JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP
Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises
(Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/43/4/MRN-3045

Production of multi enzyme preparation by *Bacillus subtilis* using mosambi peel as substrate

B. Singh[1,2], N. Garg[2]*, P. Mathur[1], S. Vaish[2] and S. Kumar[2]

[1]Amity Institute of Biotechnology, Amity University Uttar Pradesh, Lucknow-226 028, India
[2]Division of Post Harvest Management, ICAR-CISH, Lucknow-226 101, India

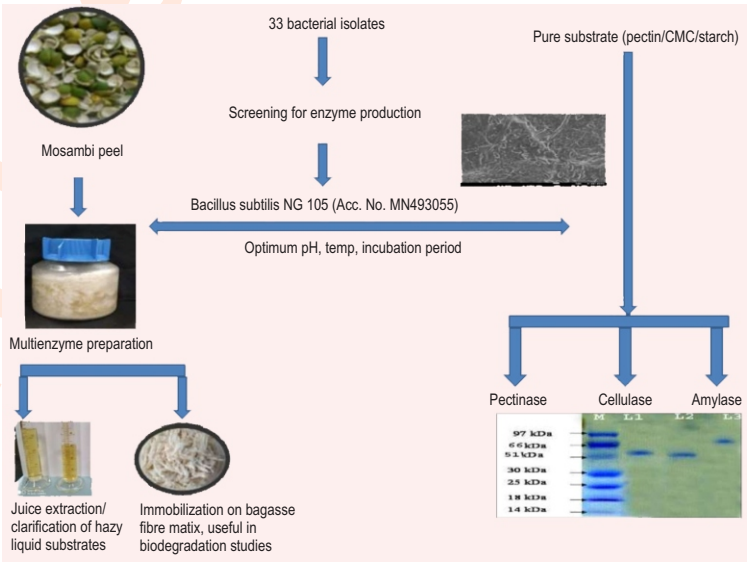
***Corresponding Author Email :** neelimagargg@gmail.com

Received: 14.09.2021 *Revised*: 03.01.2022 *Accepted*: 31.03.2022

Abstract

Aim: To determine feasibility of using mosambi peel as substrate for multi enzyme production through microbial intervention.

Methodology: Thirty-three bacterial isolates were isolated from biodegradable organic substrates and investigated *in-vitro* their biodegradable activities viz. pectinolytic, cellulolytic and amylolytic. The best performing bacterial isolate, exhibiting aforesaid activities were selected and identified using 16S rRNA technique. The factors, that influenced the fermentation, viz. temperature, pH and incubation period of bacterial culture were optimized for maximum production of multi enzymes viz. pectinase, cellulase and amylase using mosambi peel as substrate under solid-state fermentation conditions. Molecular weights of different enzymes present in multi-enzyme preparation were determined by SDS PAGE. The juice extraction efficiency of crude multi enzyme preparation was compared with that of pure commercial enzyme.



Multienzyme preparation from mosambi peel is effective in juice extraction/clarification and biodegrading.

Results: Out of thirty-three bacterial isolates, after primary and secondary screening, the best performing bacterial isolate was identified as *Bacillus subtilis* strain NG 105 (Genbank accession number MN493055). Higher enzyme activities were observed at pH 7.0, incubation temperature of 35°C and incubation period of 10 days for pectinase, cellulase and amylase using mosambi peel as substrate. The purified enzymes characterized by SDS-PAGE, revealed to have molecular mass of 65 kDa for pectinase, 50 kDa for cellulase and 55 kDa for amylase. Bagasse fibre was found to be the most suited matrix for immobilization. The juice extraction efficiency of partially purified crude multienzyme preparation was 88% of commercial pectinase.

Interpretation: Mosambi peel is a suitable substrate for multienzyme production using *Bacillus subtilis* under solid-state fermentation condition.

Key words: Amylase, *Bacillus subtilis*, Cellulase, Immobilization, Mosambi peel, Pectinase

How to cite : Singh, B., N. Garg, P. Mathur, S. Vaish and S. Kumar: Production of multi enzyme preparation by *Bacillus subtilis* using mosambi peel as substrate. *J. Environ. Biol.*, **43**, 612-621 (2022).

Introduction

India ranks second in fruits and vegetable production in the world, after China. During 2018-2019, India produced 98.56 million metric tonnes of fruits. The processing of fruits results in the generation of big quantum of waste in the form of peel, pomace, stones and seeds. Such wastes are generally rich in bio-constituents such as fibre, pectin, cellulose, starch, phenolics, pigments and other useful materials. Enzymes are one of the commercially important commodities, used in different types of industries, like food, detergent, textile and paper industries. Pectinases are widely used in textile industry in retting and degumming of plant bast fibres, scouring of cotton, extraction and clarification of fruit juices and coffee (Anand *et al.,* 2020).

Cellulase has novel application in the production of fermentable sugars and ethanol, detergents and other chemicals (Jayasekara and Ratnayake, 2019). Similarly, Amylase is widely used for biotechnology processes including starch degradation, pharmaceutical applications, food liquefaction and saccharification, clinical, medical, analytical chemistry, paper manufacturing, baking and bread industry (Ahmad *et al.,* 2019). Enzyme can be obtained from plants as well as microorganisms. Enzymes of microbial origin are preferred over plant origin due to fast, better controlled and economical production. However, the production cost of enzymes are high because of high-value of raw materials. This cost, however, can be lowered using low-value substrate industrial waste. There are reports of fermentative production of enzymes using fruit peels of mango pomegranate, apple, mosambi, banana, orange, etc. Kumar *et al.* (2014) used *Aspergillus niger* for production of amylase using mango peel as substrate. Shariq and Suhail (2019) applied yeast consortium for production of enzymes using mosambi peel as substrate.

Recently, Garg *et al.* (2021) reported *Lactobacillus plantarum* for enzyme production using fruit and vegetable waste as substrate. Citrus is one of important fruit crops of India with a production of 13.4 million metric tonnes during 2018-19. Its processing for juice generates peel (20-25%) as waste. This peel is a rich source of carbohydrates, flavonoids and oxidants. In general, pectinases, cellulases and amylases are involved in the degradation of pectin, cellulose and starch rich substrates (fruit peels) where bacteria play a crucial role in producing these enzymes (Ahlawat *et al.,* 2009; Rehman *et al.,* 2019; Dai *et al.,* 2020). Citrus peel is reported to be one of the potential substrates for production of pectinase solely. Till date there are reports available on production of single enzyme but not on bacteria mediated production of multienzyme preparation, such as pectinases, cellulases, and amylases using mosambi peel as a substrate by a single bacteria. This multienzyme preparation will be useful for juice clarification/extraction from substrates rich in pectin, fibre and starch and use of individual enzymes can be avoided. The present study reports the feasibility of using mosambi peel as substrate for multienzyme preparation including pectinase, cellulase and amylase by *Bacillus subtilis*.

Materials and Methods

All experiments were performed at Microbiology laboratory, Division of Post Harvest Management, ICAR-Central Institute for Subtropical Horticulture, Lucknow, India during the period 2018-2021. The chemicals and media were purchased from Merck, Sigma and Himedia. The carbohydrate utilization broth consisting $(NH_4)_2SO_4$ (0.2%), K_2HPO_4 (0.4%) Na_2HPO_4 (0.6%), $FeSO_4$ (0.1%), $MgSO_4$ (2%), $CaCl_2$ (0.1%), $MnSO_4$ (0.001%), $ZnSO_4$ (0.007%), $CuSO_4$ (0.005%), H_3BO_3 (0.001%) and MoO_3 (0.001%) at pH 7.2±0.2 supplemented with pectin, carboxy methyl cellulose and starch (1% w/v) was used for isolation and sub-culturing of bacteria.

Isolation of bacterial culture: The samples of degrading organic substrates were enriched for enzyme-producing bacteria in carbohydrate utilization broth using pectin/CMC/starch as carbon source. To isolate microorganisms from the collected degradable organic substrate samples, serial dilution, pour plating, and streak plating isolation techniques were used. Subsequently, the isolates were subcultured into their respective selective growth media until pure cultures were isolated.

Primary screening of bacteria: Primary screening of bacterial isolates for pectinase, cellulase and amylase production was done on the basis of clear halo zone visualization on pectin/CMC/starch utilization agar plate, respectively, by following the protocol of Sazci *et al.* (1986); Mengistu and Pagadala (2017).

Secondary screening of bacteria: The substrate (pectin/ CMC/ starch) utilization broth was inoculated with primary screened bacterial isolates for pectinase, cellulase and amylase production at pH 7.0 and temperature 35°C for 7 days (Wood and Bhat, 1988). Culture filtrate was tested for pectinase, cellulase and amylase activities following the standard protocol of (AOAC, 2005) and Miller, (1972).

Standardization of condition for enzyme assay: Conditions viz. temperature, pH and substrate concentration with respect to enzyme assay were optimized following the protocol of AOAC (2005).

Temperature- 25, 35, 45 and 55°C

Buffer pH- 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5

Substrate concentration- Pectin/CMC/Starch: 0.25, 0.5, 1 and 2%.

After optimization, the finalized enzyme assay protocol was as follows:

Enzyme assay protocol: The enzyme reaction mixture contained 0.4 ml of substrate (pectin-0.5% / CMC-1.0% / starch-1.0% dissolved in 0.2 M of acetate buffer pH- 5.5), 0.1 ml of crude enzymes and 0.5 ml of distilled water. The tubes were incubated at 35°C for 1 hr in water bath. One ml of DNS reagent was added

to stop the enzyme reaction. Subsequently, the reaction tubes were placed in water bath at 100°C for 10 min. Standards D-galacturonic acid (for pectinase) and glucose (for amylase and cellulase) were taken in the range of 1mM to 10 mM. The optical density was recorded at 550 nm an a spectrophotometer (double beam UV-VIS). Enzyme activity was expressed as the amount of enzyme that liberated 1 µmol of reducing sugar per ml per min. Enzyme activity was calculated as per the following equation:

$$\text{Enzyme activity ml}^{-1}\text{minute}^{-1} = \frac{\text{Concentration of standard } \left(\frac{\mu mol}{ml}\right)}{\text{absorbance of standard at 550nm}} X \frac{\text{Dilution X enzyme absorbence}}{\text{Time of incubation}}$$

Scanning electron microscopic image of selected bacterial isolate was followed by Talib *et al.* (2019).

Molecular identification of selected bacteria: The maximum enzyme-producing bacterial isolate was subjected to molecular characterization on the basis of 16S rDNA sequencing technique. The bacterial genomic DNA was isolated by following the earlier described protocol (Chachaty and Saulnier, 2000). The DNA was isolated from the culture and evaluated on 1.2 % agarose gel. The extracted DNA was amplified with 16S rRNA specific primer (8F and 1492R) and a single discrete PCR amplicon band of 1500 bp was observed. The PCR amplicon was enzymatically purified and further subjected to Sanger sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 704F and 907R primers using BDT v3.1 cycle sequencing kit on ABI 3730xl genetic analyzer.

The consensus sequence of 1460 bp 16S rDNA was generated from forward and reverse sequence data using aligner software. The 16S rDNA sequence was used to carry out BLAST alignment search tool of the NCBI Genbank database. Based on maximum identity score first fifteen sequences were selected and aligned using the multiple alignment software program ClustalW. The distance matrix was generated using RDP database and the Phylogenetic tree was constructed using MEGA 10 (Kumar *et al.*, 2018).

Production optimization under SSF from mosambi peels using selected bacterial isolate: Fermentation conditions *viz.* temperature (25, 35 and 45°C) and pH (4.5, 5.5, 6.5 and 7.5) were optimized. Mosambi peels, collected from wholesale fruit market, Dubagga, Lucknow were properly washed with water, chopped and air dried. To 100 g of chopped piece added water (1:1, w/v) in 500 ml capacity Erlenmeyer flask, autoclaved and inoculated with 1 ml of 24 hr old actively growing culture of selected bacterial isolate (≈ 1.0×10^7 cells ml^{-1}) and incubated at tested temperature. Samples were withdrawn at different time intervals and the culture filtrate was used for enzyme assay. Samples were precipitated with acetone and analyzed for extracellular enzyme *viz.* pectinase, cellulase and amylase activities.

Selection of suitable natural substrate on enzyme immobilization: Fibre was extracted from bagasse, rice husk, paddy straw and wheat straw by boiling with water, treating with 1N-HCl followed by 1N-NaOH treatment. Thereafter, it was washed with water, oven-dried at 60°C and sieved (60-150 mesh) (Ranganna, 2001). The extracted fibre was autoclaved, and later, the enzyme was added and incubated for 3 hrs. Un-immobilized enzyme was washed with acetate buffer, while activity was tested in enzyme immobilized on fibre.

Enzyme purification and characterization: Pure substrates (1% pectin/ CMC/ starch in carbohydrate utilization broth) inoculated with *Bacillus subtilis* were incubated at 35°C for 5 days. The culture filtrate precipitated with acetone and the protein precipitate dissolved in acetate buffer was passed through gel filtration chromatography column (1.0 × 40 cm) packed with Sephadex G-100 matrix pre-equilibrated with 0.2 M acetate buffer (pH 5.5). Two ml fractions were collected in eppendorf tubes at the flow rate of 20 ml hr^{-1} and enzyme activities were observed in each fraction. In order to determine the molecular size of pectinase, cellulase and amylase produced in respective pure substrate, enzyme precipitate was subjected to electrophoresis in 10% native polyacrylamide gel and 12% denaturating sodium dodecyl sulfate polyacrylamide gel in discontinuous buffer as per described by Laemmli (1970). After electrophoresis, the protein bands were visualized by Coomassie brilliant blue R-250 staining. A comparative study of enzymatic mango juice extraction using crude enzyme precipitate consisting of multienzymes produced by *Bacillus subtilis* against a commercial pectinase recommended for juice clarification was conducted. The extraction conditions were 100 g mango pulp having TSS 10 °B, incubated at 35°C for 180 min with 0.5 ml of crude enzyme extract/ commercial pectinase. At the end of each reaction period, the content was filtered through Whatmann No. 1 filter paper and the quantity of juice obtained was noted.

Statistical analyses: All the experiments were carried out using completely randomized design in triplicate, repeated twice for reproducibility. The analysis of experimental data with Two-way analysis of variance (ANOVA) was conduct followed by Fisher's multiple comparison test at $p<0.05$. The least significant difference (LSD) test was used to determine significant difference among the samples (Gomez and Gomez, 1984).

Results and Discussion

In total, 33 bacterial isolates were obtained from biodegrading organic substrates. For identification purpose, the isolates were designated by prefix 'B' and followed by their isolate numbers. Fifteen isolates were selected on the basis of clear halo zone diameter (Table 1). The top five isolates showing higher enzyme production on pure substrate and mosambi peel after secondary screening are depicted in Fig. 1. The results indicated that maximum production of pectinase, cellulase and amylase was observed in bacterial isolate B-1. The enzyme production by B-1 was compared with the consortium containing all five selected isolates. The results indicated that the single bacterium, B-1 performed better (518.15, 445.44 and 259.13 U ml^{-1} min^{-1}) compared to consortium (220.36, 177.63 and 105.53 U ml^{-1} min^{-1}) in production of pectinase, cellulase and amylase, respectively. This

might be due to competition among the bacterial cultures tested.

The selected bacterial isolate was subjected to molecular identification. DNA was isolated from the culture and quality was evaluated on 1.2% agarose gel, a single band of high-molecular weight DNA was observed (Fig. 2A). The molecular identification through homology search and BLAST analysis revealed that the isolated bacterial strain named NG105 belonged to *Bacillus* genera. Homology Search and BLAST analysis of 16S rDNA of selected bacterial isolate showed 99.73% similarity with *Bacillus subtilis* strain KN10B (KY511697). The phylogenetic position of this strain with other related organisms are depicted in Fig 2B. The evolutionary history was inferred using the Maximum Likelihood method and General Time Reversible model with bootstrap replications of 1000 (Nei and Kumar, 2000). The tree with the highest log likelihood is shown. The percentage

Table 1: Top 15 bacterial isolate selected after primary bacterial screening

Bacterial isolate	Pectinase	Cellulase	Amylase
B-1	22	15	25
B-2	18	07	15
B-3	15	10	19
B-4	10	05	00
B-5	10	08	10
B-6	08	02	08
B-7	08	08	18
B-8	08	09	00
B-9	08	07	21
B-10	06	10	00
B-11	05	10	10
B-12	04	05	00
B-13	00	12	00
B-14	00	08	00
B-15	00	08	08

Table 2: Optimization of fermentation pH for enzyme production (U ml^{-1} min^{-1}) by *B. subtilis* using mosambi peel as substrate

pH	Pectinase	Cellulase	Amylase
4.0	179.8±1.58	96.1±1E-14	94.4±0.54
5.0	176.6±1.58	88.7±0.60	84.9±0.37
6.0	321.1±0.60	123.4±0.60	253.1±1.38
7.0	322.6±0.02	128.2±1.18	281.2±1.93
8.0	265.2±0.28	103.2±0.80	278.9±1.81

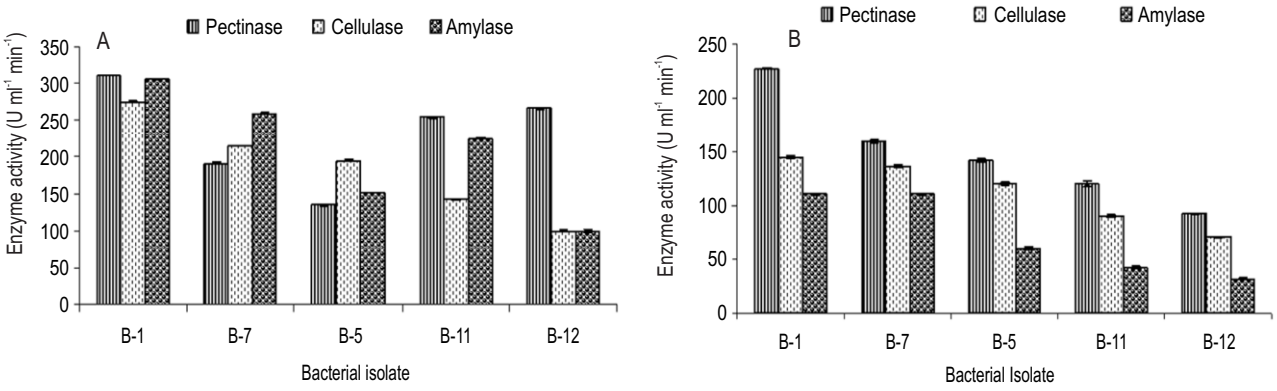


Fig.1: Secondary screening depicting top five bacterial isolates exhibiting higher enzyme production on pure (pectin, CMC and starch) substrate (a) and mosambi peel (b).

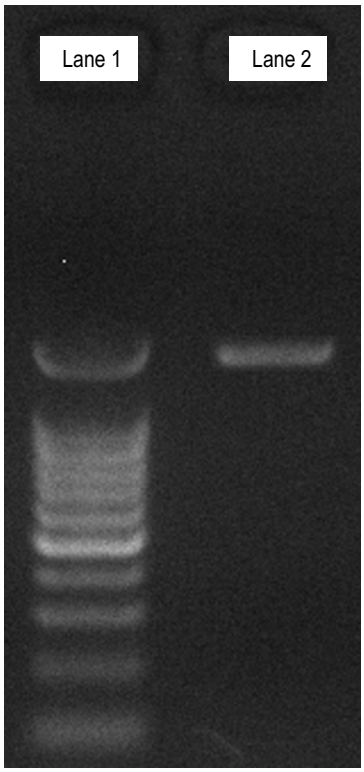


Fig. 2A: Agarose gel showing single 1500 bp of 16S rDNA amplicon. Lane 1: 100bp DNA ladder; Lane 2: 16S rDNA amplicon.

of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Further, 16S rRNA gene sequence of bacterial isolates was submitted to NCBI GenBank under the accession number Mn493055.

The scanning electron microscopic image of *Bacillus subtilis* isolate is shown in Fig. 3. Liu *et al.* (2020) reported that *Bacillus subtilis* is the key Gram-positive model bacterium widely used as a cell factory for microbial production of chemicals, enzymes, and antimicrobial materials for industry, agriculture and medicine. Sreena and Sebastian (2018) reported that optimization of physical parameters *viz.* temp., pH, type and concentration of substrate and medium components result in several fold increase in activity compared to non-optimized condition. Optimization studies indicated that higher production of pectinase, cellulase and amylase by *Bacillus subtilis* on mosambi peel substrate were observed after 10 days of fermentation (Fig. 4a). Further increase in the incubation period, reduced the enzymes production which might be due to depletion of nutrients in medium with the lapse in time, which stressed the microbial physiology resulting in inactivation of secreting machinery of enzymes. The incubation temperature greatly affects the microbial growth rate, enzyme secretion, enzyme inhibition, and protein denaturation (Singh and Mandal, 2012). An incubation temperature of 35°C was found to be optimum for enzyme production (Fig. 4b). The initial pH of fermentation medium plays a vital role in determining the level of metabolite synthesis. The maximum pectinase, cellulase and amylase production was observed at pH 7.0 (Table 2). The results indicate that near neutral to neutral pH is required for enzyme production, mean while the natural pH of mosambi peel is 4.5., therefore, adjustment of substrate pH is necessary.

The effect of variables such as incubation period, initial medium pH and incubation temperature on enzyme production varies with microorganism and substrate. Swain and Ray (2007) reported optimum incubation period, initial medium pH and temperature to be 6 days, 8.0, and 50°C respectively, for alpha-amylase production by *Bacillus subtilis* CM3 in solid-state fermentation using cassava fibrous residue. Ahlawat *et al.* (2009) reported an optimum incubation temperature of 37°C for pectinase production from *B. subtilis*, while Sreena and Sebastian (2018) reported 40°C at pH 7.0 as optimum for cellulase production. Raul *et al.* (2014) reported production and partial purification of alpha-amylase from *Bacillus subtilis* (MTCC 121) using solid-state fermentation. The maximum alpha-amylase production was found after an incubation period of 48 hr, temperature 40°C and pH 7.1. Dai *et al.* (2020) reported temperature of 50°C and pH 7.0 for maximum production of extracellular amylase and cellulase from *Bacillus subtilis* for potential application in tobacco fermentation.

Characterization of purified pectinase, cellulase and amylase by SDS-PAGE revealed three bands corresponding to 65, 50 and 55 kDa molecular size, respectively (Fig. 5). Ample diversity of alpha-amylases with respect to the molecular weights (10 to 210 kDa) has been reported (Mehta and Satyanarayana, 2016). Raul *et al.* (2014) reported molecular weight of α-amylase from *Bacillus* species ranges between 50 and 60kDa, while Rehman *et al.* (2019) detected Polygalacturonase from *Bacillus paralicheniformis* to have molecular weight of 110 kDa. Hatice *et al.* (2016) observed two protein bands belonging to pectinase of *B. subtilis* with molecular weights 60 and 64 kDa. The molecular weight of purified cellulase was determined as 80 kDa as revealed by SDS-PAGE and activity gel analysis (Gaur and Tiwari, 2015). Out of four natural fibre matrices, tested maximum immobilized enzyme yield (pectinase- 56.35%, cellulase 77.68% and amylase 59.54%) was observed on bagasse matrix on initial day (Fig. 6). The immobilized enzyme yield on bagasse fibre matrix, remained highest (pectinase- 43.12%, cellulase 74.5% and amylase 50.3%) after 5 months of storage at -20°C as compared to rice husk, wheat straw and paddy straw. Moreira *et al.* (2019) reported the bagasse treatments modify the morphological structures, influence the compositions of the minerals and the chemical groups of the fibers and enable the enzyme immobilization. The thermal stability of the immobilized enzymes is greater than that of solubilized enzymes, and it is possible to reuse the same immobilized structure for 15 cycles without a complete loss of activity.

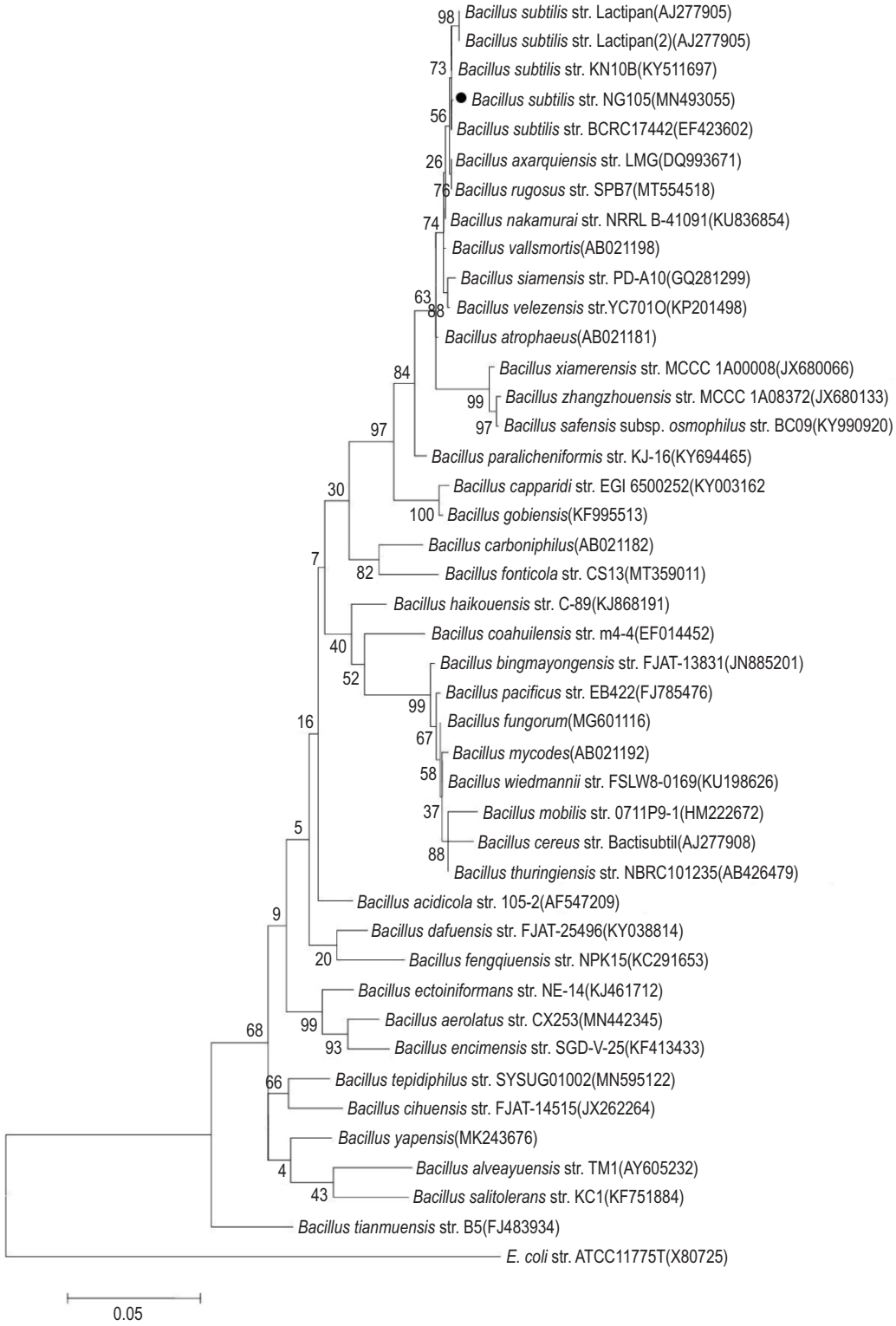


Fig. 2B: Phylogenetic tree constructed from the internal transcribe spacer 1 of 16S rDNA of strains NG105 and related organisms constructed using Maximum Likelihood algorithm from an alignment of 1370 nucleotides. Accession numbers of corresponding sequences are given in parentheses, and scale bar represents 1 base substitution per 50 nucleotide positions. The bootstrap probabilities calculated from 1,000 replications. *E. coli* Str. ATCC 11775T was taken as an out-group.

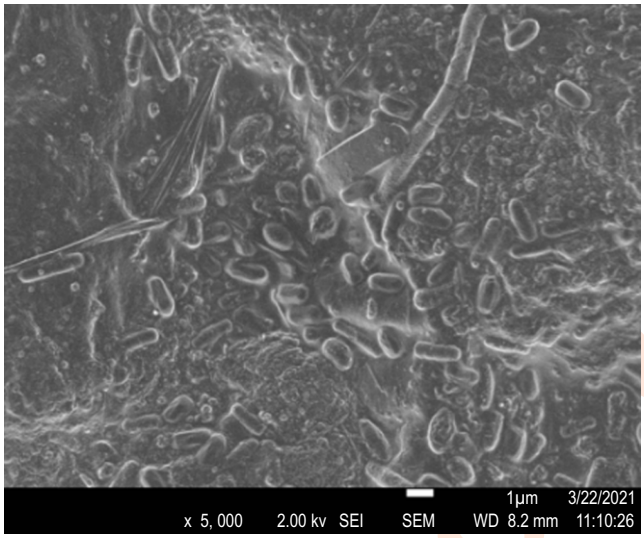


Fig. 3: SEM image of *Bacillus subtilis* 5000X.

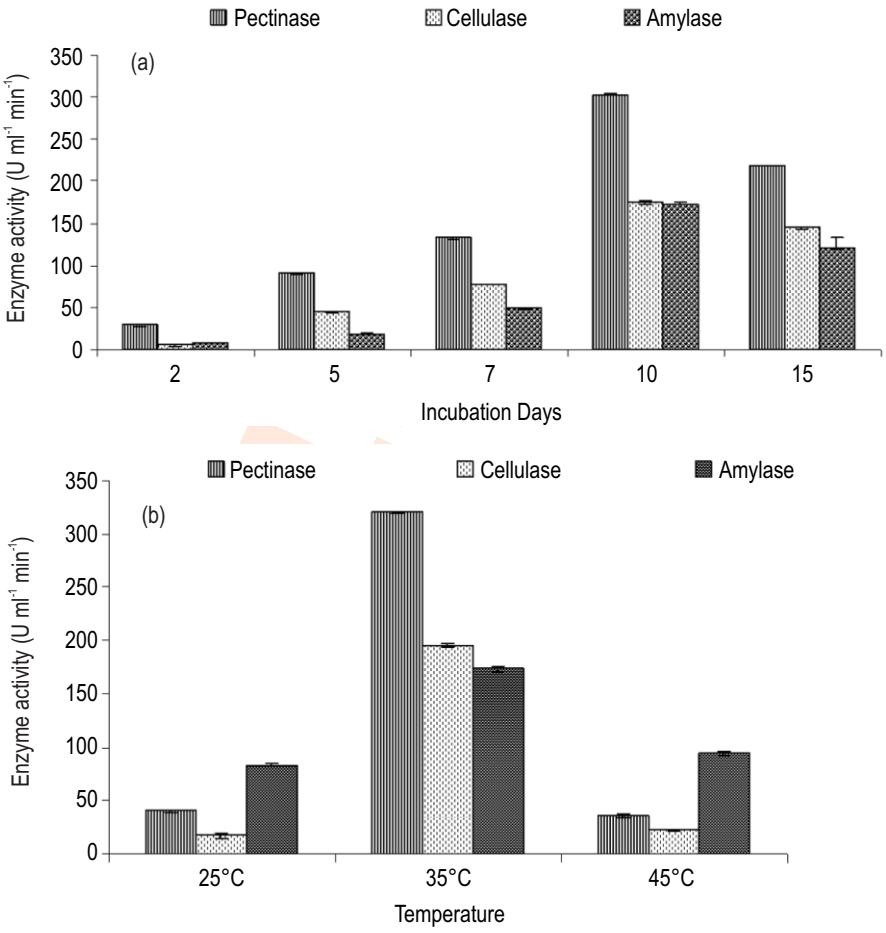


Fig. 4: Effect of (a) fermentation period and (b) incubation temperature on pectinase, cellulase and amylase using mosambi peel as substrate by *Bacillus subtilis*.

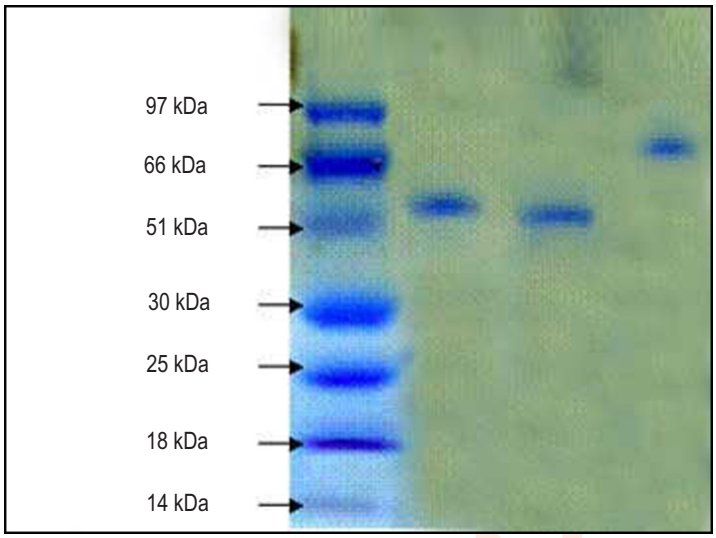


Fig. 5: SDS PAGE (12%) profile of protein marker and partially purified enzymes obtained by growing *Bacillus subtilis* on pure substrates viz. pectin, cellulose and starch. Abbreviation: Lane M- protein marker, L-1 amylase, L-2 cellulase, L-3 pectinase.

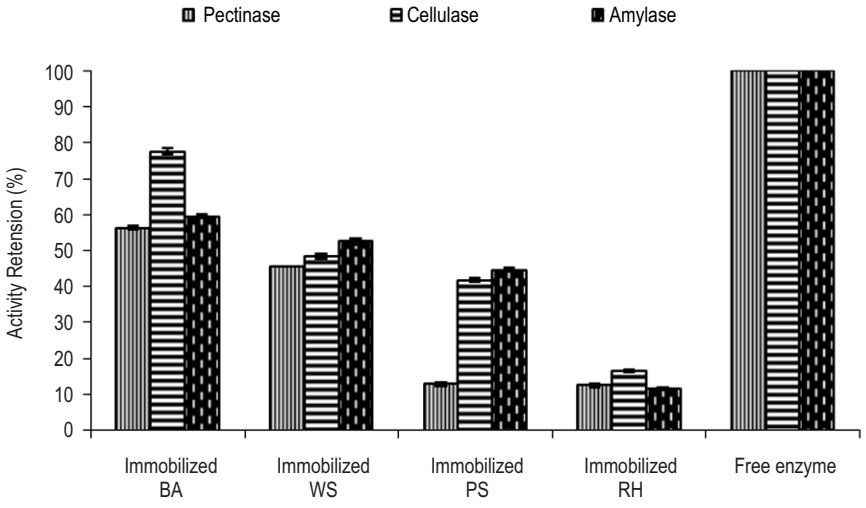


Fig. 6: Activity retention of pectinase, cellulase and amylase enzymes present in multiple enzyme preparation immobilized on different fibre matrices (BA- bagasse; WS- wheat straw; PS- paddy straw and RH-rice husk).

The turbidity and cloudiness of fruit juices are due to the presence of pectin, cellulose, hemicelluloses, lignin, starch, protein, tannin, phenols etc. The enzymatic process of fruit juice clarification is a widely accepted technology at commercial scale. A combination of the pectic group of enzymes with cellulase is used at a commercial level for juice clarification.

The cloudiness can be removed by enzymatic treatment. The addition of enzymes also results in improved yield for the juice. With the commercial pectinase enzyme, the highest juice yield obtained was 72%, while with the crude multienzyme extract from *Bacillus subtilis* was 64% whereas in control it was only 30%. The juice extraction efficiency of partially purified crude multienzyme preparation was 88% of commercial pectinase. Shrestha *et al.* (2021) reported 6% increase in apple juice recovery by use of enzyme produced by *Bacillus* sp. Rana *et al*. (2017) reported use of α-amylase produced by *Bacillus subtilis* isolated from mushroom compost, increased the clarity of apple and kiwi juice by 60% and 55% better colour, taste, flavor, and overall acceptability, respectively. Kothari *et al*. (2013), Pectinase

and amylase enzymes isolated from *Aspergillus awamori* grown on apple pomace, mosambi and orange peel as solid substrate clarified apple juice substantially.

The present study reports the feasibility of using mosambi peel as promising substrate for the production of multienzyme (pectinase, cellulase and amylase) by *Bacillus subtilis* isolate under solid-state fermentation. The enzyme can be immobilized in bagasse fibre matrix however, the activity of free enzyme was higher even after storage. The multienzyme preparation may find potential use in depolymerization of complex organic substrates and juice clarification.

Acknowledgment

The research was funded by AMAAS networking project of Indian Council of Agricultural Research, India. The authors are thankful to the Director, BSIP, Lucknow for providing scanning electron microscopy facility.

Add-on Information

Authors' contribution: N. Garg and P. Mathur: Conceived the research, designed experiments, guided during the research and manuscript preparation; **B. Singh, S. Vaish and S. Kumar:** Performed the experiments.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

References

Ahlawat, S., S.S. Dhiman, R. Battan, R.P. Mandhan and J. Sharma: Pectinase production by *Bacillus subtilis* and its potential application in bio preparation of cotton and micropoly fabric. *Process. Biochem.,* **44**, 521–526 (2009).

Ahmad, M.A., U. Isah, I.A. Raubilu and S.I. Muhammad: An overview of the enzyme: Amylase and its industrial potentials. *Bayero. J. Pure Appl. Sci.,* **12**, 352 - 358 (2019).

Anand, G., S. Yadav, R. Gupta and D. Yadav: Pectinases: From microbes to industries. *Microorga. Sustain. Environ. Hlth.,* **14**, 287-313 (2020).

AOAC: Official Method of Analysis. 18th Edn., AOAC International and Willian Harwitzed. USA (2005).

Chachaty, E. and P. Saulnier: Bacterial DNA extraction for polymerase chain reaction and pulsed-field gel electrophoresis. In: Nucleic Acid Protocols Handbook (Eds.: R. Rap;ey). Springer protocols handbooks. Human Press, totowa, N.J. (2000). 10.1385/1-59259-038-1:33.

Dai, J., A. Dong, G. Xiong, Y. Liu, M.S. Hossain, S. Liu and D. Qiu: Production of highly active extracellular amylase and cellulase from *Bacillus subtilis* ZIM3 and a recombinant strain with a potential application in tobacco fermentation. *Front. Microbiol.,* **11**, 1539 pages (2020).

Garg, N., B. Singh, S. Vaish and S. Kumar: Enzyme production from different fruit and vegetable waste using lactic acid fermentation. *J. Eco-friendly Agricul.,* **16**, 74-76 (2021).

Gaur, R. and S. Tiwari: Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. *BMC Biotechnol.,* **15**, 19 (2015).

Gomez, K.A. and A.A. Gomez: Statistical Procedures for Agricultural Research. 2nd Edn., John Wiley & Sons, NewYork (1984).

Hatice, A.M.T. and U.T. Filiz: Extracellular pectinase production and purification from a newly isolated *Bacillus subtilis* strain. *Int. J. Food Prop.,* **19**, 2443-2450 (2016).

Jayasekara, S. and R. Ratnayake: Microbial cellulases: An overview and applications. In: Cellulose (Eds.: Alejandro Rodríguez Pascual and María E. Eugenio Martín). Intech Open., 84531 (2019). DOI: 10.5772/intechopen. Available from: https://www.intechopen.com/chapters/66517.

Kothari, M.N., J.A. Kulkarni, P.M. Maid and M.M.V. Baig: Clarification of apple juice by using enzymes and their mixture. *World Res. J. Biotechnol.*, **1**, 29-31 (2013).

Kumar, M.S., S.K. Singh, N. Garg, P. Rahini and M.R.K. Rao: Production of amylase from fruit peel using *Aspergillus niger* by solid-state fermentation. *Der. Pharmacia. Lettr.,* **6**, 173-177 (2014).

Kumar, S., G. Stecher, M. Li, C. Knyaz and K. Tamura: MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.,* **35**, 1547-1549 (2018).

Laemmli, U.K.: Cleavage of structure protein during the assembly of the head of bacteriophase T4. *Nature*, **227**, 680-685 (1970).

Liu, S.Y., C.H. Fang and D. Zhang: *Bacillus subtilis*: A universal cell factory for industry, agriculture, biomaterials and medicine. *Microb. Cell Fact.,* **19**, 173 (2020).

Mehta, D. and T. Satyanarayana: Bacterial and Archaeal α-Amylases: diversity and amelioration of the desirable characteristics for industrial applications. *Front. Microbiol.,* **7**, 1129 pages (2016).

Mengistu, F. and V.K. Pagadala: Isolation and screening of amylase producing thermophilic spore-forming *Bacilli* from starch-rich soil and characterization of their amylase activity. *Afr. J. Microbiol. Res.,* **11**, 851-859 (2017).

Miller, G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugars. *J. Anal. Chem.,* **3**, 426-428 (1972).

Moreira, A.A., A.C.V Ferreira, J.B. Silva, and M.L.L Ribeiro: Treatment of sugarcane bagasse for the immobilization of soybean - glucosidase and application in soymilk isoflavones. *Braz. J. Food Technol.,* **22**, 1-17 (2019).

Rana, N., N. Verma, D. Vaidya and B. Dipta: Production of Amylase from Bacillus thuringiensis J2 Using Apple Pomace as Substrate in Solid State Fermentation. *Int. J. Curr. Microbiol. App. Sci.* **6**, 3465-3474 (2017).

Ranganna, S.: Handbook of Analysis and Quality Control for Fruits and Vegetable Products. 7th Edn., Tata McGraw Hill Book Co., New Delhi, pp. 594-625 (2001).

Raul, D., T. Biswas, S. Mukhopadhyay, D.S. Kumar and S. Gupta: Production and partial purification of alpha-amylase from *Bacillus subtilis* (MTCC 121) using solid-state fermentation. *Biochem. Res. Int.,* **2014**, 1–5 (2014).

Rehman, M.D., S. Yoon, S. Choi, K.K. Young, P. Chulhwan and C.Y. Jin: Production of Novel polygalacturonase from *Bacillus*

paralicheniformis CBS32 and application to depolymerization of Ramie fibre. *Polymers* (Basel), **11**, 1525 (2019).

Sazci, A., A. Radford and K. Erenlerr: Detection of cellulolytic fungi by using Congo red as an indicator: A comparative study with the dinitro salicylic acid reagent method. *J. Appl. Microbiol.,* **61**, 559-562 (1986).

Shariq, M. and M. Suhail: Citrus limetta peels: A promising substrate for the production of multienzyme preparation from a yeast consortium. *Bioresour. Bioproc.,* **6**, 43 pages (2019).

Shrestha, S., J.R. Khatiwada, X. Zhang, C. Chio, A.L.M. Kognou, F. Chen, S. Han, X. Chen and W. Qin: Screening and molecular identification of Novel pectinolytic bacteria from forest soil. *Fermentation*, **7**, 40 pages (2021).

Singh, S. and S.K. Mandal: Optimization of processing parameters for production of pectinolytic enzymes from fermented pineapple residue of mixed *Aspergillus species*. *Jordan J. Biol. Sci.,* **5**, 307–314 (2012).

Sreena, C.P. and D. Sebastian: Augmented cellulase production by *Bacillus subtilis* strain MU S1 using different statistical experimental designs. *J. Genet. Eng. Biotechnol.,* **16**, 9–16 (2018).

Swain, M.R. and R.C. Ray: Alpha-amylase production by *Bacillus subtilis* CM3 in solid-state fermentation using cassava fibrous residue. *J. Basic Microbiol.,* **47**, 417–425 (2007).

Talib, N., N.E. Mohamad, S.K. Yeap, Y. Hussin, M.N.M. Aziz, M.J. Masarudin, S.A. Sharifuddin, Y.W. Hui, C.L. Ho and N.B. Alitheen: Isolation and characterization of *Lactobacillus* spp. from Kefir Samples in Malaysia. *Molecules*, **24**, 2606 pages (2019).

Wood, T.M. and K.M. Bhat: Methods for measuring cellulase activities. *Metho. Enzymol. Biom. part A-cellulose Hemicellul.,* **160**, 87-112 (1988).