ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

ISOLATION AND CHARACTERIZATION OF MANNANOLYTIC BACTERIA FOR THE PRODUCTION OF PREBIOTIC MANNOOLIGOSACCHARIDES FROM COPRA MEAL

Monika Rana[1], Aditya Kumar[1], Deepak Kumar[1], Koushik Mazumder[2], Naveen Gupta*[1]

[1]*Department of Microbiology, Panjab University, Chandigarh, India,*

[2]*Food & Nutritional Biotechnology, National Agri-Food Biotechnology Institute, Mohali, Punjab, India,*

**Corresponding author: ng.puchd@gmail.com*

ABSTRACT

Present study was aimed to isolate a bacterium producing mannanase using copra meal as substrate for the production of mannooligosaccharides (MOS) with effective prebiotic characteristics. In this regard, isolation and screening of the bacterial isolates was done on the basis of mannanase yield, ability to hydrolyze copra meal, and growth promoting effect of MOS in the hydrolysate on the gut bacteria. Isolate MAN-7 was selected and identified as *Bacillus subtilis* MAN7 using 16Sr RNA sequencing. Other than the mannanase, bacterium was also found to produce an array of hydrolytic enzymes including lipase, pectinase, laccase, amylase, cellulase, xylanase, lignin peroxidase and protease; increasing horizons for its enzymes applicability in various biocatalysts based industrial processes. Mannanase produced from *Bacillus subtilis* MAN7 was used for the production of MOS from copra meal. MOS of varying degree of polymerization (DP) produced were detected using thin layer chromatography (TLC). MOS produced from copra represents an opportunity for the development of a cheaper prebiotics in coming future.

Keywords: Mannanase, Oligosaccharides, Mannooliosaccharides, Copra meal, Prebiotic, Agro-waste, Hydrolytic enzymes, Mannanolytic

1. INTRODUCTION

Prebiotics are "Selectively fermented ingredients which allow specific changes, both in the composition and activity of the gastrointestinal microflora that confers benefits of host well being and health" [1]. Non-digestible oligosaccharides (NDO) is a well known class of prebiotic including fructooligosaccharides [2], maltooligosaccharides, isomaltooligosaccharides [3], galactooligosaccharides [4], xylooligosaccharides [5], chitin-oligosaccharides [6], mannooligosaccharides (MOS) [7]. MOS is one of the important class of NDO known to have beneficial effects on the growth of intestinal bacteria especially *Lactobacilli* and *Bifidobacteria* and limiting the growth of enteric pathogens [8]. However, for their commercial viability, MOS with effective prebiotic characteristics and cost effective production is required. Use of agro-waste for their production is one of the viable alternatives. Copra meal, an agro-waste widely available in India, is the left over residues obtained after the extraction of oil from the well dried kernel of coconut fruit (*Cocos nucifera*). Copra meal contains galactomannan, hence is a suitable substrate for MOS production in an economical manner.

Depolymerising enzyme involved in the MOS production is β-mannanases which randomly catalyze the cleavage of β-1, 4-D mannosidic linkages present in β-mannan and heteromannan [9] and known to be produced by numerous bacteria, fungi, actinomycetes, plants, and animal species [10]. Quantity and spectrum of MOS i.e. their degree of polymerization (DP) depend on the mannan source, microbial producer of mannanase, and the hydrolysis conditions employed [11].

In this regard, a bacterial strain producing high yields of mannanase and MOS having effective prebiotic characteristics was isolated. Selected isolate was characterized and MOS production was done from cheaper agro-waste *i.e.* copra meal using bacterial mannanase.

2. MATERIAL AND METHODS

2.1. Chemicals and microorganisms

Locust bean gum (LBG), 3, 5-dinitrosalicylic acid (DNSA), guaiacol, casein etc. were purchased from Sigma Aldrich, USA. Carboxymethyl cellulose (CMC), starch, yeast extract, De Man, Rogosa and Sharpe (MRS) agar, luria broth agar etc. were purchased from Hi-

media, India. TLC aluminium sheets silica gel 60 F_{254} were purchased from Merck, US. 1, 4-β-D-mannooligosaccharides standards *viz.* mannobiose (M2), mannotriose (M3), mannotetraose (M4), mannopentaose (M5), mannohexaose (M6) were purchased from Megazyme, Ireland. All other chemicals were of analytical grade.

Lactobacillus casei (MTCC No. 1423) and *Escherichia coli* (MTCC 119) were procured from Microbial Type Culture Collection (MTCC), Chandigarh.

2.1.1. Copra meal

Copra meal was procured from Calicut, Kerala, India and it was pre-treated to remove fat, protein and free sugars etc. Treated copra meal was designated as CM_T.

2.2. Isolation and screening of extracellular mannanase producing bacteria

Soil and coconut waste samples were collected from the area near coconut oil extraction mill and landfill sites. Enrichment of the samples was done in minimal medium (MM) containing $FeSO_4 \cdot 7H_2O$ (0.002%), $CaCl_2 \cdot 2H_2O$ (0.005%), $MgSO_4 \cdot 7H_2O$ (0.002%), KH_2PO_4 (0.23%), K_2HPO_4 (0.7%) supplemented with 0.5% (w/v) LBG (pH 8.0) at 37ºC, 120-150rpm. Enriched samples were spread plated on to the MM agar plates. Isolates of different colony morphology were selected and replica plating was done on MM plates containing LBG. Mannanase positive isolates were indicated by zone of clearance around colony after staining the plates with congo red [12].

2.2.1. Mannanase production and assay

Isolates showing good mannanase production on plate were inoculated into liquid minimal medium containing 1.0% (w/v) CM_T. Culture supernatants were obtained after 24h and mannanase activity was determined using using 0.5% (w/v) LBG as substrate (pH 8.0) at 60˚C [11]. Mannanase activity was expressed in International Unit per ml ($IUml^{-1}$). One IU was defined as the amount of mannanase enzyme releasing 1µmol of mannose equivalent in one minute under standard assay conditions.

2.3. Secondary screening and selection of the isolate

Secondary screening of the isolates was done as described by Titapoka *et al.*, [13] with some modifications. Cultural supernatant of the shortlisted bacterial isolates were referred as CM-hydrolysate. MRS and M9 media were prepared for the growth of *Lactobacillus casei* and *Escherichia coli* respectively. Primary cultures of both bacteria were prepared (absorbance value of 0.5 at 600nm) and used as 1% (v/v) to inoculate two set of media *i.e.* one containing 5% (v/v) CM-hydrolysate and other without it for their growth in the MRS and M9 media lacking carbon source followed by their incubation at 37˚C for 4h. Appropriate dilutions of cultures of *L. casei* and *E. coli* were spread plated on MRS and LB agar respectively. Effect on growth of the organisms (log $CFUml^{-1}$) was determined by calculating the difference in the cell count obtained from media with and without hydrolysate [13].

2.4. Identification of the selected isolate

The cellular morphology of the bacterial isolate was examined by Gram staining. Physiological and biochemical characterization was done according to Bergey's Manual of Determinative Bacteriology [14]. For Scanning Electron Microscope (SEM) analysis, gold coating of bacterial cells suspension was done using JEOL ion sputter (JFC-1100) followed by examination under SEM (Model JSM6100, JEOL). Further, identification of the bacterium was carried out using 16S rRNA sequencing [15]. Multiple sequence alignment was done and phylogenetic tree was constructed by Maximum Likelihood method using Molecular Evolutionary Genetics Analysis software version 10 (MEGA X) [16]. The evolutionary history was concluded using Maximum Likelihood method and Tamura-Nei model [17].

2.4.1. Enzyme profile of the selected isolate

Selected bacterial isolate was grown on MM containing 1.0% (w/v) of birch wood xylan, CMC, starch, pectin, casein, tributyrin agar plates for the evaluation of enzymes viz. xylanase, cellulase, amylase, pectinase, protease, lipase respectively. Screening for laccase was done by streaking the selected strain on M162 plates containing 2mM guaiacol [15]. For lignin peroxidase (LiP), MM agar plates with 0.0125% (w/v) methylene blue dye was used. All the inoculated plates were incubated at 37˚C for 72h. For the presence of xylanase, cellulase, amylase and pectinase; staining was done by Gram iodine solution. For LiP production, de-colorization zone against the colonies was checked.

2.5. Mannooligosaccharides production from copra meal

Hydrolysis of copra meal (CM_T) by mannanase from *Bacillus subtilis* MAN7 was carried out by using 2% (w/v)

of CM_T, mannanase dose 20.0IUml^{-1} at pH 8.0, 60˚C for 2h. Enzymatic reaction was stopped by boiling it for 10min. Unhydrolyzed material was removed and MOS were extracted from the supernatant by adding two volumes of chilled ethanol (-20˚C for 2h). MOS generated were detected by thin layer chromatography (TLC). MOS were applied on TLC plate as spots. Mobile phase contain Butanol: glacial acetic acid: Water (2:1:1) and MOS were detected by spraying 0.3% (w/v) N-(1-Naphthyl)ethylenediamine dihydrochloride solution prepared in ethanol: sulphuric acid (10:1) followed by heating the plate at 100˚C for 5-10 min.

3. RESULTS AND DISCUSSION

3.1. Isolation and screening of extracellular mannanase producing bacteria

Soil and coconut waste sample from coconut oil extraction mill were enriched and plated on minimal media (MM) agar plate. Forty isolates showing different colony morphology were obtained and were replicated on MM containing locust beam gum (LBG). Out of forty, eleven isolates showed the significant mannanolytic activity, indicated by zone of clearance after congo red staining. These isolates were tested for their ability to hydrolyze pre-treated copra meal (CM_T) and production of mannanase. Four isolates *viz*. MAN-1, MAN-7, MAN-18 and MAN-24 with the best mannanase yield were shortlisted for secondary screening (Fig. 1).

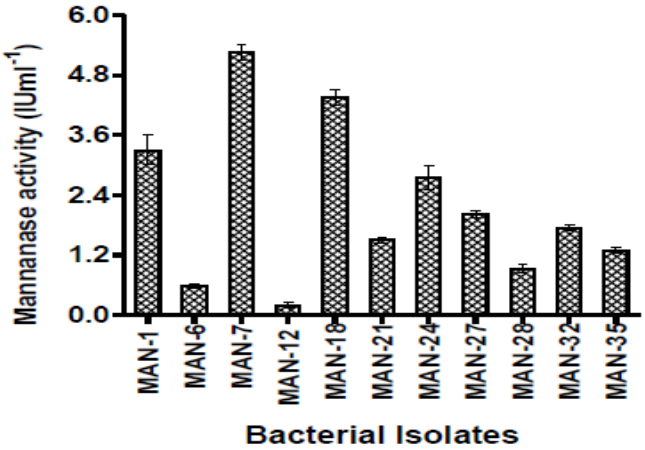


Fig. 1: Screening for extracellular mannanase production in minimal media containing copra meal

3.2. Secondary screening and selection of the isolate

To classify any molecule as prebiotic, one of the important criteria is its ability to enhance the growth of beneficial organisms and to suppress the growth of pathogenic organisms of the gut [1]. Prebiotic attributes of mannooligosaccharides (MOS) varies with their quantity and spectrum in terms of their degree of polymerization (DP). MOS spectrum is dependent on mannan source, hydrolysis conditions and most importantly enzyme source [11]. There are very limited studies where screening and selection of the mannanolytic organisms for MOS production have been done on the basis of their ability to produce oligosaccharides with effective prebiotic characteristics [13].

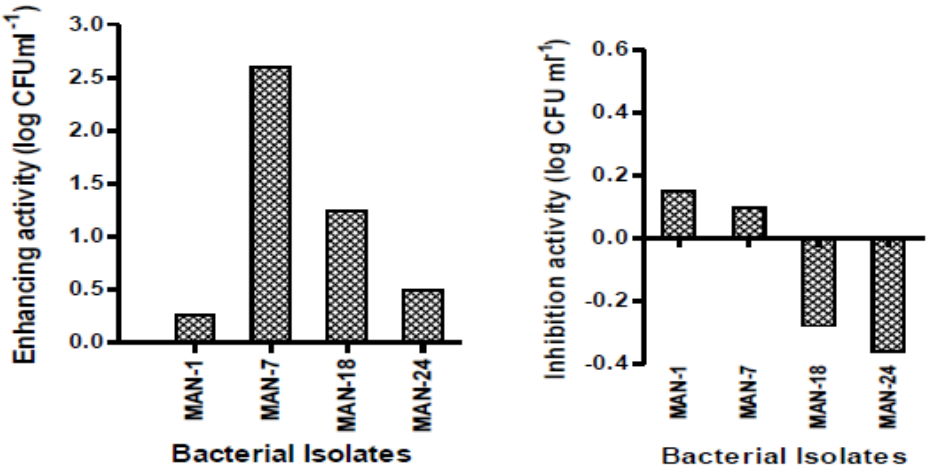


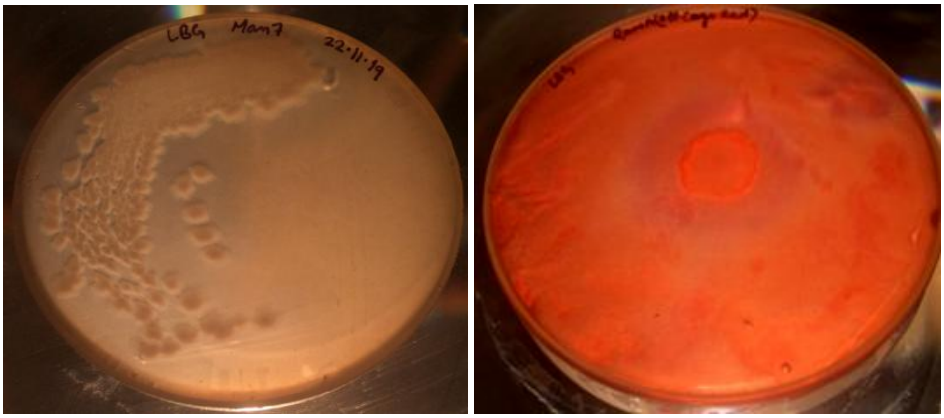
Fig. 2: Effect of copra hydrolysate containing mannooligosaccharides on the growth of gut organisms:***A- Lactobacillus casei B- Escherichia coli***

Therefore, secondary screening of the shortlisted isolates was done by evaluating the prebiotic characteristics of their culture supernatant *i.e.* copra hydrolysate obtained after the growth of isolates in the media supplemented with CM_T.

Copra hydrolysates from all the isolate supported the growth of *Lactobacillus casei*; highest growth enhancing activity was observed with Isolate MAN-7 (Fig. 2). When effect of hydrolysates on the growth of *Escherichia coli* was monitored, MAN-18 and MAN-24 promoted its growth, whereas no significant growth promotion was observed with Isolate MAN-1 and MAN-7 (Fig.2). From results it was concluded that MOS present in the hydrolysate of Isolate MAN-7 promoted the growth of beneficial bacteria and didn't support or marginally inhibited the growth of pathogenic bacteria. Similar results have previously been reported [13, 20]. Therefore, Isolate MAN-7 with the highest mannanase yield with copra meal and hydrolysate showing significant prebiotic characteristics was selected for further studies.

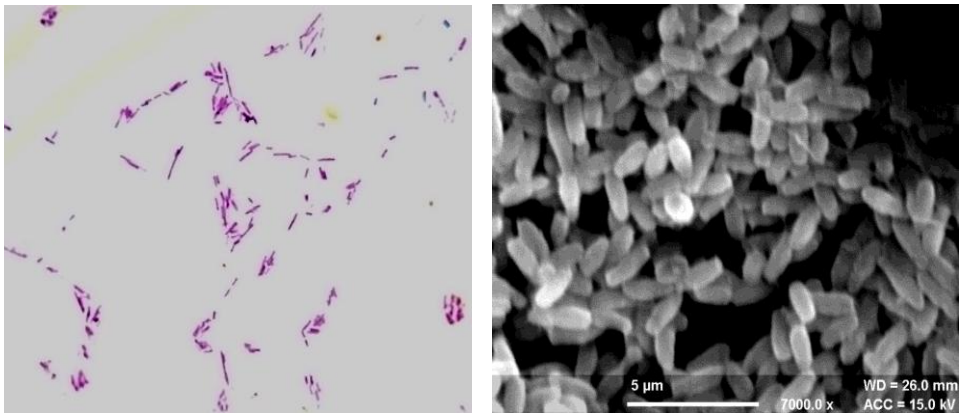
3.3. Identification of the selected isolate

Colonies of Isolate MAN-7 were off-white, irregular, rough, non- sticky with undulate edges; zone of hydrolysis around the 72h old bacterial growth indicated the production of mannanase (Fig. 3). Light microscopic and scanning electron micrographs of the bacterial cells showed Gram positive, non-capsulated, short rods (Fig. 4). Bacteria were found to be motile in logarithmic phase; growth in wide temperature and pH range and was able to grow in high NaCl concentration (~16% (w/v) (Table 1).



A B

Fig. 3: Cultural characteristics of Isolate MAN7 on MM+0.5%LBG agar plate: A- Colony morphology B- Zone of mannanolytic activity after Congo-red staining



A B

Fig. 4: Micrographs of Isolate MAN-7: A-Light microscopic micrograph at 100X after Gram staining, B- Scanning electron micrograph at 7000X

Table 1: Morphological, cultural, physiological, and biochemical characteristics of Isolate MAN-7

Test	Result
Morphological Characteristics	
Gram staining	Gram-positive
Appearance	Short rods
Endospores	+
Arrangement	Placed singly
Capsule formation	Non-capsulated
Cultural Characteristics	
Colony color	Off-white
Colony shape and texture	Irregular and rough
Colony edges	Undulate edges
Stickiness	Non-sticky
Pigment formation	None
Motility	Motile
Physiological Characteristics	
Optimum pH for growth	7.0
Optimum temperature for growth	37°C
NaCl Tolerance	up to 16.0%
Biochemical Characteristics	
Catalase	+
Oxidase	-
D-Glucose	+/ Gas
Nitrate reduction	+
Triple Sugar Iron (TSI)	K/A
H_2S production	-
Sucrose	-
Mannose	+
Mannitol	+
Xylose	+
Indole production	-
Methyl Red (MR)	-
Voges–Proskauer (VP)	-
Citrate utilization	+
Urease	+
Phenyl pyruvic acid (PPA)	-
Lysine	-
Ornithine decarboxylase	-
Arginine dehydrolase	-

+ positive; - negative; K/A Alkaline slant/acidic butt (glucose fermentation only)

3.3.1. Phylogenetic analysis

16S rRNA sequence of the strain was submitted to GenBank database with Accession no MK559542 and aligned through BLASTn search. Phylogenetic tree of 16rRNA sequences of the Isolate MAN-7 and other closely matched species was constructed (Fig. 5). Phylogenetic analysis inferred that it is most closely related to *Bacillus subtilis*. Therefore, it was designated as *Bacillus subtilis* MAN7 and deposited in MTCC, Chandigarh, India with MTCC No. 12896.

Many bacteria including *Bacillus* spp. have been reported to produce mannanase(s) [18]. Mannanase(s) from different *Bacillus* spp. viz. *Bacillus* sp. CFR1601 [8], *Bacillus* sp. MR10 [19], *Bacillus circulans* NT 6.7 [20], *Bacillus pumilus* GBSW19 [21] have been shown to produce MOS using different mannan sources.

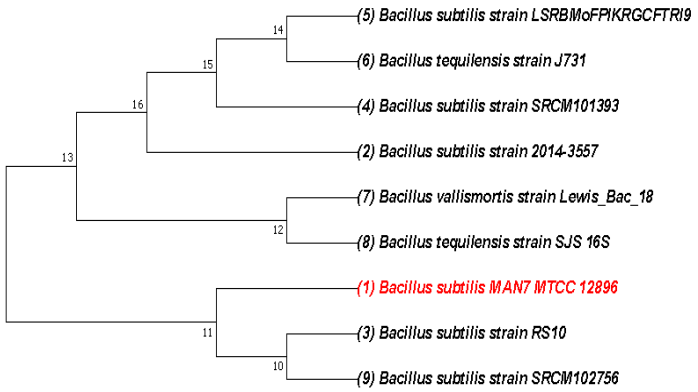


Fig. 5: Maximum Likelihood phylogenetic tree constructed using 16S rRNA sequence of *Bacillus subtilis* MAN7 and other species of related genera

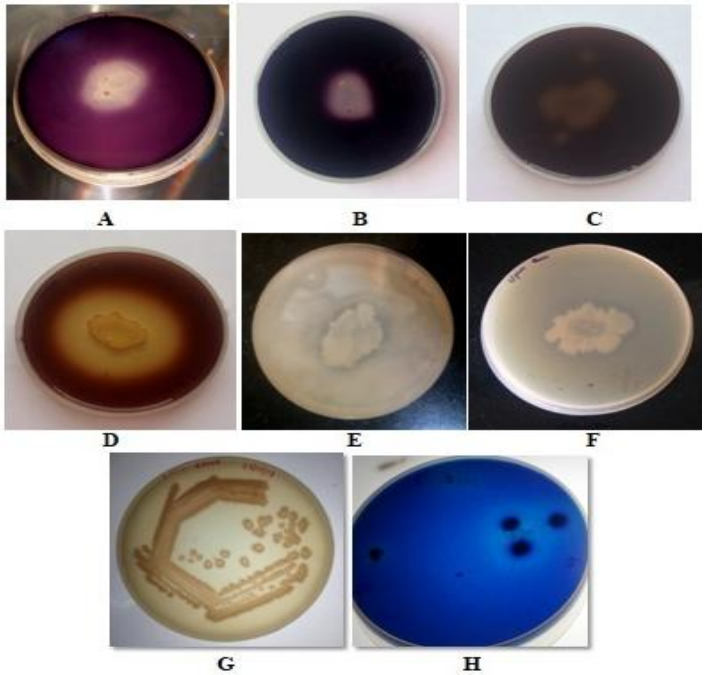


Fig. 6: Enzyme profile of *Bacillus subtilis* MAN7: A-Cellulase; B-Amylase; C-Xylanase; D-Pectinase; E-Protease; F-Lipase; G- Laccase; H- Lignin peroxidase

3.3.2. Enzyme profile of Bacillus subtilis MAN7

Other than mannanase, *Bacillus subtilis* MAN-7 also produced number of other industrially important enzymes like xylanase, cellulase, amylase, pectinase, lipase, laccase and lignin peroxidase indicated by the zone of clearance on growth medium supplemented with substrates for respective enzymes (Fig. 6).

These enzymes can be exploited individually or their concoctions in various industrial processes such as dehairing of leather [22], pulp bio-bleaching [23], detergents [24], biofuels [24], cosmetics formulation [15] etc. to make them more eco-friendly. Because of the production of wide array of these biocatalysts, *Bacillus subtilis* MAN7 can be exploited in numerous bioprocesses and can be further explored for its utility in various industrial sectors.

3.4. Mannooligosaccharides production from copra meal

MOS generated by the hydrolytic action of bacterial mannanase on CM_T were detected by TLC. Formation of MOS of varying degree of polymerization i.e. M2-M6 was observed whereas no monomeric sugar was detected on TLC plate (Fig. 7).

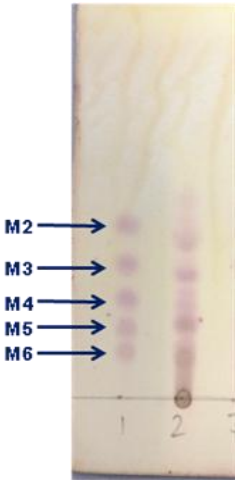


Fig.7: Thin layer chromatography analysis: (1) Mannooligosaccharides standards (2) Mannooligosaccharides from copra meal

Production of MOS with varying DP have been reported in some studies. M3-M6 have been reported by Pangsri et al., 2015 [20], M1-M4 by Thongsook and Chaijamrus, 2018 [25]. There are very few reports which have shown MOS production with wide degree of polymerization. Formation of MOS with wide DP using mannanase from *Bacillus subtilis* MAN7 without the production of monomeric sugar make this enzyme a good candidate for the production of MOS.

4. CONCLUSION

Bacillus subtilis MAN7 was isolated from the waste of coconut oil extraction mill. Isolate produced high yields of mannanase and copra hydrolysate obtained from the isolate showed good prebiotic characteristic. Mannanase from *Bacillus subtilis* MAN7 was used for the production of mannooligosaccharides (MOS) from agricultural waste. MOS of varying degree of polymerization were produced without the formation of monomeric sugar. Further, optimization studies for the hyperproduction of MOS from copra meal and their characterization is being carried out in order to produce cheaper prebiotics to understand their chemistry as well as to explore their bioactive properties.

5. ACKNOWLEDGEMENTS

Authors are thankful to UGC-SAP (F3-4-5/2016 DRS-I), New Delhi for providing financial support. Authors are thankful to Sophisticated Analytical Instrumentation Facility (SAIF), Panjab University, Chandigarh for providing SEM facility.

6. REFERENCES

1. Gibson GR, Hutkins R, Sanders ME, Prescott SL, et al. *Nat Rev Gastroenterol Hepatol*, 2017; **14**:491-502.
2. Sangeetha PT, Ramesha MN, Prapulla SG. *Trends Food Sci Technol*, 2005; **16(10):**442-457.
3. Kothari D, Patel S, Goyal A. *J Food Sci*, 2015; **79(8).**
4. Gosling A, Steven GW, Barber AR, Kentish SE, Gras SL. *Food Chem,* 2010; **121(2):**307-318.
5. Brienzo M, Carvalho W, Adriane MF, Milagres. *Appl Biochem Biotechnol*, 2010; **162**:1195-1205.
6. Kumar A, Kumar D, George N, Sharma P, Gupta N. *Int J Biol Macromol,* 2018; **109**:263-272.
7. Ghosh A, Verma AK, Tingirikari JR, Shukla R, Goya A. *Mol Biotechnol,* 2014; **57(2):**111-127.
8. Srivastava PK, Panwar D, Prashanth KVH, Kapoor M. *J Agr Food Chem*, 2017; **65(13):**2827-2838.
9. Intaratrakula K, Nitisinpraserta S, Nguyen TH, Haltrichc D, Keawsomponga S. *Protein Expres Purif,* 2017; **139**:29-35.
10. Chauhan PS, Puri N, Sharma P, Gupta N. *Appl Microbiol Biotechnol*, 2012; **93(5):**1817-1830.
11. Mikkelson A, Maaheimo H, Hakala TK. *Carbohydr Res*, 2013; **372**:60-68.
12. Chauhan PS, Soni SK, Sharma P. *Int J Pharma Bio Sci,*

2014; **5(1):**237-251.
13. Titapoka S, Keawsompong S, Haltrich D, Nitisinprasert S. *World J Microb Biot,* 2008; **24(8):**1425-1433.
14. Holt JG, Krieg NR, Sneathm PHA, Staley JT, Williams ST. *Bergey's manual of determinative bacteriology*. 9th ed. Williams and Williams, Baltimore; 1994.
15. Kumar D, Kumar A, Sondhi S, Sharma P, Gupta N. *3 Biotech*, 2018; **8(3):**1-10.
16. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. *Mol Biol Evol*, 2018; **35(6):**1547-1549.
17. Tamura K, Nei M. *Mol Biol Evol*, 1993; **10(3):** 512-526.
18. Chauhan PS, George N, Sondhi S. *Int J Pharma Bio Sci,* 2014; **5**(1): 176-192.
19. Chaikaew S, Kanpiengjai A, Intatep J, Unban K, Wongputtisin P, Takata G, Khanongnuch C. *Prep Biochem Biotech,* 2017; **47(4):**424-433..
20. Pangsri P, Piwpankaew Y, Ingkakul A, Nitisinprasert S, Keawsompong S. *Springerplus*, 2015; **4**:771.
21. Zang H, Xie S, Wu H, Wang W, Shao X, Wu L, et al. *Enzyme Microb Tech*, 2015; **78:**1-9.
22. George N, Sondhi S, Soni SK, Gupta N. *Indian J Microbiol*, 2014; **54(2):**139-142.
23. Fantahun W, Virk AP, Naveen G, Prince S. *J Microbiol Biotechn,* 2013; **3(4):**32-41.
24. Chapman J, Ismail AE, Dinu CZ. *Catalysts*, 2018; 8(238):1-26.
25. Thongsook T, Chaijamrus S. *J Food Sci Technol*, 2018; **55(9):**3721-3730.