown in cane molasses and juice diluted to 6% sucrose, giving higher levan production (11.32 g/L and 4.81 g/L, respectively) after 12 h (Vaidya and Prasad, 2012). Hydrolase activity in cane molasses drastically decreased after 6 h, whereas levan production continued up to 15 h and remained almost constant (Fig. 11c). In cane juice, levan formation showed increasing trend till 12 h (Fig. 11d) and correlated with decrease in levansucrase activity. Thus, it was evident that with decreasing hydrolytic activity the transfructosylation activity increased which was also seen with sucrose as substrate. However, maximal levan production obtained using sucrose (8.5 g/L) was higher than that of cane molasses (1.94 g/L) and cane

juice (1.0 g/L). Further clarification of media is necessary for production level levan yield. *Halomonas* sp. was grown in sugar beet and starch molasses pre-treated by different methods. The levan yield in sugar beet after the clarification and pre-treatment (1.31 g/g of dry cell weight) was greater than before (0.72 g/g of dry cell weight) (Kucukasik *et al.*, 2011). *Microbacterium laevaniformans* was used for levan production (10.4 g/L in 48 h) using date syrup as source of sucrose and the results suggest that increase in fermentation time caused a decrease in levan yield (Moosavi-Nasab *et al.*, 2010). Higher levan formation in cane molasses might be due to the presence of readily available fructose in the medium unlike cane juice. Levansucrase from *Halomonas* sp. effectively synthesized levan from sugar beet (4.19 g/L) but not from starch molasses (3.68 g/L) as source of sucrose and was attributed to readily available sucrose content in sugar beet, a limitation in starch molasses (Kucukasik *et al.*, 2011).
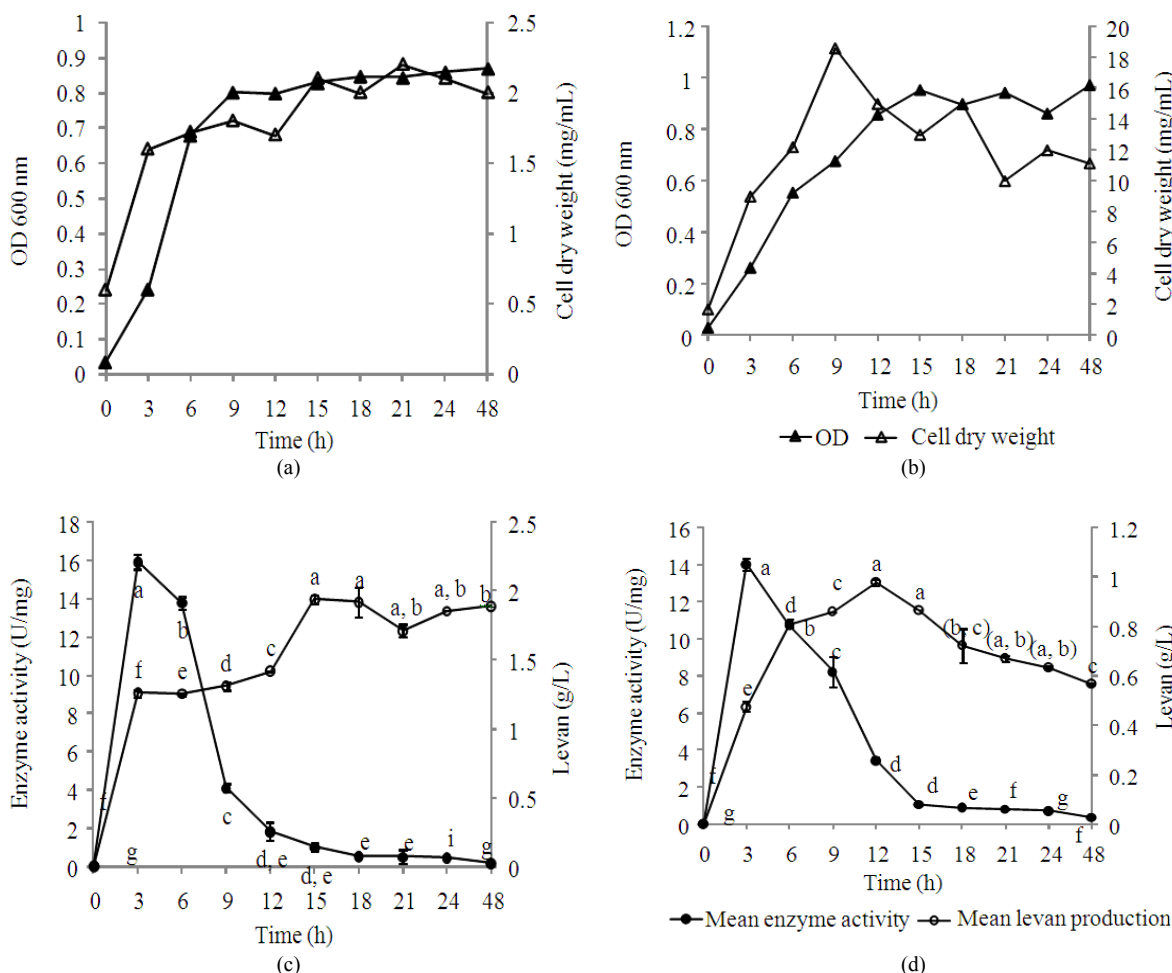

Fig. 11. Study of *E.coli* expressed FTF activity using fortified cane molasses (a, c) and cane juice (b, d). Determination of OD_{600nm} and cell dry weight (a, b); levansucarse activity and levan content (c, d). Points with superscripts a-g differ from each other significantly at $p<0.05$

Conclusion

The present study reports the functional characterization of a thermostable levansucrase from *B.subtilis* BB03 expressed in *E.coli*. The recombinant enzyme was stable over a wide pH range and showed considerable activity in cane molasses and juice. Several studies reported lower levan yield using crude low-cost sources as compared to sucrose (Han and Watson, 1992; Moosavi-Nasab *et al.*, 2010; Oliveira *et al.*, 2007) with wild type system, this study was an attempt to obtain levan production in a heterologous system using *sacB* gene with its own promoter without IPTG induction. Further studies are needed for analyzing different media formulations which may include better clarification strategies for cane molasses and juice, in order to obtain higher levan production. However, this study provides useful knowledge about the functional and molecular properties of a thermostable levansucrase from *Bacillus* expressed in a heterologous system and demonstrate the potential use of low cost sucrose sources like cane molasses and juice for levan yield.

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Author's Contributions

Dr. D. Theertha Prasad: Project was conceived, planned and co-ordinated.

Ms Vinithi Vaidya: The experiments were executed.

Dr. GR Prabhu: Bioinformatics analysis.

Ethics

The authors declare that there is no conflict of interests regarding the publication of this paper.

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