Current Trends in Biotechnology and Pharmacy
Vol. 15 (6) 131 -136, 2021, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2021.6.23

Morphological and Molecular Based Identification of Pectinase Producing *Staphylococcus scuiri* from Tuber

Sonia Sharma, Neeraj Wadhwa*
Department of Biotechnology, Jaypee Institute of Information Technology, Noida, Uttar Pradesh, India*
*Corresponding author:neeraj.wadhwa@jiit.ac.in

Abstract

Endophytic microorganisms (both bacteria and fungi) are excellent sources of bioactive natural products inhabit inside plants having the potential of being exploited for their beneficial properties as well as application in agriculture, health, food sectors. Further, the optimal cultivation conditions for the enhanced production of pectinolytic enzyme from bacterial strain *Staphylococcus sciuri* found in corm of *Amorphophallus paeoniifolius* was investigated. We report that the maximal pectinolytic enzyme production is in the modified PSAM medium where *Staphylococcus sciuri* could utilize was lactose(5gm) that replaced Pectin, Tryptone (3gm) that replaced peptone at 25oC with 10% inoculum, at pH 5. We could recover 12.61U of enzyme activity in crude cell free supernatant. Pectinase was partially purified by ammonium sulphate precipitation followed by DEAE sephadex chromatography. We could recover 66.0 units of enzyme in 30-60% Ammonium sulphate fraction 100mM DEAE sephadex.16S rRNA sequencing of isolated bacteria and Molecular Evolutionary Genetics Analysis version 5.2(MEGA5.2) was used to construct phylogenetic tree and Nucleotide Similarity and Distance calculated.

Keywords endophyte, tubers, *Staphylococcus sciuri*, *Amorphophallus paeoniifolius,* Pectinase,

Introduction

Pectinases (pectinolytic enzymes) is a group complex enzymes that catalyse the hydrolysis of pectin-bringing about de-polymerization, de-methylation, and de-esterification reactions. This enzyme separate polygalacturonic acid into monogalacturonic acid by opening glycosidic linkages. Pectinase enzyme is broadly classified into three types: pectin esterase, hydrolases and lyases. Hydrolases group catalyses the hydrolytic cleavage of α-1,4-glycosidic linkage in pectic acid and pectin they are also termed as Polygalacturonases and Polymethylgalacturonases .Pectin esterase brings aboutde-esterification of the methoxyl pectin whereas, Lyases (Polygalacturonate Lyase andPolymethylgalacturonate Lyase) catalyse the breakdown of α-1,4-glycosidic linkage in pectic acid and pectin, respectively by trans-elimination reaction and resulting information of unsaturated galacturonates [1,2]

Pectinase enzymes are widely distributed in nature. They mainly occur in plants, bacteria, fungi, yeasts, insects, nematodes and protozoa. Extracellular pectinase find commercial importance in biotechnology and various industries like pharmacy, paper , oil extraction, food processing, coffee and tea fermentation and increase the juice yield. Acidic pectinases are applied during pressing , straining, removing suspended particles to make the juice and beverages from apples, pear, grape juice, strawberry, blackberry and raspberry sparkling clear[3,4,5,6,7] Majority of pectinases for this purpose have been isolated from microbial sources of genus Bacillus and Cocci . Alkaline pectinases are mainly used in the degumming and retting of fiber crops, biopesticide industry and pretreatment of wastewater of fruit juice industries [8,9,10]. The present study involves isolation, identification and optimization of process parameters for single enzyme-(i.e., pectinase alone) assisted hydrolysis (enzyme concentration, pH, temperature,incubation time)

Materials and Methods

Isolation of Endophytic bacteria

Plant parts corm of *Amorphophallus paeoniifolius Ipomoea batata, Colocaesia esculenta* were cut into small pieces, surface sterilized and were isolated endophytes. Surface sterilization was done by washing the pieces with water followed by sodium hypochloride 0.1% and 70% ethanol for 1 min. Tissue extract was made in sterile water in ratio of 1:10 and 100ul of sample was spread on nutrient agar plates. Bacterial growth was observed and separated on NAM plates. Primary identification was done by gram staining [11]

Qualitative screening of bacterial strain for pectinase production

Bacterial isolates of corm and peel of selected underground tubers were cultivated in pectinase screening agar medium (PSAM) (nutrient agar containing 1.5% pectin pure). Media was sterilized by autoclaving at 121°c for 15 min. Bacterial isolates were streaked on plates the pectin containing agar plates was checked for pectin utilization which can be detected on flooding the culture plates with freshly prepared Iodine-Potassium Iodide solution (iodine-1.0g,potassium iodide-5.0g in 330ml distilled water) this solution gives color to the medium containing pectin, which results in a translucent halo region where pectin is degraded, which indicates the

presence of pectinolytic activity. From three pectinase producing isolates, two strains produced greater the zone diameter of the clearing zone and these can be considered as good pectinase producer [12]

Quantitative screening of bacterial stain for pectinase production

All pectinase producing endophytes were inoculated in 500 ml Erlenmeyer flask having 250ml of production media comprising of composition peptone 3g, yeast extract-0.5g, KH2PO4-0.15g, CaCl2- 0.001g, pectin -0.5g, Na2CO3-0.5g at pH 8 with 1% (v/v) inoculum (106spores/ml) and incubated at 30°C under shaking condition (150 rpm) for 7days. [13,14] The culture broth was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was used to evaluate enzyme activity.

Determination of amount of protein

The culture broth was centrifuged at 10000 rpm , 10 min and supernatant containg protein was extracted. The amount of protein produced was assayed by Bradford test.[15]

Determination of extracellular enzyme activity

This assay determines the reducing sugars produced as a result of enzymatic hydrolysis of pectin by Dinitrosalicylic acid reagent (DNS) method [16] For enzyme assay, culture broth was centrifuged and supernatant (100 L) from the culture broth was treated as source of enzyme. The substrate for enzyme was prepared by mixing 0.5% (w/v) citrus pectin in 0.1M of pH 7.5 phosphate buffer.

Enzyme was incubated with substrate (0.5%w/v) citrus pectin at 50°C, for 10 min Reaction was stopped by addition of 200ul of DNS reagent followed by heating in water bath (92°C) for further 10 min Enzyme blank was considered as blank.. One enzyme unit is defined as the amount of enzyme that catalyzes mol of galacturonic acid per minute (molmin−1) under the assay conditions. Enzyme activity was measured at 540 nm [17]

Identification of Bacterial strains producing Pectinase

The identification of selected bacteria was performed on the basis of Morphological, Biochemical and Molecular characteristics

Morphological Characterisation

Morphological characteristics such as colony, morphology (color,shape) and cell morphology (shape, gram reaction, arrangement) of the selected bacterial strain were studied for identification.

Biochemical Characterisation

The bacterial strain was subjected to various biochemical test including IMVIC tests, carbohydrate tests, starch hydrolysis, casein hydrolysis, high temperature, low pH, urease test and catalase, acetate utilization test, nitrate test, motility test, high salt.

Molecular characterization

All the seven endophytic bacteria were classified by 16S rRNA identification. Sequencing was done by Sanger method in ABI 3500xL genetic analyzer (Life Technologies, USA) at NCIM PUNE

Effect of various nutritional and physiological parameter on pectinase production

The effect of carbon and nitrogen sources on pectinase production was analysed by incubating in production media containing different carbon and nitrogen sources through one variable at a time approach [18,19]

Effect of nutritional parameters on pectinase production

Effect of substrate on pectinase production was analysed by growing the culture with different carbon and nitrogen sources. All the pectinase positive colonies were grown with different carbon (pectin, glucose, lactose, maltose, PGA, sucrose), nitrogen (tryptone, peptone, KNO3, NH4SO3

Effect of physiochemical parameters on pectinase production

The effect of pH was analysed by incubation the culture in media with different, pH.(4,6,,8,10,12). the production of pectinase was noted at different temperature(25°C,32°C,37°C,42°C,47°C,50°C) with different inoculums (2,4,6,8,10) concentration

Media optimization

Different parameters like carbon, nitrogen, pH, temperature,inoculum were selected and optimized for the better production of pectinase enzyme

Scale up procedure for pectinase producing microorganism in modified optimized media and enzyme production

All the pectinase producing cultures (3 nos) were inoculated in media with composition peptone 3gm, yeast extract-0.5g, KH2PO4-0.15gm, CaCl2-0.001g,pectin -0.5gm, Na2CO3-0.5gm at pH 8 for 2days , Following which the 10% inoculum was added in modified optimized media with replacement conditions as mentioned below in Table 1.

The cultures were further allowed to grow in the modified media for 72 hours and enzyme was purified .

Table 1: Composition of Modified optimised media

S.no	Replacement Condition	Staphylococcus sciuri	Exiguobacterium acetylicum	Exiguobacterium indicum
1	Carbon	Lactose	Pectin	Pectin
2	Nitrogen	Tryptone	KNO3	KNO3
3	pH	8	10	4
4	Temperature	37°C	42°C	50°C
5	Innoculum percentage	10%	10%	10%

Enzyme purification

Bacillus sp *Staphylococcus sciuri* pectinase was purified from one litre of culture broth (growth conditions: 0.01gm a $CaCl_2$, KH_2PO_4(1.5gm), Yeast extract(5gm), Na_2CO_3 (5 gm)., Peptone (3 gm) Pectin (5gm) 37° C, 110 rev/ min, 24 h) Modified medium was used for growth of S*taphylococcus sciuri* where lactose 5g, replaced Pectin, Tryptone 3gm replaced peptone. After 72 hrs of further incubation in the modified medium cell-free supernatant was collected (spun at 10,0000 rpm for 15 minutes). This cell free supernatant was saturated with $(NH4)_2SO_4$ to three cut of ammonium sulphate (0 -30%, 30 - 60%, 60-80% saturation). The precipitates were dissolved in minimum amount of Tris HCl buffer (0.01 M, pH 8) and dialysed against the same buffer, Dialysed fractions were further loaded on equilibrated on DEAE-Sephadex columns,

Ion exchange chromatography

Anion exchanger, DEAE-Sephadex, was packed into a glass column (15 _ 0:55 cm, 10 ml-bed volume). The column was equilibrated with Tris HCl buffer (10 mM,pH 8) and 2.0 ml sample was loaded on to it. Bound protein was eluted with Tris HCl buffer containing 0, 100, 200 and 400 mM NaCl concentrations. Fractions (1ml) were collected. The protein content pectinase activity was assayed of the fractions by method described earlier The fractions showing pectinase activity were pooled, concentrated and saved for further analysis

Results and Discussion

Several bacterial strains were isolated from different tuber from different areas of Ghaziabad and Delhi (see Table 2).Out of these strain in serial nos 1-3 were from *Amorphophallus paeoniifolius,* serial nos 4-5 were from *Colocasia esculenta* and serial nos 6-7 from Ipomoea batatas. All strain were maintained on nutrient agar medium

Table 2: Bacterial strain from different indigenous sources

S.no.	Bacterial strain	Source
1	A	*Amorphophallus paeoniifolius (Corm)*
2	B	*Amorphophallus paeoniifolius (Corm)*
3	C	*Amorphophallus paeoniifolius (Peel)*
4	D	*Colocasia esculenta*
5	E	*Colocasia esculenta*
6	F	*Ipomoea batatas*
7	G	*Ipomoea batatas*

Pure bacterial culture were assessed for presence of pectinolytic activity by growing at 37oC for 24hrs in screening agar medium (PSAM) (nutrient agar containing 1.5% pectin pure).. Pectinolytic activity was detected by visualizing a clear around the colony using potassium-iodide flooding method [6]. Among these isolates only

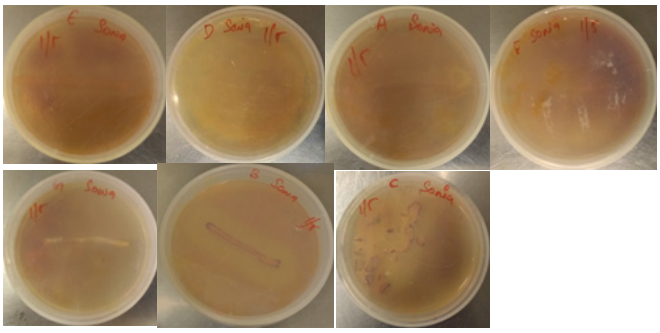

Figure 1 Qualitative screening of bacterial strains for pectinolytic activity on pectin agar medium. Strains A, B , F show clear halo and confirm that they are pectinase producers

three A,B,F showed pectinolytic activity. (See Fig 1)

The bacterial strain were further screened for pectinase production using batch fermentation by growing in a production medium containing peptone 3g, yeast extract-0.5g, KH_2PO4-0.15g, $CaCl_2$-0.001g,pectin -0.5g, Na_2CO_3-0.5g at pH 8 It was observed that the pectinase production varied from strain to strain (See Table 3) the strain A showed was a better producer of pectinase as compared to B and F.

The identification of selected bacteria strains was done on the basis of Morphological, Biochemical, Molecular characteristics as well as 16SrDNA sequence analysis. On the basis of morphology, physiology and biochemical tests, The selected strains was identified Strain A is that of *Staphylococcus scuiri*, B is *Exiguobacterium acetylicum,* F is *Exiguobacterium indicum*.

The details of biochemical reaction characteristics of pectinase producing strain are given in Table 4

The pure culture was sent to NCIM Pune for 16S rDNA sequencing. The nucleotide sequence of 16S rDNA was submitted in Gene Bank Database under accession number A as MK106142. The 16S rDNA sequence of bacterial strain was aligned with available 16SrDNA sequence in Gene Bank Database and finally phylogenetic tree was constructed Fig.3. The phylogenetic tree indicated that bacteria A belong to genus staphylococcus and the pattern of tree determined that the strain is closely related to other *Staphylococcus*

Table 3: Production of pectinase from different bacterial strain

Bacterial strain	Specific activity (umol/mgmin)	Relative activity (%)
Staphylococcus scuiri (strain A)	*12.61*	*100*
Exiguobacterium acetylicum (strain B)	*8.26*	*65.5*
Exiguobacterium indicum (strain F)	6.81	54.00

Table 4: Morphological,physiological and biochemical characteristics of maximum pectinase producing bacterial strain

Cellular characteristics	Staphylococcus scuiri	Exiguobacterium acetylicum	Exiguobacterium indicum
Gram staining	Positive	Positive	positive
Morphology	Cocci	Cocci	long
Motility	Non motile	Non motile	Non motile
Fermentation reaction			
Mannitol	+	+	+
Lactose	-	-	-
Glycerol	-	+	+
Sorbitol	+	+	-
Maltose	+	+	+
Starch	-	-	+
Dextrose	+	+	+
Sucrose	+	+	+
Fructose	+	+	-
Xylose	+	-	+
Biochemical reaction			
Indole	-	-	-
Methyl red	+	-	-
Voges proskauer	+	-	-
Citrate	+	-	-
Nitrate reduction	-	-	-
Catalase	+	+	+
Urease	-	-	+
Starch hydrolysis	-	+	+
Gelatin hydrolysis	+	-	-
Oxidase	-	-	-
pH 5	+	-	+

scuiri strain with100% 16s rDNA similarity. See Fig 2

Carbon, nitrogen, pH and temperature are the major element required for structural and metabolic activities of living organism .These sources has a profound effect on the production of enzymes by microorganism. The effect of carbon sources on the production of pectinase was determined by performing the fermentation in the production media with different carbon sources. *Staphylococcus scuiri* produced higher concentration of pectinase in the medium containing lactose as a source of carbon The sources of nitrogen cause significant impact on microbial enzyme production. The influence of various nitrogen sources on pectinase production by bacteria was determined by performing the fermentation in the production media with different nitrogen sources. observed that the *Staphylococcus scuiri* produced more pectinase in the medium with organic nitrogen sources like tryptone and peptone as compared to medium with inorganic nitrogen sources. Fig 3 a, b,

Table 5: Purification table for enzyme pectinase from *Staphylococcus sciuri*

PURIFICATION TABLE	TOTAL PROTEIN	TOTAL ACTIVITY	TOTAL SPECIFIC ACTIVITY	FOLD PURIFICATION	YIELD (%)
Crude	1300.00	2500.00	1.92	1.0	100.0
(NH4)2SO4 Precipitation (0-30%)	1484.60	641.33	0.43	0.2	25.7
30-60%	2076.30	67.50	0.03	0.0	2.7
60-80%	1995.04	584.60	0.29	0.2	23.4
DEAE -Cellulose 0-30 (400mM)	5.76	117.70	20.43	10.6	4.7
30-60(100mM)	0.26	13.57	52.18	27.1	0.5
60-80(400mM)	0.10	6.67	66.00	34.3	0.3

Along with nutritional parameter physiological parameter also play a major role the in pectinase production by *Staphylococcus scuiri.* The influence of different temperature pH, and inoculum was determined by performing the fermentation medium with different temperature, pH, and inoculum. The optimum pH for Staphylococcus scuiri was found to be 8 and temperature was found to be 37 °C when 10% inoculums was used. (Fig 4,5,6).. For the partially purified enzyme, We could recover 66.0 units of enzyme in 30-60% Ammonium sulphate fraction followed by elution with 100mM NaCl on DEAE sephadex chromatography at pH 8, and we report that the optimum temperature preferred by the partially purified enzyme is 25 °C (Table 5). Hence, this enzyme proves to be a promising candidate for commercial applications, where lower temperatures may be energy saving and faster reaction will be economically viable process which can be easily achieved as well as meet the increasing demand of the product where alkaline pectinase is required.

Conclusions

Staphylococcus sciuri is reported animal-associated bacterial species, commonly present on skin and mucosal surfaces of a wide range of pets and farm and wild animals and environmental reservoirs, such as soil, sand, water, and marsh grass but its clinical relevance for humans is increasing [20]. Few

subvarieties of S. sciuri also are frequently colonizing hospital environment, and are also anti biotic resistant[21[. We have isolated this strain from tuber which is found underground and would have come into the corm tissues during its development; hence the Molecular identification of the isolates to the species and subspecies levels is very important. Secondly, It is also well known that use of pectinase enzyme in commercial application is seen in everyday life and in industry level for example production lines including fruit juices and wine brewing. Alkaline Pectinase like the one found in our study on *Staphylococcus sciuri* have found application in retting of bast fibres, pulp and paper making also The study on application of this enzyme needs to be performed. We would also like to mention that there is requirement of temperature withstanding abilities for the enzyme. We propose that there is need of a mutant enzyme pectinase, that maybe more resistant to higher temperatures as compared to the enzyme that is found in this study and also the strain should pass the antibiotic susceptibility testing. This will ensure safety and unnecessary unintentional spread of the bacteria in the environment causing health issues.

Acknowledgement

Wish to acknowledge with thanks expert suggestions, advice and technical support provided by DPMAC Members at Jaypee Institute of Information Technology , JIIT for chemicals and lab space to the research scholar. Would also like to express sincere thanks to NCIM, Pune for their technical help on 16s rRNA technology sequencing.

References

1. Fogarty, M.V. & Kelly, C.T. (1983), In Microbial Enzymes and Biotechnology. ed.. London & New York: Elsevier Applied Science Publishers. 13-182

2. Gainvors, A., Belarbi, A.(1995), Detection methods for polygalacturonase producing strains of Saccharomyces cerevisae.Yeast ,10:1311-1319

3. D.R Kashyap., Chandra S. K. A.,Tewari R., (2000),Production, purification and characterization of pectinase from a Bacillus sp. DT7. World J. Microbiol. Biotech.16:277−282

4. D. D. Rokade, S. L. Vaidya, N. M. A. Rehman, and P. P. Dixit, . (2015) “Screening of pectinase producing bacteria, isolated from Osmanabad Fruit Market soil,” Int. J. Interdiscip. Multidiscip. Stud., vol. 2, no. 6, pp. 141–145.

5. Angayarkanni J, Palaniswamy M,Murugesan S and Swaminathan K(2002), Improvement of tea leaves fermentation with Aspergillus spp. pectinase. J. Biosci. Bioengg.,94:299-303.

6. O. J. Oumer and D. Abate, (2018), “Screening and Molecular Identification of Pectinase Producing Microbes from Coffee Pulp,” Biomed Res. Int., vol. 2018, no. April, pp. 1–7.

7. Ceci L, and Loranzo J (2008), Determination of enzymatic activities of commercial pectinases for the clarification of apple juice, Food Chemistry, 61: 237-241.

8. Bajpai,P.(1999).,Application of enzymes in the pulp and paper industry. Biotechnol prog, 15: 147-157.

9. Bruhlmann F, Kim KS, Zimmerman W , Fiechter A (1994) ,Pectinolytic enzymes from actinomycetes for the degumming of ramie bast fibers,Appl. Envt. Microbiol. 60:2107-2112.

10. Horikoshi, K.(1972), Production of alkaline enzymes by alkalophilic microorganisms Part III. Alkaline pectinase of Bacillus No P-4- N. Agricultural and Biological Chemistry 36:285-293.

11. G. A. Aaisha and D. L. Barate, (2016), “Isolation and Identification of Pectinolytic Bacteria from Soil Samples of Akola Region, India,” Int. J. Curr. Microbiol. Appl. Sci., vol. 5, no. 1, pp. 514–521.

12. H. A. MercimekTakcı 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.

13. Namasivayam, E., Ravindar, J.D., Mariappan, K., Akhil, J., Mukesh, K. and Jayaraj, R., (2011). Production of extracellular pectinase by Bacillus cereus isolated from market solid waste. J Bioanal Biomed, 3(3), pp.70-75.

14. Priya V and Sashi V, (2014), “Pectinase Enzyme Producing Microorganisms,” Int. J. Sci. Res. Publ., vol. 4, no. 3, pp. 1–4.

15. Bradford, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry, 72(1-2), pp.248-254

16. G. L. Miller (1959) Use of Dinitrosalicylic Acid Reagent for Determination of Reducing SugarAnalytical Chemistry 31 (3), 426-428.

17. Roosdiana A, Prasetyawan S, Mahdi C, Sutrisno X (2013) Productionand characterization of Bacillus firmus pectinase. J Pure Appl Chem Res 2:35–41.

18. Guan Y, Yin D, Du X, Ye X (2018a) Functional metabolomics approach reveals the reduced biosynthesis of fatty acids and TCA cycle is required for pectinase activity in Bacilluslicheniformis. J Ind Microbiol Biotechnol 45:951–960.

19. Guan Y, Yin D, Du X, Ye X (2018b) Metabolomics approach used for understanding temperature-related pectinase activity in Bacillus licheniformis DY2. Fems Microbiol Lett 365:23.

20. Kloos, W. E. (1980). Natural populations of the genus Staphylococcus. Annu. Rev. Microbiol. 34:559-592.

21. Dakić, I., Morrison, D., Vuković, D., Savić, B., Shittu, A., Jezek, P., Hauschild, T., & Stepanović, S. (2005). Isolation and molecular characterization of Staphylococcus sciuri in the hospital environment. Journal of clinical microbiology, 43(6), 2782–2785.