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Production, Characterization of Pectinase from *Bacillus inaquosorum* and its applications

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KEYWORDS

Immobilization, Oil extractions, Pectinase, response surface methodology, Plackett-Burman design

ABSTRACT:

The study focuses on isolating, screening, and characterizing extracellular polysaccharide-degrading enzymes, with particular emphasis on pectinase production, which has vast applications across industries such as pharmaceuticals, food, dairy, textiles, and plant fiber processing. The primary aim was to identify novel bacterial strains capable of producing pectinase and to optimize various parameters for maximizing enzyme production. Natural resources like industrial waste, decayed fruit waste, fruit peels, and rhizospheric soil were used as sample sources for microbial isolation. In total, hundred bacterial isolates were obtained from twenty-three different samples collected from various regions of Gujarat, India. One particular bacterial strain, identified as Bacillus inaquosorum, showed significant potential for pectinase production and was selected for further study. Submerged fermentation was employed to enhance enzyme production, followed by optimize pectinase production, the study employed the one-factor-at-a-time (OFAT) approach, where in key physicochemical factors such as pH, inoculum size, temperature, and incubation time were varied to determine their impact on enzyme production. Subsequent statistical optimization of media components was conducted using the Plackett-Burman design, which identified significant factors influencing enzyme production. Among the media components, glucose and pectin were identified as the best carbon sources, while ammonium sulfate and potassium nitrate (KNO_3) were the best nitrogen sources. In the next phase, further optimization was performed using the Response Surface Methodology (RSM) through a Central Composite Design (CCD). This optimization yielded a significant improvement in enzyme production, with the highest pectinase activity recorded at 1.35 ± 0.04 U/mL/min, representing an increase of 0.545 ± 0.02 U/mL/min compared to the unoptimized medium. Enzyme purification through a three-step process involving ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration chromatography using a Sephadex G-75 column. The purification process achieved a 60.86% yield of pectinase. Characterization of the purified enzyme revealed that the highest pectinase activity occurred at a pH of 6 and a temperature of 50°C. Additionally, the presence of Fe^{2+} ions further enhanced the enzyme's activity. The stability of the enzyme in the presence of surfactants and inhibitors suggests its potential for industrial applications, particularly in sectors requiring pectin degradation. The kinetic parameters of the enzyme were determined, with the Km value recorded as 166.53 M and the Vmax as 769.24 µM/ml, indicating the enzyme's efficiency in catalyzing reactions. The optimal conditions were found to be a pH of 6.0, inoculum size of 3 mL, temperature of 37°C, and an incubation time of 48 hours. The results demonstrate that optimizing medium components and fermentation conditions can significantly enhance pectinase production, with potential applications in industries such as food processing, particularly for juice clarification and plant fiber degradation.

1. Introduction:

nzymes play pivotal roles in various metabolic activities within living cells, and their industrial applications have surged due to advancements in biotechnology. Derived from plants, fungi, yeast, actinomycetes, and bacteria, enzymes such as amylase, pectinase, cellulase, and xylanase are vital for numerous industrial processes (Al-Zuhair et al., 2015).

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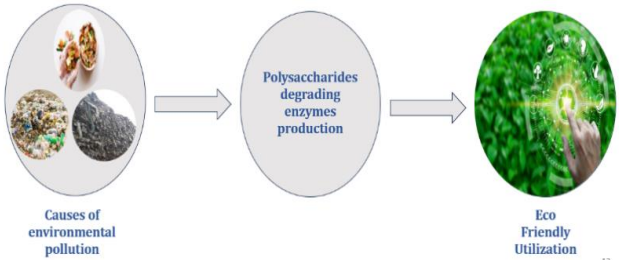
Amylase facilitates the conversion of starch into sugars and finds application in diverse industries including detergents, paper, and food production (Upadhyay et al., 2021). Pectinase, specialized in breaking down pectin, is utilized in animal feed, wastewater treatment, pharmaceuticals, and pulp and paper manufacturing (Salbu et al., 2012). Cellulases and xylanases are crucial for the degradation of cellulose and xylan, respectively, supporting applications in biofuel production, animal nutrition, and food and beverage manufacturing (Ejaz et al., 2021).

Isolating and screening microbes from natural sources like soil aids in obtaining enzyme sources. Selective media facilitate the growth of desired microbes, with enrichment media selectively isolating and growing microbes using specific carbon sources (Luang-In et al., 2019). Optimization of fermentation conditions and media components influences bacterial enzyme production, often achieved through statistical experimental designs like Plackett-Burman designs and Response Surface Methodology (Doan et al., 2021).

Techniques such as electrophoresis are employed for enzyme protein purity analysis, while immobilization techniques like sodium alginate composite beads enhance enzyme stability and reusability (Poturcu et al., 2017).

Enzymes are indispensable tools in various industries due to their specificity, speed, and eco-friendly nature, replacing traditional chemical processes that often contribute to pollution. With the continuous advancement of biotechnology, novel applications of industrial enzymes are emerging rapidly, predicted to become integral to catalyzed factory processes and household use in the future (Chapman et al., 2018).



Enzymes like pectinase, commercially utilized since 1930, serve diverse industrial purposes including wine and fruit juice preparation, cotton scouring, wastewater treatment, and paper bleaching.

In summary, enzymes, particularly pectinases, offer immense potential across diverse industries due to their specificity and efficiency. Advances in microbial screening, optimization of production processes, and purification techniques contribute to the expanding industrial applications of enzymes, driving innovation and sustainability in various sectors.

2. Material and methods:

2.1. Isolation and screening of microbes producing extracellular enzyme:

In this study, soil and rotten fruit samples were processed to isolate bacteria and fungi for analysis. Serial dilutions were performed up to 10^{-6}, and 0.1 mL from each dilution was inoculated onto nutrient agar plates using the spread plate method. The inoculated plates were incubated at 37°C for 24 to 48 hours to promote bacterial growth. After incubation, bacterial colonies were observed and isolated (Luang-In et al., 2019).

The bacteria were then screened for hydrolytic enzyme production by growing them on solid media containing specific substrates and observing zones of hydrolysis around the colonies. Various agar media were employed for this screening, and incubation was carried out at 37°C for 24 to 48 hours. Hydrolysis zones were visualized by flooding the plates with 50 mM potassium iodide-iodine solution. The formation of clear zones around the colonies indicated the presence of hydrolytic enzymes (Reddy & Saritha, 2016). Quantitative enzyme estimation was conducted for selective isolates using a specific formula (Kaur & Gupta, 2017).

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

2.2. Identification of pectin degrading microbes:

Microscopic observation, particularly through Gram's staining, is crucial in microbial characterization. It aids in investigating cellular morphology, arrangement, and staining properties of isolates (Murugan et al., 2021).

Microbial identification via the 16S rRNA gene involves DNA isolation, PCR amplification, gel visualization, purification, and sequencing. Sequences are compared to databases like GenBank using BLAST for similarity. These techniques enable accurate microbial identification, aiding in taxonomy and functional trait understanding.

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2.3. Enzyme assay:

A pure culture of the extracellular polysaccharide degrading microorganism is selected and grown on a suitable agar medium. After overnight-grown culture of the respective isolates was used as an inoculum for the fermentation process(Patel & Shah, 2024b). cell-free supernatant was used as crude enzyme. The products formed after enzymatic reaction (reducing sugars) were measured using DNSA method (Mercimek Takcı & Turkmen, 2016).

2.4. Optimization of Production Medium:

The study aimed to identify optimal media components for pectinase production through a one-factor-at-a-time (OFAT) approach, systematically varying growth conditions while keeping other variables constant. The effects of various factors on enzyme production were investigated, including carbon sources, nitrogen sources, incubation period, pH, temperature, and inoculum concentrations (Yadav et al., 2017) .

In media optimization for pectinase production, the Plackett-Burman design was employed to identify key factors significantly impacting enzyme activity. Traditional optimization methods informed the selection of parameters such as incubation temperature, inoculum size, incubation period, and pH. Carbon and nitrogen sources were evaluated using the Plackett-Burman design with Design Expert software version 13.0. Four different medium components, serving as carbon and nitrogen sources, were screened using a 12-run experiment. Each variable was examined at two concentration levels: -1 (low level) and +1 (high level). This approach allowed for the evaluation of factors effects on pectinase activity (Gajdhane et al., 2016).

Response Surface Methodology (RSM) utilizes quantitative data from factorial designs for regression analysis, commonly applied in optimization. In media optimization, RSM focused on pectin, glucose, and ammonium sulfate from Plackett-Burman Design. These factors were encoded at five levels ($-\alpha$, -1, 0, 1, α) (refer to Supplementary data Table-3), and a matrix design comprising 20 experimental runs was employed (Liu et al., 2020). RSM aids in concurrently addressing multiple equations, determining optimal values for variables affecting a specific process or response through the construction of three-dimensional surface plots based on experimental data.

2.5. Bioreactor cultivations:

A 5-L stirred tank bioreactor made of stainless steel and featuring a double-jacket was employed (BioEngineering, Wald, Switzerland), with an operational volume of 3 L. Equipped with two 6-bladed impellers, the stirrer facilitated mixing. Sterilization was conducted at 121°C for 30 minutes. Throughout the cultivation, agitation was maintained at 200 rpm, and sterile air was introduced for aeration. pH control was managed by adjusting the culture to a pH of 6, achieved through a pH controller linked to an acid/base feeding peristaltic pump containing 5 M HCl and 5 M NaOH solutions.

2.6. Purification of enzyme:

The partial purification of the enzyme involved ammonium sulfate precipitation and dialysis. The cell-free extract was saturated with different concentrations of ammonium sulfate, followed by centrifugation to collect the pellets. These pellets were then suspended in a tris buffer and dialyzed overnight (Rakaz et al., 2021). Total protein amount was determined using the Lowry method. The dialyzed enzyme was loaded onto a DEAE-sephadex ion exchange column and eluted with a NaCl gradient (Carrasco et al., 2019). Subsequently, the enzyme concentrate was applied to a Sephadex G-75 column and eluted with tris buffer. Fractions were collected, assayed, and stored at 4°C (Kohli & Gupta, 2019). Total protein estimation was again performed using the Lowry method. This purification process allowed for the enrichment of the enzyme and separation from other cellular components, facilitating further analysis and characterization (Patel & Shah, 2024a).

2.6.1. Determination of molecular weight and zymogram analysis:

The determination of the molecular weight of enzymes was conducted via SDS-PAGE according to the Laemmli method with slight modifications. Protein samples were loaded onto stacking and separating gels, followed by electrophoresis. The gel was then stained with Coomassie Brilliant Blue and destained to visualize protein bands (Champasri et al., 2015).

Zymogram analysis was performed to assess enzymatic activity, particularly that of pectinase, using a gel-based technique. Protein samples were loaded onto gels containing the respective substrate and subjected to electrophoresis. After soaking the gel in specific buffers conducive to enzyme activity, it was stained to visualize hydrolysis zones. The appearance of distinct bands or zones of hydrolysis indicated active pectinase enzymes,

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providing insights into enzyme production and activity under tested conditions (Jalil & Ibrahim, 2021).

MALDI-TOF mass spectrometry analyzes ribosomal proteins in microorganisms, ionizing them into charged molecules to measure mass to charge ratio. Proteins are mixed with an energy absorbent matrix and ionized by a laser. The resulting mass spectrum, called Peptide Mass Fingerprint, identifies genera and species based on unique molecular weights (Amin et al., 2019).

Characterization of purified enzymes:

The purified enzymes were analysed to define their characterization by studying pH, temperature, metal ions, surfactant, and enzyme kinetics.

2.7. Immobilization:

Enzyme immobilization involves attaching enzymes to a support matrix to enhance stability and reusability. Various methods such as physical adsorption, covalent binding, and encapsulation can be employed. Alginate-based immobilization is a practical technique, where sodium alginate is mixed with water and added dropwise to calcium chloride to form beads. Mechanical stability, storage stability, and temperature stability of immobilized pectinase were evaluated.

Mechanical stability was assessed by applying shear force, while storage stability was tested at 4°C over 7 days (Bié et al., 2022). Temperature stability was determined by exposing enzymes to 50°C and monitoring activity using a DNS assay. Immobilized enzymes showed improved stability and reusability compared to free enzymes, making them valuable for various industrial applications. This alginate-based immobilization method offers a practical means of enzyme recovery and utilization, enhancing efficiency in industrial processes.

2.8. Application of pectinase

2.8.1. Clarification of fruit juice:

The clarification process of fruit juice involves several meticulous steps to ensure the production of a high-quality, visually appealing product. Fresh, ripe fruits are carefully selected, washed, and rinsed to eliminate any external contaminants. Following this, the fruits undergo grinding to achieve a homogenous juice consistency. Pasteurization at 85°C for 3 minutes is then applied to deactivate natural fruit enzymes, with subsequent cooling to room temperature.

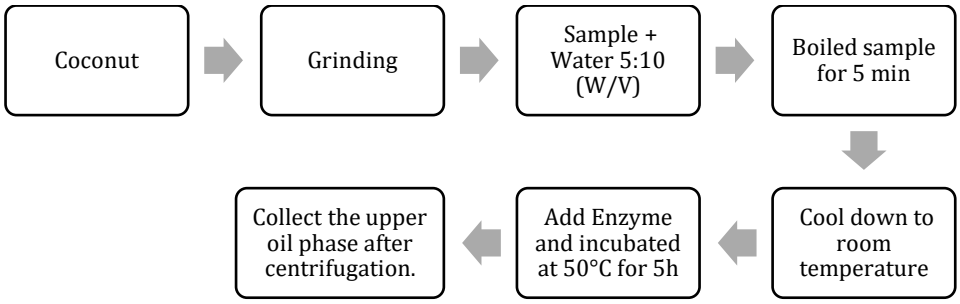
Optimization experiments are conducted to determine the most effective enzyme concentration and incubation time for the enzymatic treatment process. Using 25ml of juice, varying incubation times between 30 to 180 minutes at a constant temperature of 50°C are tested. After enzymatic treatment, the juice is subjected to a heating step at 90°C for 5 minutes to deactivate the enzyme.

The clarity of the juice is evaluated using a spectrophotometer to measure the percentage transmittance at a wavelength of 660 nm. Distilled water serves as a reference blank during measurements. The clarity percentage is calculated using a specific formula based on the difference in transmittance levels.

$$\textbf{Clarity \%} = \frac{\boldsymbol{Treated(OD) - Untreated\,(OD)}}{\boldsymbol{Untreated(OD)}} * \mathbf{100}$$

2.8.2. Oil Extraction from coconut:

a) By enzymatic method



b) Cold extraction:

Cold extraction (Smedes 1999) was performed with few modifications. Briefly, 5 g of grated coconut, 2.4 mL of propan-2 ol and 3 mL of cyclohexane were mixed and the samples kept overnight under refrigeration after a brief agitation under vortexing. The nonlipid material was removed by washing with aqueous 0.9% NaCl once using one fifth of the collected volume.

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c) Soxhlet Extraction:

The grated coconut (5.0 g) was fed to a soxhlet extractor fitted with a 0.25 L round bottom flask and a condenser. The extraction was carried out for 12 h with 0.15 L of petroleum ether (40 to 50°C). After extraction, petroleum ether was distilled off under vacuum using a rotary evaporator at 45°C. The oil obtained was stored under refrigeration (4°C), until used for further analyses.

d) DETERMINATION OF ACID VALUE AND FREE FATTY ACID:

The determination of acid value and free fatty acids in oils or fats involves titrating the material with aqueous alkali solution in an alcoholic medium. For acid value determination, a weighed sample is mixed with hot ethyl alcohol and phenolphthalein indicator, boiled, and titrated with standard alkali solution. The acid value is calculated using the formula (56.1 * V * N) / W, where V is the volume of alkali solution used, N is the normality of the alkali solution, and W is the weight of the sample. Free fatty acids are often expressed as a percentage, calculated based on oleic, lauric, ricinoleic, or palmitic acids, depending on the type of oil or fat. The free fatty acid percentage is determined using the formula (28.2 * V * N) / W, where V, N, and W have the same meanings as in the acid value calculation (Ajayi et al., 2021).

e) DETERMINATION OF IODINE VALUE:

The material is treated, in carbon tetrachloride medium, with a known excess of iodine monochloride solution in glacial acetic acid (Wijs solution). The excess of iodine monochloride is treated with potassium iodide. The liberated iodine estimated by titration with sodium thiosulphate solution.

$$\text{Iodine value} = \frac{12.69(B-S)N}{W}$$

B = Volume in ml of standard sodium thiosulphate solution required for the blank, S = volume in ml of standard sodium thiosulphate solution required for the sample, N = normality of the standard sodium thiosulphate solution, W = weight in g of the material taken for the test.

3. Result and Discussion:

3.1. Sample collection:

Sample of rhizospheric soil, root nodules, decomposed waste and decomposing fruits were collected from different located areas, Gujarat, India. The soil samples were kept in polythene bag at 4°C for further use (Oumer & Abate, 2018).

3.2. Isolation and Screening of polysaccharides degrading microbes:

The study aimed to isolate and screen microbes capable of degrading polysaccharides to assess their potential for extracellular hydrolytic enzyme production. Microorganisms were cultured on screening agar media containing different polysaccharides as the sole carbon source, allowing for the evaluation of their hydrolysis abilities. Out of 92 microbial isolates, 32 were selected based on their hydrolytic potential. These selected isolates, labeled DA1 to DA11, DC1 to DC8, and DP1 to DP12, exhibited clear hydrolysis zones on the agar plates, indicating strong enzyme activity. The screening for four key hydrolytic enzymes xylanase, cellulase, pectinase, and amylase was conducted on these isolates. Among the 32 selected isolates, 25 demonstrated activity for all three enzymes, while seven exhibited activity for two enzymes. Five isolates (DA1, DC3, DC7, DP8, and DP10) were chosen for further study based on their high hydrolysis capacity on pectin, starch, and cellulose substrates.

Subsequently, submerged fermentation was carried out under controlled conditions to further evaluate the enzyme production of these isolates. Enzyme assays were performed on crude enzyme extracts from the five selected isolates to measure the activity of amylase, pectinase, and cellulase. The results showed varying levels of enzyme production among the isolates. Pectinase activity was measured in the range of 0.038 to 0.250 Units/ml, with isolate DP8 showing the highest activity at 0.250 Units/ml. Isolates DC7 and DA1 also demonstrated strong pectinase activity. Overall, pectinase production was excellent across all isolates tested. Amylase activity showed more variation, with DP8, DC7, and DC3 producing moderate activity levels at 0.137, 0.107, and 0.111 Units/ml, respectively. DA1, however, exhibited the lowest amylase activity at 0.031 Units/ml. For cellulase, the enzyme activity was generally lower compared to pectinase and amylase. DC7 and DP8 displayed the highest cellulase activity at 0.097 and 0.095 Units/ml, respectively, while DA1 showed the lowest cellulase activity at 0.022 Units/ml.

In their study on microbial production of pectinase, Kaur and Gupta reported pectinase activity in the range of 0.028 to 0.256 U/ml from various bacterial isolates. The study focused on optimizing pectinase production using submerged fermentation. The maximum activity was observed under controlled pH and temperature

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conditions, with *Bacillus sp.* showing the highest pectinase activity of 0.256 U/ml.

Fig -1 The zone of hydrolysis on the different screening agar medium after flooding with Lugol's iodine. (A)-Starch hydrolysis, (B)-Pectin hydrolysis, (C)- Cellulose hydrolysis by DC7 and DC8 isolates.

Reddy and Saritha explored the production of cellulase by fungal isolates obtained from soil samples. They reported cellulase activity ranging from 0.06 to 0.12 U/ml, with *Aspergillus niger* showing maximum cellulase activity of 0.12 U/ml. The study also emphasized the influence of incubation time and temperature on enzyme production.

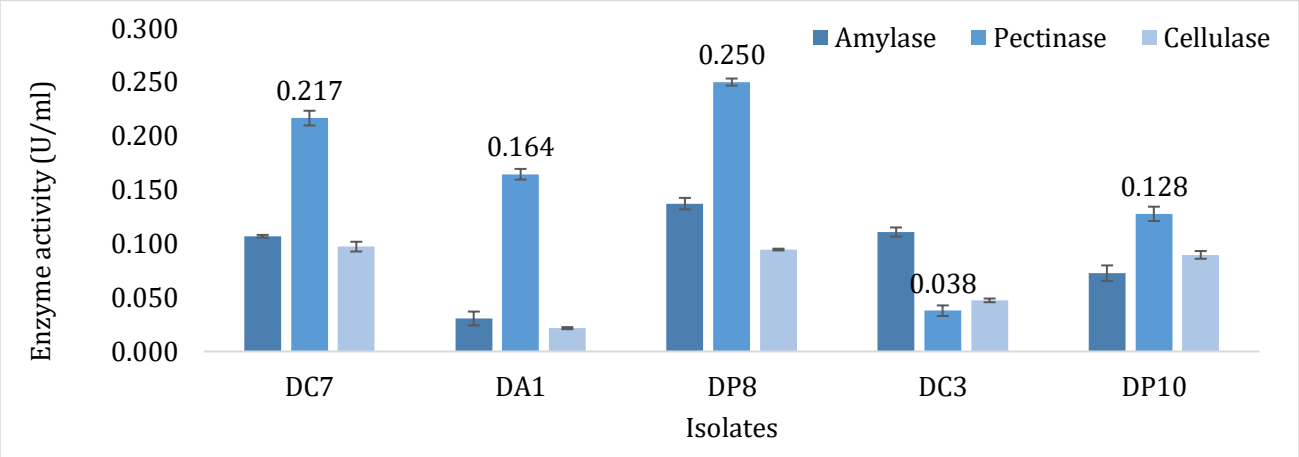

Fig -2 Result of study related to fermentative production of enzymes from selected isolates.

3.3. Identification of enzyme producing microbes:

The identification of enzyme-producing microbes utilized a polyphasic approach, considering morphological, cultural, and molecular characteristics as evaluative parameters. The results of cultural and microscopic examinations, detailed in tables-4, contribute to the comprehensive characterization of the enzyme-producing microbes, laying the foundation for further molecular identification.

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Table-4 Morphological characteristics of selected isolates.

Isolate	Gram's reaction	Shape	Size	Arrangement
DC7	Positive	Rod, Bacillus	Big	Single, Pair
DA1	Positive	Rod, Bacillus	Big	Pair, single
DP8	Positive	Bacillus	Big	Single, Chain
DC3	Positive	Bacillus	Big	Single, Chain
DP10	Positive	Rod	Big	Pair, single

Following secondary screening, isolates DP8 was prioritized for molecular identification due to their significant production of pectinase and amylase. DNA isolation confirmed purity, with high-molecular-weight DNA observed on agarose gel. PCR amplification of the SSU-rRNA gene was conducted, and subsequent sequencing and BLAST analysis revealed these isolates to be closest to *Bacillus spp*.

Several bacterial species have previously been identified as potential enzyme sources for bio-based industries through 16S rRNA sequencing (Prajapati et al., 2021; Kaur & Gupta, 2017). Additional bacterial genera known for their pectinolytic activity include species from Pseudomonas (Sohail & Latif, 2016) and Streptomyces (Ramirez-Tapias et al., 2015). According to Bergey's bacterial identification manual and 16S rRNA results, the bacterium was concluded to be *Bacillus inaquosorum.*

Phylogenetic Tree

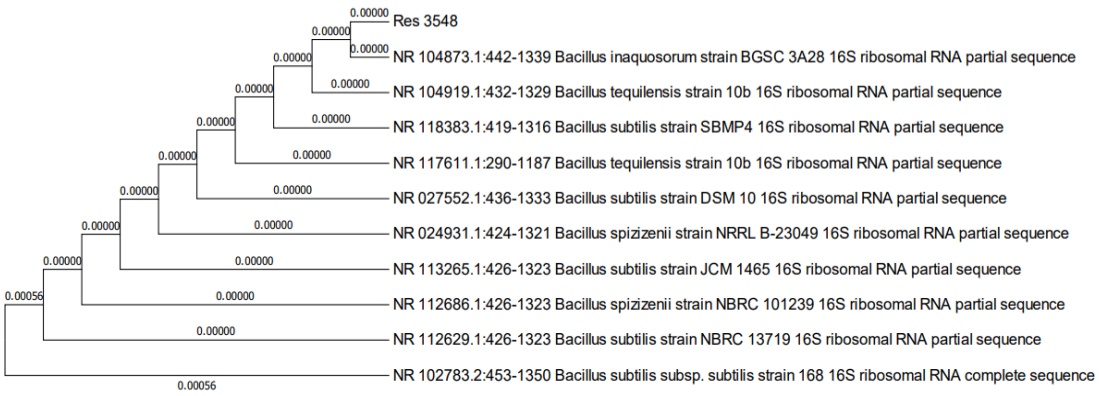

Fig-3 Evolutionary relationships of taxa for (DP8) *Bacillus inaquosorum* strain BGSC 3A28

3.4. Optimizing media and cultural conditions for pectinase activity:

3.4.1. One Factor at a Time (OFAT): The One Factor at a Time (OFAT) approach was employed to assess the impact of various factors on pectinase activity. Results indicated that optimal pectinase activity was achieved with dextrose DP-8 as the carbon source, ammonium sulfate as the nitrogen source, and specific pH, temperature, incubation time, and inoculum size.

Table-5 Result of One factor at time for pectinase.		
Isolates	DP-8 (***Bacillus inaquosorum)***	
Carbon sources (1%)	Glucose	(0.497 ± 0.001)
Nitrogen sources (0.5%)	Ammonium sulphate	(0.505 ± 0.001)

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pH	7	(0.495 ± 0.03)
Temperature (ºC)	35	(0.515 ± 0.05)
Incubation time (Hour)	48	(0.507 ± 0.03)
Inoculum size (%)	3%	(0.545 ± 0.02)

Similarly, research by Singh et al. in 2019 supported these findings, showing that temperatures exceeding 40°C led to enzyme denaturation and reduced activity. Research by Patel et al. in 2018 corroborated these findings, showing that an inoculum size of 2-6% (v/v) was ideal for *Bacillus subtilis* in submerged fermentation. Furthermore, a 2022 study by Kaur et al. explored the effects of varying incubation times on pectinase production in thermophilic fungi.

3.4.2. Screening of parameters using Plackett-Burman design:

The Plackett-Burman design was utilized to screen various nutrients affecting pectinase production by Bacillus sp. (Reddy & Saritha, 2016). The experiment identified pectin, glucose, and ammonium sulfate as significant factors for pectinase production with a confidence level exceeding 95%. Conversely, KNO_3 was deemed insignificant. These findings guided further investigation into the concentration requirements and interaction effects of the significant components. In a study conducted by Oumer et al., the production of pectinase was compared between solid-state fermentation and submerged fermentation using *Bacillus inaquosorum.*

Table-6 Statistical analysis of components for pectinase production by *Bacillus inaquosorum*

Component	Effect	Std Error	t value	P-value	coefficient (%)
Glucose	0.276	0.050	5.514	0.000	99.990
Pectin	-0.136	0.050	-2.719	0.011	98.910
KNO_3	0.122	0.050	2.431	0.102	89.790
Ammonium sulphate	0.246	0.050	4.924	0.000	99.990

Furthermore, Plackett-Burman design (PBD) analysis revealed that satkara peel (Citrus macroptera), urea, and $(NH_4)_2SO_4$ had a significant impact on pectinase production by *Aspergillus niger* ATCC 1640 during solid-state fermentation(Ahmed et al., 2021).

3.4.3. Response surface method:

The central composite design (CCD) was employed to explore interactions among significant factors affecting pectinase production by *Bacillus inaquosorum*. Twenty trials were conducted to examine various combinations of 0.4 g glucose, 0.15 pectin, and 0.11 g ammonium sulphate concentrations.

Table -7 Analysis of variance of quadratic model for glucoamylase production using *Bacillus inaquosorum*

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.03	9	0.2255	12.02	0.0003	Significant
A-Glucose	0.3136	1	0.3136	16.72	0.0022	
B-Pectin	0.3461	1	0.3461	18.46	0.0016	
C-Ammonium sulphate	0.8112	1	0.8112	43.26	< 0.0001	
AB	0.1879	1	0.1879	10.02	0.0101	
AC	0.0196	1	0.0196	1.05	0.3307	

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BC	0.0648	1	0.0648	3.46	0.0927	
A^2	0.0129	1	0.0129	0.6853	0.4271	
B^2	0.0348	1	0.0348	1.86	0.2029	
C^2	0.2361	1	0.2361	12.59	0.0053	
Residual	0.1875	10	0.0188			
Lack of Fit	0.1522	5	0.0304	4.31	0.0674	not significant
Pure Error	0.0353	5	0.0071			
Cor Total	2.22	19				

The regression equation, derived post-analysis of variance (ANOVA), provides an estimation of pectinase production level dependent on concentrations of pectin, glucose, and ammonium sulfate. The model's accuracy in predicting pectinase production was validated under conditions estimated by the model, yielding a predicted pectinase production of 1.18 U/ml. Subsequent experiments with optimized medium components confirmed maximum pectinase activity of 1.35 U/ml, validating the model's predictions. The CCD facilitated identification of significant levels influencing pectinase production, highlighting the importance of carbon and nitrogen sources in enzyme production.

Bacillus sphaericus (MTCC 7542) demonstrated polygalacturonase activity ranging from 4.3 to 6.2 U/mL when cultivated in a synthetic medium. Furthermore, a maximum PG activity of 5.60 U/mL was achieved through submerged fermentation of *B. subtilis* using hazelnut shell hydrolysate. In this study, co-culturing Bacillus species led to a significant enhancement in pectinase activity, increasing from 5.60 to 11.40 IU/mL.

3.5. Scale up of optimized media:

After meticulous optimization, focusing on critical factors like glucose, pectin, and ammonium sulfate, scale-up experiments were conducted in 3-liter volumes to validate the efficacy of the optimized media in larger batches. The study assessed pectinase activity in fermentation media at volumes of 100 mL and 3000 mL over durations of 24, 48, and 72 hours. Conversely, the 3000 mL media consistently exhibited higher pectinase activity across all time points, peaking at 2.237 U/mL at 48 hours. Achieved a 2- fold increase in enzyme activity in a 2000 mL scale-up but faced difficulties in maintaining pH stability (Garcia-Martinez et al., 2019).

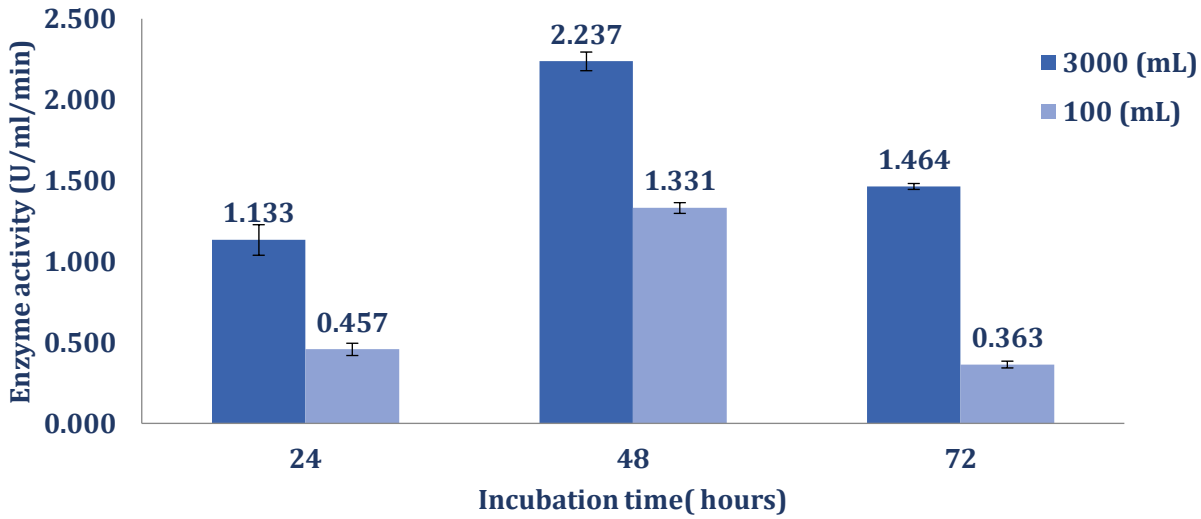

Fig - 4 Result of scale up study.

3.6. Purification of pectinase:

The purification of pectinase involves several techniques aimed at removing impurities and isolating the enzyme to enhance its purity and activity for industrial applications. The molecular weight determined by MALDI-TOF was 54842.917 Da (Figure 5). Detailed results of the enzyme purification can be found in Table 8, providing valuable insights into the efficiency of the purification methods.

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Table – 8 Result of purified pectinase from *Bacillus inaquosorum*					
Purification steps	Total activity (U/ml/min)	Protein Conc. (mg/ml)	Specific activity (U/mg)	Fold	Yield (%)
Crude	2.226 ± 0.04	0.102 ± 0.03	21.865 ± 0.05	1 ± 0.00	100 ± 0.00
Ammonium sulphate (30%)	2.014 ± 0.02	0.088 ± 0.05	22.888 ± 0.03	1.047 ± 0.04	90.460 ± 0.02
Ion Exchange Chromatography	1.629 ± 0.02	0.037 ± 0.01	44.027 ± 0.04	2.014 ± 0.01	73.167 ± 0.04
Gel permeation chromatography	1.355 ± 0.05	0.015 ± 0.03	90.333 ± 0.05	4.131 ± 0.03	60.861 ± 0.03

Following four purification steps, the pectinase was purified to achieve a 4.131 -fold increase in purity, resulting in a 60.86 % yield and a specific activity of 90.33 U/mg protein. In the SDS PAGE molecular weight found 52KDa. In fig-4 labeled as Lane M, molecular weight marker; lane (1) crude pectinase; lane (2) ammonium sulphate concentrated and dialysed protein sample; lane (3) partially purified protein sample after DEAE-cellulose ion exchange chromatography; lane (4) purified pectinase after Gel permeation chromatography.

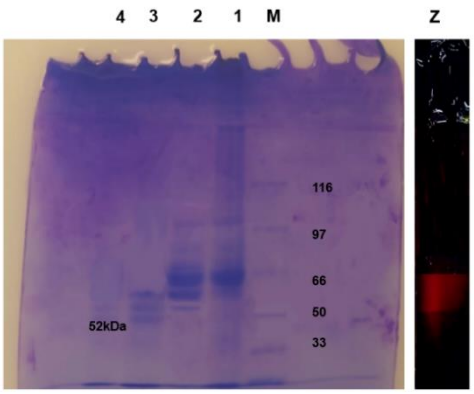

Fig - 4: SDS-PAGE profile of pectinase.

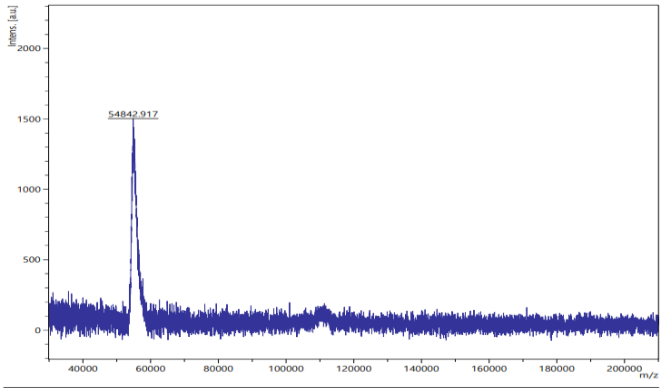

Fig-5 Molecular weight determination by MALDI-TOF analysis of purified enzyme

Pectinase from *Bacillus licheniformis* was purified using a strategy that included ammonium sulfate precipitation at 70% saturation, followed by dialysis and gel filtration chromatography. This purification process achieved a 7.1-fold enhancement in enzyme purity, with a yield of 38%. The specific activity of the enzyme increased significantly from 18 U/mg to 128 U/mg (Kumar & Verma, 2018).

3.6.1. Characterization of purified enzyme:

The characterization of the purified enzyme, pectinase, involved several key experiments to understand its properties and activity: Optimal activity was observed at pH 6.0, with a significant increase in production up to pH 5.0 and a decline beyond pH 7.0. Conversely, at pH 10.0, the production values were significantly lower. The temperature range of 30°C to 90°C was explored, with the highest production of pectinase observed at 50°C. Among tested metal ions, Fe^{+2} slightly enhanced pectinase production compared to the control, while other metal salts showed no significant impact. Surfactants like tween-20 and tween-80 did not enhance enzyme activity, while presence of mercaptoethanol, SDS, and EDTA decreased pectinase activity notably. Non-regression analysis showed a strong positive correlation between substrate concentration (Citrus Pectin) and enzyme activity. The K_m and V_{max} values were determined as 166.53 mM and 769.24 µMol/min, respectively, indicating the enzyme's affinity for its substrate and maximum catalytic efficiency.

3.7. Immobilization of enzyme:

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The immobilization of enzymes offers advantages in terms of stability and reusability compared to their free forms. In this study, pectinase was immobilized, and its activity was compared to the free enzyme. The enzyme activity of free and immobilized was measured, the free enzyme activity was 2.226 Units/ml/min, and immobilized beads activity was found to be 1.19 Unit/ml/min.

Mechanical Stability:

The immobilized beads demonstrated high mechanical stability, with 95% of the beads remaining undamaged after being subjected to shearing force for 5 hours. This indicates that the alginate beads used for immobilization are robust and can withstand bioprocessing conditions (Behram et al., 2023).

3.7.1. Storage Stability and Reusability:

Immobilization significantly improved the storage stability of the enzyme. The comparison between free and immobilized enzyme activity over time, stored at 4°C, showed that the immobilized enzyme retained higher activity levels, suggesting prolonged shelf life and reusability potential. According to Singh et al. (2019), the immobilization of pectinase in sodium alginate resulted in a 70% retention of its initial activity.

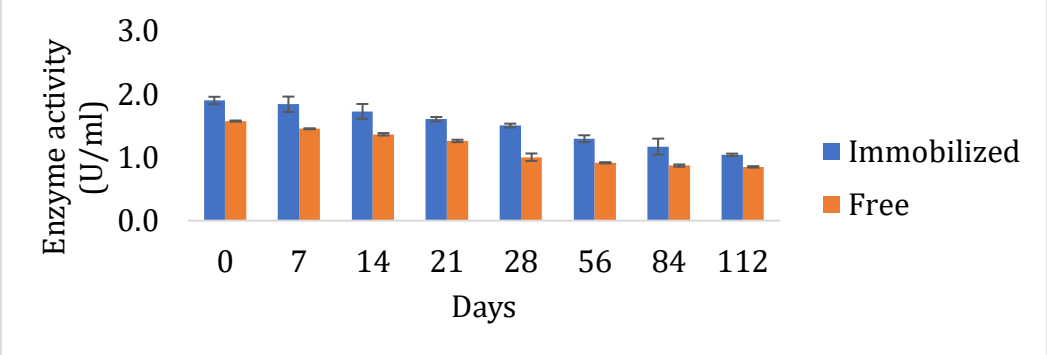

Fig-6 Storage stability of immobilization enzyme

3.7.2. Thermal Stability:

Immobilization also conferred greater thermal stability to the enzyme. While the free enzyme lost about 51% of its activity after exposure to 50°C for 120 minutes due to denaturation, the immobilized enzyme retained approximately 55% of its activity under the same conditions. This indicates that immobilization protects the enzyme from thermal denaturation to some extent.

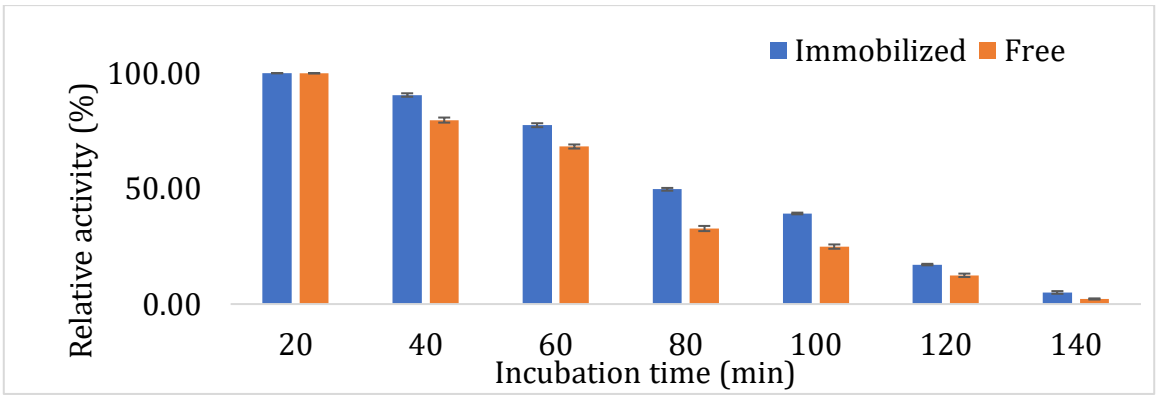

Fig-7 Thermal stability of immobilization enzyme

In research by Li et al. (2020), the immobilized pectinase demonstrated high reusability, retaining 75% of its initial activity after ten cycles of reuse. This was attributed to the protective environment provided by the sodium alginate matrix, which shields the enzyme from denaturation and degradation. Chen et al. (2021) demonstrated that immobilized pectinase retained 80% of its initial activity after 30 days of storage at 4°C,

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whereas the free enzyme showed a significant reduction in activity under the same storage conditions.

3.8. Studies related to fruit juice clarification by pectinase:

Preliminary experiments were performed to determine the optimum condition like enzyme concentration and incubation time for maximum yield and clarity of fruit juice. For the optimization of the enzyme treatment, 10ml of guava juice and mango were measured, treated with different concentrations, and incubated at a temperature of 50°C for different incubation times**.**

Table-9 Result of fruit juice clarification by pectinase

Incubation time (min)	Clarity (%)	
	Guava juice	Beetroot juice
0	0.46 ± 0.01	1.10 ± 0.008
30	48.01 ± 0.004	23.08 ± 0.005
60	90.49 ± 0.01	67.40 ± 0.004
90	90.95 ± 0.008	88.73± 0.008
120	90.94 ± 0.01	88.83± 0.009

Increasing enzyme concentration and Increasing time, the treated juice showed an increase in the clarity and yield. The maximum juice clarity of guava and beetroot were obtained at an incubation time of 60 min and 120 min by pectinase.

Table-10 characterization of fruit juice clarification by pectinase

Fruit Juice	pH	Viscosity (%)	Clarity (%)	Reducing sugar (U/ml)
Guava	**4.0**	**9.2**	**90.95 ± 0.008**	**1.64**
Beetroot	**4.0**	**1.2**	**88.83± 0.008**	**0.19**

Similarly, Ahmed et al. (2020) found that for beetroot juice, an enzyme concentration of 1.5% (v/v) resulted in 87% clarification. According to Gupta et al. (2019), the optimal temperature for pectinase activity in guava juice clarification was 45°C, with an incubation time of 2 hours. Under these conditions, the enzyme achieved an 89% reduction in turbidity.

4.4. Oil extraction by enzyme:

The comparison of oil extraction methods—enzymatic treatment, cold extraction, and Soxhlet extraction-reveals significant differences in oil yield and efficiency. Cold extraction stands out with the highest oil yield (65.83 ± 0.052%), attributed to its mechanical pressure method, which minimizes thermal degradation and efficiently disrupts cell walls for oil release. Enzymatic treatment, while offering specificity and gentleness, yields a moderate oil amount (51.11 ± 0.045%), suggesting less efficiency compared to cold extraction. Soxhlet extraction provides the lowest yield (25.89 ± 0.032%) due to solvent-based extraction, which might not fully dissolve all oil components, leading to lower recovery and potential solvent residues. A study by Kumar et al. (2019) found that combining pectinase treatment with mechanical pressing increased the oil yield to 88%, showing the synergistic effect of enzyme pre-treatment.

5. Conclusion:

The isolation and screening of polysaccharide-degrading microbes involved culturing microorganisms on screening agar media containing various polysaccharides as the sole carbon sources. Out of 92 isolates, 32 were selected based on their hydrolytic capacity, with five isolates exhibiting the highest hydrolysis capacity on pectin, starch, and cellulose substrates. Further identification using a polyphasic approach revealed two

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isolates as *Bacillus inaquosorum*, indicating their potential for enzyme production in industrial applications.

Optimization of media and cultural conditions for pectinase activity was conducted using the One Factor at a Time (OFAT) approach and Plackett-Burman design. The OFAT approach identified glucose, ammonium sulfate, pH, temperature, incubation time, and inoculum size as critical factors affecting pectinase activity. The Plackett-Burman design highlighted pectin, glucose, and ammonium sulfate as significant factors for pectinase production. Response Surface Methodology (RSM) further optimized these factors, resulting in enhanced pectinase production.

Scale-up experiments validated the efficacy of the optimized media in larger batches, demonstrating higher pectinase activity in 3000 mL media compared to 100 mL media. Purification of pectinase involved multiple steps, resulting in a 4.131-fold increase in purity, a 60.86 % yield, and a specific activity of 90.33 U/mg protein. Characterization of the purified enzyme revealed optimal pH and temperature ranges, metal ion effects, and kinetic parameters.

The immobilization of pectinase showed promising results in terms of stability and reusability compared to its free form. The immobilized enzyme exhibited high mechanical stability, improved storage stability, and greater thermal stability. Furthermore, studies related to fruit juice clarification by pectinase demonstrated its efficacy in enhancing juice clarity and yield.

In addition, a comparison of oil extraction methods highlighted cold extraction as the most efficient method, yielding the highest oil percentage. Enzymatic treatment showed moderate oil yield, while Soxhlet extraction provided the lowest yield due to solvent-based extraction.

Overall, this study provides valuable insights into the isolation, screening, and optimization of enzyme-producing microbes, along with the application of enzymes in various industrial processes such as fruit juice clarification and oil extraction. The findings contribute to the development of efficient and sustainable bioprocessing methods with potential applications in diverse industries.

References:

1. Ahmed, T., Rana, M. R., Zzaman, W., Ara, R., & Aziz, M. G. (2021). Optimization of substrate composition for pectinase production from Satkara (Citrus macroptera) peel using Aspergillus niger-ATCC 1640 in solid-state fermentation. *Heliyon*, *7*(10), e08133. https://doi.org/10.1016/j.heliyon.2021.e08133
2. Ajayi, A. A., Lawal, B., Salubi, A. E., Onibokun, A. E., Oniha, M. I., & Ajayi, O. M. (2021). Pectinase Production by Aspergillus niger using Pineapple Peel Pectin and Its Application in Coconut Oil Extraction. *IOP Conference Series: Earth and Environmental Science*, *655*(1). https://doi.org/10.1088/1755-1315/655/1/012014
3. Al-Zuhair, S., Ramachandran, K. B., Farid, M., Aroua, M. K., Vadlani, P., Ramakrishnan, S., & Gardossi, L. (n.d.). *Enzyme Research Enzymes in Biofuels Production*.
4. Amin, F., Bhatti, H. N., & Bilal, M. (2019). Recent advances in the production strategies of microbial pectinases—A review. In *International Journal of Biological Macromolecules* (Vol. 122, pp. 1017–1026). Elsevier B.V. https://doi.org/10.1016/j.ijbiomac.2018.09.048
5. Behram, T., Pervez, S., Nawaz, M. A., Ahmad, S., Jan, A. U., Rehman, H. U., Ahmad, S., Khan, N. M., & Khan, F. A. (2023). Development of Pectinase Based Nanocatalyst by Immobilization of Pectinase on Magnetic Iron Oxide Nanoparticles Using Glutaraldehyde as Crosslinking Agent. *Molecules*, *28*(1). https://doi.org/10.3390/molecules28010404
6. Bié, J., Sepodes, B., Fernandes, P. C. B., & Ribeiro, M. H. L. (2022). Enzyme Immobilization and Co-Immobilization: Main Framework, Advances and Some Applications. In *Processes* (Vol. 10, Issue 3). MDPI. https://doi.org/10.3390/pr10030494
7. Carrasco, M., Rozas, J. M., Alcaíno, J., Cifuentes, V., & Baeza, M. (2019). Pectinase secreted by psychrotolerant fungi: Identification, molecular characterization and heterologous expression of a cold-active polygalacturonase from Tetracladium sp. *Microbial Cell Factories*, *18*(1). https://doi.org/10.1186/s12934-019-1092-2
8. Champasri, C., Champasri, T., & Woranam, K. (2015). Purification, biochemical characterization of a Macrotermes gilvus cellulase and zymogram analysis. *Asian Journal of Biochemistry*, *10*(5), 190–204. https://doi.org/10.3923/ajb.2015.190.204
9. Chapman, J., Ismail, A. E., & Dinu, C. Z. (2018). Industrial applications of enzymes: Recent

advances, techniques, and outlooks. In *Catalysts* (Vol. 8, Issue 6). MDPI. https://doi.org/10.3390/catal8060238

10. Doan, C. T., Chen, C.-L., Nguyen, V. B., Tran, T. N., Nguyen, A. D., Wang, S.-L., & Vn (, A. D. N. (2021). *Conversion of Pectin-Containing By-Products to Pectinases by Bacillus amyloliquefaciens and Its Applications on Hydrolyzing Banana Peels for Prebiotics Production*. https://doi.org/10.3390/polym
11. Gajdhane, S. B., Bhagwat, P. K., & Dandge, P. B. (2016). Response surface methodology-based optimization of production media and purification of α-galactosidase in solid-state fermentation by Fusarium moniliforme NCIM 1099. *3 Biotech*, *6*(2). https://doi.org/10.1007/s13205-016-0575-7
12. Jalil, M. T. M., & Ibrahim, D. (2021). Partial purification and characterisation of pectinase produced by aspergillus niger LFP-1 grown on pomelo peels as a substrate. *Tropical Life Sciences Research*, *32*(1), 1–22. https://doi.org/10.21315/tlsr2021.32.1.1
13. Kaur, S. J., & Gupta, V. K. (2017). Production of pectinolytic enzymes pectinase and pectin lyase by Bacillus subtilis SAV-21 in solid state fermentation. *Annals of Microbiology*, *67*(4), 333–342. https://doi.org/10.1007/s13213-017-1264-4
14. Kohli, P., & Gupta, R. (2019). Application of calcium alginate immobilized and crude pectin lyase from Bacillus cereus in degumming of plant fibres. *Biocatalysis and Biotransformation*, *37*(5), 341–348. https://doi.org/10.1080/10242422.2018.1564745
15. Liu, C., Hao, L., Chen, F., & Yang, C. (2020). Study on extraction of peanut protein and oil bodies by aqueous enzymatic extraction and characterization of protein. *Journal of Chemistry*, *2020*. https://doi.org/10.1155/2020/5148967
16. Luang-In, V., Yotchaisarn, M., Saengha, W., Udomwong, P., Deeseenthum, S., & Maneewan, K. (2019). Isolation and identification of amylase-producing bacteria from soil in Nasinuan community forest, Maha Sarakham, Thailand. *Biomedical and Pharmacology Journal*, *12*(3), 1061–1068. https://doi.org/10.13005/bpj/1735
17. Mercimek Takcı, H. A., & Turkmen, F. U. (2016). Extracellular Pectinase Production and Purification from a Newly Isolated Bacillus subtilis Strain. *International Journal of Food Properties*, *19*(11), 2443–2450. https://doi.org/10.1080/10942912.2015.1123270
18. Murugan, T., Deepika, P., Kowsalya, A., Sivasubramanian, K., Rejisha, R. P., Murugan, M., & Wins, J. A. (2021). Production and characterization of extracellular pectinase from a newly isolated Bacillus species from fruit waste soil. *Materials Today: Proceedings*, *45*, 2087–2090. https://doi.org/10.1016/j.matpr.2020.09.607
19. Oumer, O. J., & Abate, D. (2018). Screening and molecular identification of pectinase producing microbes from coffee pulp. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/2961767
20. Patel, D., & Shah, H. (2024a). Bulletin of Environment, Pharmacology and Life Sciences Purification and characterization of pectinase from Bacillus sp. and its application in fruit juice clarification. In *Env. Pharmacol. Life Sci* (Vol. 13).
21. Patel, D., & Shah, H. (2024b). Isolation And Screening of Polysaccharide Degrading Microbes from Various Natural Sources. *Adv. Biores*, *15*(1), 76–84. https://doi.org/10.15515/abr.0976-4585.15.1.7684
22. Poturcu, K., Ozmen, I., & Biyik, H. H. (2017). Characterization of an Alkaline Thermostable Pectin Lyase from Newly Isolated Aspergillus niger _WHAK1 and Its Application on Fruit Juice Clarification. *Arabian Journal for Science and Engineering*, *42*(1), 19–29. https://doi.org/10.1007/s13369-016-2041-6
23. Rakaz, M. A., Hussien, M. O., & Ibrahim, H. M. (2021). Isolation, Extraction, Purification, and Molecular Characterization for Thermostable α - Amylase from Locally Isolated Bacillus Species in Sudan. *Biochemistry Research International*, *2021*. https://doi.org/10.1155/2021/6670380
24. Reddy, M. P. chandra, & Saritha, K. V. (2016). Effects of the culture media optimization on pectinase production by Enterobacter sp. PSTB-1. *3 Biotech*, *6*(2), 1–11. https://doi.org/10.1007/s13205-016-0502-y
25. Salbu, L., Bauer-Brandl, A., Alderborn, G., & Tho, I. (2012). Effect of degree of methoxylation and particle size on compression properties and compactibility of pectin powders. *Pharmaceutical*

Development and Technology, *17*(3), 333–343. https://doi.org/10.3109/10837450.2010.535831

26. Upadhyay, D., Universiy, P., Andhare, P., & Universiy, P. (2021). *Issn : 2277 – 4998 a Study on Lipase : Review*. *10*(June), 333–340.
27. Yadav, S., Maurya, S. K., Anand, G., Dwivedi, R., & Yadav, D. (2017). Purification, characterization and retting of Crotolaria juncea fibres by an alkaline pectin lyase from Fusarium oxysporum MTCC 1755. *3 Biotech*, *7*(2). https://doi.org/10.1007/s13205-017-0750-5