Advances in Bioresearch
Adv. Biores., Vol 15 (1) January 2024: 76-84

Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.15.1.7684

Advances in Bioresearch

ORIGINAL ARTICLE

Isolation And Screening of Polysaccharide Degrading Microbes from Various Natural Sources

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ABSTRACT
Polysaccharide degrading enzymes occupies huge stack of application in pharmaceutical, food, dairy, textile, plant fiber processing and related industries. The main purpose of this work was isolation, screening and characterization of extracellular enzymes from various microbial sources eg. Bacillus spp. etc. Particularly focusing on which samples could be natural resources such as industrial waste, decade fruits waste, fruits peels and rhizospheric soil etc. In the current study, nineteen bacteria were isolated from eight different samples collected from various regions of Gujarat, India. These isolates were allowed to grow in media containing various polysaccharides substrate to assess their potential to form showed the clear zone around the colonies.
Keywords: *Polysaccharides, Enzymes, Pectinase, Cellulase, Amylase, Xylanase, Zymography*

Received 24.09.2023 Revised 11.10.2023 Accepted 30.12.2023

How to cite this article:
Dhvani Patel, Hardik Shah. Isolation And Screening of Polysaccharide Degrading Microbes from Various Natural Sources. Adv. Biores., Vol 15 (1) January 2024: 76-84.

INTRODUCTION

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units such as glucose link together by glycosidic bonds. A polysaccharide can be a *homopolysaccharide*, made up of similar monosaccharides, or a *heteropolysaccharide* containing more varieties of monosaccharides. Each polysaccharide is degraded by its specific enzyme by breaking down glycosidic bond forming mono, di or oligo saccharides. Amylase catalyses is the reaction of conversion of starch into sugar. Amylase was discovered in 1815 by Kirchhoff. Amylase is obtained from plants and microorganisms and plays an important role in different industrial processes such as detergent industry, paper industry, food and pharmaceutical industries [1]. Pectinase specifically breakdown pectin [2] which is an acidic heteropolysaccharide with high molecular weight that is predominantly made up of α (1−4) linked D-galacturonic acid residues. Pectinase is used in a variability of industries related to animal feed, wastewater treatment, pharmaceutical, and pulp and paper [3]. Cellulases are responsible for cellulose degradation by hydrolyzing the β-1,4-glycosidic bonds. These are inducible enzymes produced by a several microorganisms during their growth on cellulosic materials. Therefore, cellulose is converted to simple sugar, glucose, which can be fermented into cellulosic biofuels [4]. Xylanase are a crucial group of depolymerizing enzymes used for the hydrolysis of the xylan that is major components of hemicellulose. There is a need for depolymerization of this complex polymer for its efficient utilization in different industrial applications [5]. Recently, there has been much interest in xylanase for bioethanol production, animal nutrition, manufacture of food and beverages. In this present study, primarily aim was to isolate the bacteria from soil with ability to hydrolyse complex polysaccharides followed by screening on the basis of ability to form clear zone of hydrolysis around the colonies in solid media and identify the isolate using polyphasic approach. The enzyme was purified by ammonium sulphate precipitation and isopropyl alcohol, to achieve future studies.

MATERIAL AND METHOD

Sample collection:

As stated in table 1, rhizospheric soil samples and decomposing fruits sample were collected from different located areas, Gujarat, India. The samples were stored at 4 °C for further use [6].

Table-1 Details of samples used in research study.

Samples	Number of isolates	Location	Geographical coordinates of the sampling sites	
			N Latitude	E Longitude
Rhizospheric soil near Lemon tree	8	Vadavali, Patan, Gujarat	23.646	72.144
Rhizospheric soil near Beetroot nodules	4	Vegetable market, Mehsana, Gujarat	23.594	72.384
Decomposed mix fruites	1	Vegetable market, Mehsana, Gujarat	23.594	72.384
Rotten lemon	6	Kherva, Mehsana, Gujarat	23.544	72.442
Rotten Orange	1	Vegetable market, Mehsana, Gujarat	23.594	72.384

Preparation of sample suspension:

Soil suspension was prepared by adding 1 gm sample in 100 ml of sterile N-saline. The rotten fruits were rinsed with distilled water to remove dust. After that, the peel from infected area were separated from fruits and cut into the small pieces [7]. Samples were suspended by agitation for 30 min at 120 rpm on a rotary shaker incubator to obtain homogenous suspension.

Isolation of bacteria:

For current work, exclusive focus was given on bacteria, the serial dilutions were performed up to 10^{-6} from sample suspension. From all the dilutions, 0.1 ml was inoculated by the spread plate method in the respective solidified media like nutrient agar and incubated at 37°C for 24 - 48 hours. These isolates were sub-cultured on the selective media and maintained at 4°C for further studies [8].

Screening of bacteria producing extracellular enzymes:

Screening of bacteria producing different hydrolytic enzymes was carried out on the basis of their ability to grow on solid media containing particular substrate and formation of zone of hydrolysis on solid media. The assessment of pectin hydrolysis activity for selective isolates was carried out using pectin screening agar medium [9]. Plates were incubated at 37°C for 24-48 hours followed by flooding with 50Mm potassium iodide-iodine solution (Lugol's iodine) to observe the clear zone of hydrolysis around the colonies [6]. The quantitative estimation of the enzyme was carried out for only selective isolates using following formula [10].

$$\text{Hydrolysis capacity} = \frac{\textit{Diameter of clearing zone}}{\textit{Colony diameter}} \qquad (1)$$

Similarly, assessment of ability to degrade the polysaccharides such as starch, cellulose and xylan was carried out using solid media containing respective substrates.

Identification of pectin degrading microbes:

Cultural characterization: The isolated colonies observed in the nutrient agar medium were consider for morphological studies. The aspects covered for characterization includes size, shape, margin, elevation, consistency, texture, odor, opacity, pigmentation.

Microscopic observation: Gram's staining was performed for selected isolates followed by microscopic observation using oil immersion lens in binocular microscope (LABOMED, Vision 2000) [11].

Microbial Identification using 16S rRNA Gene:

DNA was isolated from the bacterial culture using Qiagen kit. Fragment of 16S rRNA gene was amplified by PCR primer 357F and 1391R. A single discrete PCR amplicon band was observed when resolved on Agarose. The PCR amplicon was purified by column purification to remove contaminants. DNA sequencing reaction of PCR amplicon was carried out with 357F 3'(TACGGGAGGCAGCAG)5' & 1391R 3'(GACGGGCGGTGTGTACA)5' primers sequence using BDT v3.1 Cycle Sequencing Kit on ABI 3500xl Genetic Analyzer. The sequence obtained was used to carry out BLAST with the database of GenBank, NCBI. Based on maximum identity score first ten sequences were selected and aligned using multiple sequence alignment software program MEGA7.

Pectinase production under submerged fermentation:

Based on the results obtained during screening, further work was focused on pectinase production. Primarily pectinase was produced in submerged fermentation under unoptimized conditions. Overnight grown was culture of respective isolates was used as an inoculum. 5% inoculum was added to each of the flask containing pectinase broth (pH-7) incubated in rotary shaker 120 rpm at 37°C. After fermentation production medium was centrifuged at 10,000 rpm for 30min at 4°C. The clear supernatant was used for as crude enzyme pectinase assay.

Enzyme assay:

As mentioned in previous section, cell-free supernatant was used as crude enzyme. The products formed after enzymatic reaction (reducing sugars) were measured using DNSA method. One unit of enzyme was defined as the amount of enzyme that release one µM of reducing sugar per minute under standard assay conditions and is expressed as U/ml/min**.** Standards for DNSA method varied with the substrates. For starch, maltose was used as standard whereas for pectin and cellulose, glucose was used as standard. Concentration of standard was adjusted as 100 µg/ml in all cases. [12].

Zymogram analysis:

For the zymography, the protein samples (crude enzyme) were loaded onto 12% separating gel supplemented with 0.1% (w/v) respective substrate and electrophorased for 120 min. After completion of electrophoresis, the gel soaked in appropriate buffer for 60 min so, that the enzyme can hydrolyse substrate present into the gel prior to staining with Lugol's iodine solution. 0.1 M sodium phosphate butter (pH- 7.0), 0.1M acetate buffer (pH- 5.5) and 0.1M citrate buffer (pH- 5.0) were used to soak the gel for amylase, pectinase and cellulase respectively. In the case of cellulase, gel required destaining with 2 M NaCl until the clear area of cellulose hydrolysis was observed.

Partial Purification of pectinase:

Crude enzyme was brought to 20-80 % saturation with solid ammonium sulphate. The mixture was stirred overnight with magnetic stirrer at 4 °C for to allow proper protein precipitation. The precipitates were separated by centrifugation at 12,000 rpm for 15 min which was followed by resuspension in 50 mM sodium phosphate buffer (pH 7.0) for further study. The partially purified enzyme was dialyzed overnight against the same buffer (Rakaz et al. 2021) and investigated for enzyme activity as stated in 2.7. Estimation of total protein was performed by Lowry method using bovine serum albumin (BSA – 200 µg/ml) as a standard [15].

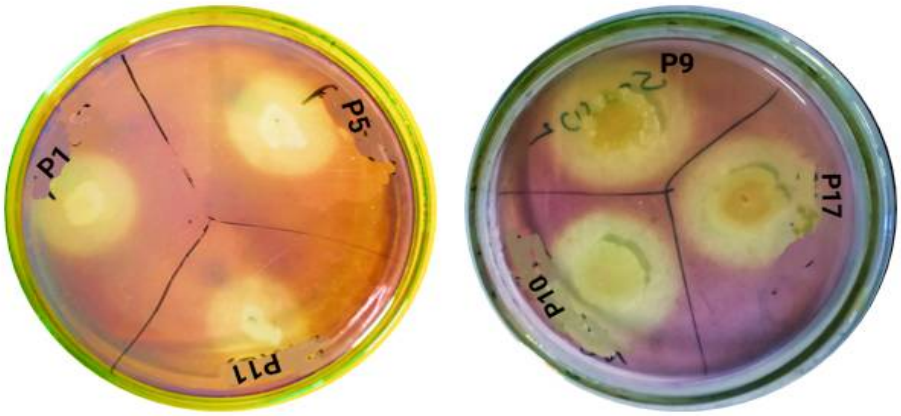
RESULT AND DISCUSSION

Isolation and screening of pectinase-producing microbes:

Since microorganisms were grown on pectin screening agar media containing pectin as the sole source of carbon, their pectinase-producing ability can be assured from the value of hydrolysis capacity as indicated equation. The details of bacteria isolated from various samples are summarized in section table-2.

Table- 2 Observation of pectinase activity on solid media

Source of samples	Isolates	Zone diameter (mm)	Colony diameter (mm)	Hydrolysis Capacity
Rhizospheric soil near Lemon tree	P1	17	8	21.25
	P2	5	3	16.67
	P3	17	10	17.00
	P4	13	7	18.57
	P5	15	5	30.00
	P6	5	3	16.67
	P7	6	3	20.00
	P8	13	10	13.00
Rhizospheric soil of Beetroot nodules	P9	20	5	40.00
	P10	15	2	75.00
	P11	24	10	24.00
	P12	4	3	13.33
Decomposed mix fruits	P13	20	14	14.29
Rotten lemon	P14	0	0	0.00
	P15	07	5	14.00
	P16	35	23	15.22
	P17	15	3	50.00
	P18	28	18	15.56
Rotten orange	P19	25	15	16.66



(Figure 1: The zone of hydrolysis on the pectin screening agar medium after flooding with Lugol's iodine. A – Pectin hydrolysis by isolates P1, P5, P11 and B – Pectin hydrolysis by isolates P9, P10, P17)
Polyphasic approach was utilized for identification where morphological, cultural, and molecular characteristics were considered as evaluative parameters.

Out of 19, six isolates were selected for further study on the basis of hydrolysis capacity. The potential isolates displayed clear zone of hydrolysis on pectin screening agar. These isolates, namely P1, P5, P9, P10, P11 and P17 exhibited maximum hydrolytic capacity[12]. After incubation time, the plates were flooded with Lugol's iodine and observed for zone of hydrolysis in fig-1.

Cultural characterization :

Colonies of the isolates were observed in a range of small to big in size. Shape was mostly round, except for P1 and P17, which was elliptical and irregular respectively. In texture, generally the colonies were seen with smooth surface. Table-3 represent cultural characteristics based on the observation of colonies on nutrient agar plates.

Table- 3 Cultural characteristics of microbes

Isolate	P1	P5	P9	P10	P11	P17
Size	Small	Small	Big	Intermediate	Intermediate	Intermediate
Shape	Elliptical	Round	Round	Round	Round	Irregular
Margin	Entire	Entire	Undulate	Entire	Entire	Undulate
Elevation	Flat	Convex	Raised	Convex	Raised	Convex
Consistency	Moist	Moist	Butyrous	Butyrous	Butyrous	Butyrous
Texture	Smooth	Smooth	Punctate	Smooth	Smooth	Smooth
Odor	No	No	No	No	No	No
Opacity	Transparent	Transparent	Translucent	Transparent	Transparent	Transparent
Pigmentation	No	No	Pale yellow	No	Yellow	Pale yellow

Microscopic examination:

To observe morphological characteristics like size, shape and arrangements, microscopy is a necessary tool to be used with and without stain. The esult of microscopy after gram staining is shown in Table.

Table-4 Morphological characteristics

Isolate	Gram's reaction	Shape	Size	Arrangement
P1	Positive	Bacillus	Big	Pair, single
P5	Positive	Rod, Bacillus	Big	Pair, single
P9	Positive	Rod	Big	Pair, single
P10	Positive	Rod, Bacillus	Big	Single, Chain
P11	Positive	Rod, Bacillus	Big	Single, Pair
P17	Positive	Rod	Big	Single, Chain

Molecular Identification:

Widespread use of this gene sequence for bacterial identification and taxonomy followed a body of pioneering work by Woese, who defined important properties. The degree of conservation is assumed to

result from the importance of the 16S rRNA as a critical component of cell function. Based on the results of secondary screening focus was concentrated on isolate P5 which was able to produce pectinase and amylase in remarkable quantity. As part of molecular identification DNA was isolated from bacteria and it's purity was confirmed on 1% agarose gel where a single band of high-molecular-weight DNA was observed. The fragment of SSU-RNA gene was amplified by PCR on ABI 3500 x1 genetic analyzer. The full-length sequence of 16S rRNA gene was used to carry out BLAST. Based on the results obtained during morphological and molecular characterization isolate P5 was found closest to *Bacillus subtilis*. Earlier, many bacteria have been reported as a potential source for enzymes that have applications in bio-based industries identified using 16S rRNA sequencing for the production of thermal and acid-stable pectinase from *Bacillus subtilis* strain [17] and this is the first report of the production pectin-degrading enzymes by *B. subtilis* SAV-21 in SSF using Coccount fiber [10]. Some bacterial genera were reported successfully by the 16S rRNA identification for pectinolytic properties which include species of *Pseudomonas* [12] and *Streptomyces* [11]. According to Bergey's bacterial identification manual and 16S rRNA results, the bacterium was concluded to be *B. subtilis*.

Growth on various substrates:

The objective of the present work was to explore the production of extracellular hydrolytic enzymes by microbes associated with rotten fruits and rhizospheric soil. Total nineteen isolates were screened for the presence of three different hydrolytic enzymes viz. xylanase, cellulase, and amylase among which sixteen isolates were found to display at least one of three activities (fig-2) while three isolates were unable to exhibit any of these activities. The cellulolytic activity was found to be the most common hydrolytic activity detected in thirteen isolates, very closely followed by the xylanolytic and amylolytic activity detected in nine of the isolates. Three isolates viz. P4, P11, P12 were able to express all three hydrolytic enzymes. Two different enzyme activities were detected in nine of the isolates and four other isolates expressed only one hydrolytic enzyme. In Hosseini Abari et al. 2021 only single isolate was observed to give positive result of pectinase and amylase. From the sugarcane bagasse [18] and various hot spring [19] were reported for the archive of essential enzymes such as amylase, cellulase, xylanase, pectinase, etc.

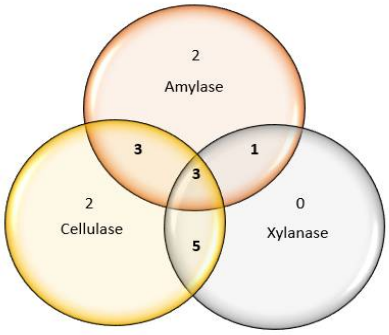
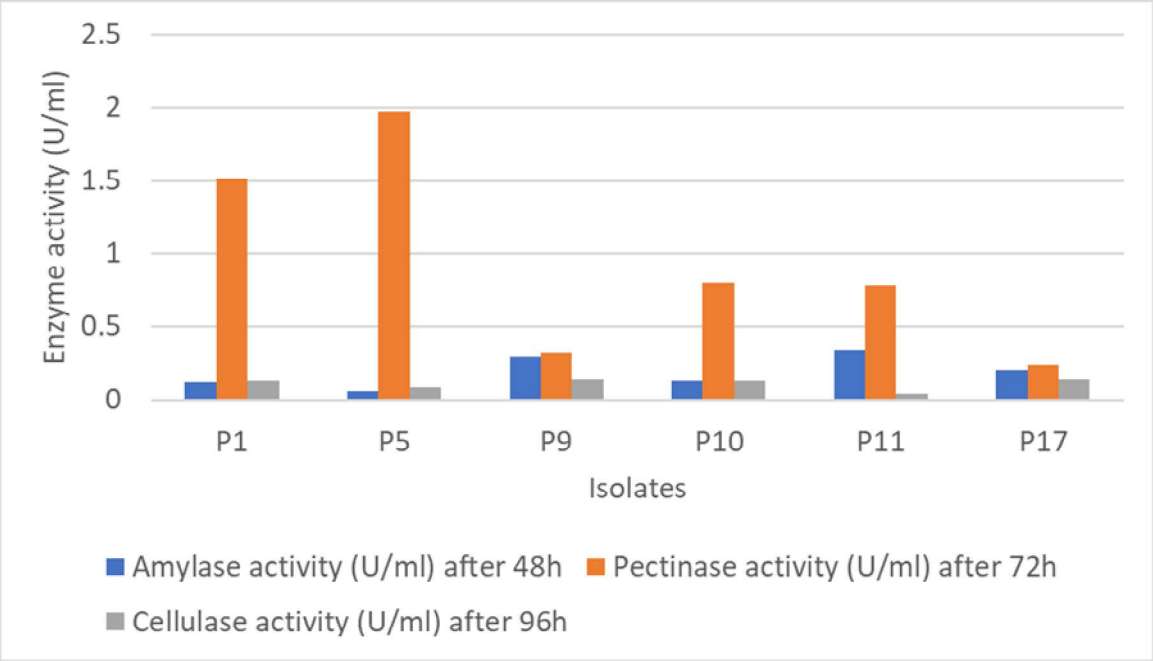


Fig -2 Hydrolytic enzyme profiling of the isolates.

Study on production of different enzymes:

After incubation enzyme assay was performed from crude enzyme. The results are shown in fig-3. There results of enzymes assay indicates amount of enzyme produced during submerged fermentation under controlled parameters. Enzyme producing ability of the isolates was determined by using process based on estimation of product liberated after enzymatic reaction. This assay was modified accordingly to evaluate the amylase, pectinase and cellulase activity of crude enzymes from six different isolates. The six isolates exhibiting ability to produce all three enzymes were selected for further study. The amylase activity of six different isolates eg. P1, P5, P9, P10, P11 and P17 was observed as 0.12, 0.06, 0.30, 0.13, 0.34 and 0.20 Unit respectively. The amylase activity of isolate P11 was observed maximum, followed by P9 and P17. Isolates P1 and P10 has moderate activity while isolate P5 has lowest amylase activity as compared to other isolates. All isolates were found to be excellent producers of pectinase as seen in fig-3. Pectinase activity of all six isolates was observed in the range of 0.24 to 1.97 Unit. Isolate P5 showed highest pectinase activity (1.97 U/ml) than other selected isolates and lowest activity (0.24 U/ml) was

observed in P17. Cellulase activity for all the selected isolates was observed very low. Isolates P9 and P11 showed 0.14U/ml cellulase activity while 0.13 U/ml cellulase activity was observed in P1 and P10.



(Fig -3 Result of study related to fermentative production of enzymes from selected isolates)

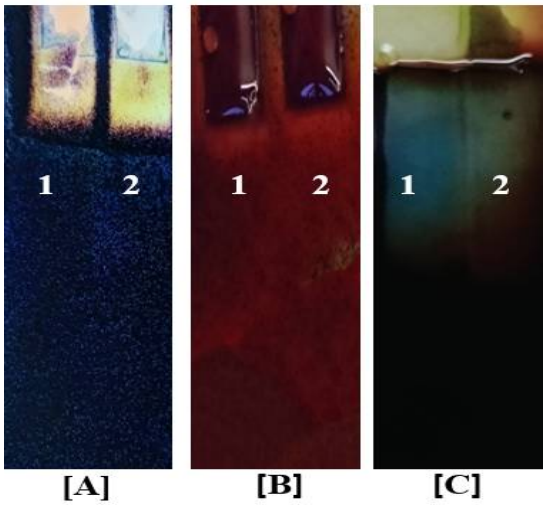
Zymography:

In zymography is usually utilized to detect enzyme activity after separation of enzymes in native PAGE. Zymography of hydrolytic enzymes results in hydrolysis of particular substrate present in the gel which shows either clear band in coloured background or coloured band in transparent background. The clear band obtained during zymogram confirms the presence of respective hydrolytic enzyme in these sample. The results of zymography are shown in table - 5 and fig - 4.

Table 5: Results of the zymography analysis

		Substrate used during fermentation by isolate P1			Substrate used during fermentation by isolate P5		
		Starch	Pectin	carboxymethyl cellulose	Starch	Pectin	carboxymethyl cellulose
Substrate used with native PAGE	Starch	+	+	-	-	+	-
	Pectin	-	+	-	-	+	-
	Cellulose	-	-	+	-	-	+

In case of both the isolates, results indicates that substrate used in submerged fermentation media served as inducer for production of its specific enzyme which can be readily observed in the fig - 5. While grown on pectin, both isolates also displayed positive results for amylase. It may be due to ability of enzyme to breakdown the α- 1,4 glycosidic linkage in starch.



(Fig- 4 Zymogram analysis of polysaccharides degrading enzymes from bacteria. [A] Native PAGE containing starch: Lane 1: enzyme derived from P1 Lane 2: P5. [B] Native PAGE containing pectin: Lane 1: P1, Lane 2: P5. [C] Native PAGE containing carboxymethyl cellulose Lane 1: P1, Lane 2: P5. Observed the clearance area indicates the presence of hydrolysis the substrates.)

Partial purification:

Ammonium sulphate precipitation was used for partial purification to enhance the purity of crude enzyme. Result of enzyme assay after partial purification shows that increase in yield and purified fold. The crude enzyme obtained from P1 isolate had specific activity of 2.36 U/mg which was increased after ammonium sulphate precipitation to 3.38 U/mg. In case of P5 isolates specific activity -14 U/mg was observed which is comparatively higher than the specific activity 3.1 U/mg in similar study done by (Datta et al. 2014). In isolates P5 observed fold was 1.09 U/mg which was less than P1. Isopropyl alcohol shows better result in terms of yield as compared to ammonium sulphate. For isolates P1 and P5, using isopropyl alcohol yield increase up to 16.45% and 15.73% as compared to ammonium sulphate precipitation.

(Table – 6 Partially Purification of enzyme from microbes)

Purification step	Isolates	Enzyme activity (Unit)	Total protein (mg)	Specific activity (Unit/mg)	Fold purified	% Yield
Crude enzyme	P1	1.52	0.65	2.36	1	100
	P5	1.97	0.15	12.79	1	100
Ammonium sulfate precipitation	P1	1.25	0.37	3.38	1.46	82.23
	P5	1.54	0.11	14	1.09	78.17
Iso propyl alcohol	P1	1.50	0.45	3.33	1.41	98.68
	P5	1.85	0.15	12.33	0.96	93.90

CONCLUSION

The current study successfully resulted in isolation of bacteria from rhizospheric soil and decaying fruits which served as valuable sources for these bacteria having ability to degrade a wide spectrum of complex polysaccharides such as starch, pectin and cellulose. Higher specific activity was obtained by protein precipitation employing used 60% ammonium sulphate in the case of both isolates gaining 82.23 and 78.17-fold for P1 and P5 respectively. The possibility of employing microbes as valuable sources of industrially important enzymes has sparked interest in studying extracellular enzymes in a variety of microorganisms in recent years. In today's era, pectinase play a curial role in diverse application such as extraction of fruit juice, food sector, clarification of wine, tea, cocoa, coffee, vegetable oil extraction, jam and jellies preparation etc. These enzymes are also applied in the paper and pulp industries for, paper bleaching, cotton bio-scouring. As the need for industrially important enzymes is expanding at a rapid rate, current work can be possibly extended in the direction of commercialization after completing in depth study related to fermentative production and detailed characterisation of pectinase.

ACKNOWLEDGMENTS
The research was non-funded. The authors are thankful to the Mehsana Urban Institute of Sciences, Faculty of Science, Ganpat University, Gujarat for providing a laboratory facility.

FUNDING
The authors disclosed that the research is non-funded.

CONFLICT OF INTEREST
The authors disclosed no potential conflicts of interest, financial or otherwise.

REFERENCES
1. D. Upadhyay, P. Universiy, P. Andhare, and P. Universiy, (2021): "Issn : 2277 – 4998 a Study on Lipase : Review," vol. 10, no. June, pp. 333–340.
2. S. Haile and A. Ayele,(2022): "Pectinase from Microorganisms and Its Industrial Applications," *Sci. World J.*, vol. 2022, doi: 10.1155/2022/1881305.
3. R. Singh, M. Kumar, A. Mittal, and P. K. Mehta,(2016): "Microbial enzymes: industrial progress in 21st century," *3 Biotech*, vol. 6, no. 2, doi: 10.1007/s13205-016-0485-8.
4. U. Ejaz, M. Sohail, and A. Ghanemi, (2021): "Cellulases: From bioactivity to a variety of industrial applications," *Biomimetics*, vol. 6, no. 3, pp. 1–11, doi: 10.3390/biomimetics6030044.
5. N. Bhardwaj, B. Kumar, and P. Verma, (2019): "A detailed overview of xylanases: an emerging biomolecule for current and future prospective," *Bioresour. Bioprocess.*, vol. 6, no. 1, doi: 10.1186/s40643-019-0276-2.
6. O. J. Oumer and D. Abate, "Screening and molecular identification of pectinase producing microbes from coffee pulp," *Biomed Res. Int.*, vol. 2018, 2018, doi: 10.1155/2018/2961767.
7. A. Ahmed, U. Ejaz, and M. Sohail, (2020). "Pectinase production from immobilized and free cells of Geotrichum candidum AA15 in galacturonic acid and sugars containing medium," *J. King Saud Univ. - Sci.*, vol. 32, no. 1, pp. 952–954, doi: 10.1016/j.jksus.2019.07.003.
8. V. Luang-In, M. Yotchaisarn, W. Saengha, P. Udomwong, S. Deeseenthum, and K. Maneewan, (2019)."Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand," *Biomed. Pharmacol. J.*, vol. 12, no. 3, pp. 1061–1068, doi: 10.13005/bpj/1735.
9. M. P. chandra Reddy and K. V. Saritha, (2016). "Effects of the culture media optimization on pectinase production by Enterobacter sp. PSTB-1," *3 Biotech*, vol. 6, no. 2, pp. 1–11, doi: 10.1007/s13205-016-0502-y.
10. S. J. Kaur and V. K. Gupta, (2017). "Production of pectinolytic enzymes pectinase and pectin lyase by Bacillus subtilis SAV-21 in solid state fermentation," *Ann. Microbiol.*, vol. 67, no. 4, pp. 333–342, doi: 10.1007/s13213-017-1264-4.
11. T. Murugan *et al.*, (2021)."Production and characterization of extracellular pectinase from a newly isolated Bacillus species from fruit waste soil," *Mater. Today Proc.*, vol. 45, no. xxxx, pp. 2087–2090. doi: 10.1016/j.matpr.2020.09.607.
12. H. A. Mercimek Takcı and F. U. Turkmen, (2016). "Extracellular Pectinase Production and Purification from a Newly Isolated Bacillus subtilis Strain," *Int. J. Food Prop.*, vol. 19, no. 11, pp. 2443–2450 doi: 10.1080/10942912.2015.1123270.
13. C. Champasri, T. Champasri, and K. Woranam,(2015). "Purification, biochemical characterization of a Macrotermes gilvus cellulase and zymogram analysis," *Asian J. Biochem.*, vol. 10, no. 5, pp. 190–204, 2015, doi: 10.3923/ajb.2015.190.204.
14. M. A. Rakaz, M. O. Hussien, and H. M. Ibrahim, (2021). "Isolation, Extraction, Purification, and Molecular Characterization for Thermostable α -Amylase from Locally Isolated Bacillus Species in Sudan," *Biochem. Res. Int.*, vol. 2021, doi: 10.1155/2021/6670380.
15. P. Kohli and R. Gupta, (2019). "Application of calcium alginate immobilized and crude pectin lyase from Bacillus cereus in degumming of plant fibres," *Biocatal. Biotransformation*, vol. 37, no. 5, pp. 341–348,, doi: 10.1080/10242422.2018.1564745.
16. S. N. Yassin, T. M. Jiru, and M. Indracanti, (2021). "Screening and Characterization of Thermostable Amylase-Producing Bacteria Isolated from Soil Samples of Afdera, Afar Region, and Molecular Detection of Amylase-Coding Gene," *Int. J. Microbiol.*, vol. doi: 10.1155/2021/5592885.
17. J. Prajapati, P. Dudhagara, and K. Patel, (2021). "Production of thermal and acid-stable pectinase from Bacillus subtilis strain BK-3: Optimization, characterization, and application for fruit juice clarification," *Biocatal. Agric. Biotechnol.*, vol. 35, no. March, p. 102063,doi: 10.1016/j.bcab.2021.102063.
18. J. Li *et al.*, "Synergism of cellulase, xylanase, and pectinase on hydrolyzing sugarcane bagasse resulting from different pretreatment technologies," *Bioresour. Technol.*, vol. 155, pp. 258–265, 2014, doi: 10.1016/j.biortech.2013.12.113.
19. A. P. Pathak and S. R. Jadhav, (2019). "Production and characterization of a thermo-ph stable pectinase from bacillus licheniformis unp-1: A novel strain isolated from unapdev hot spring," *Indian J. Geo-Marine Sci.*, vol. 48, no. 5, pp. 670–677.
20. G. Datta Tripathi, Z. Javed, and A. Kumar Singh, (2014). "Pectinase production and purification from Bacillus

subtilis isolated from soil," *Pelagia Res. Libr. Adv. Appl. Sci. Res.*, vol. 5, no. 1, pp. 103–105, [Online]. Available: www.pelagiaresearchlibrary.com.