M.P.Kusuma et al. / Journal of Pharmacy Research 2014,8(2),106-112

Research Article
ISSN: 0974-6943

Available online through
http://jprsolutions.info

Optimization of Polygalacturonase using isolated *Bacillus subtilis* C4 by submerged fermentation

M.P.Kusuma * and D.Sri Rami Reddy
Centre for Biotechnology, Faculty of Chemical Engineering and Technology, College of Engineering, Andhra University, Visakhapatnam, Andhra Pradesh, India.

Received on:21-11-2013; Revised on: 08-12-2013; Accepted on:12-01-2014

ABSTRACT

Aim: Pectinases are an enzyme group that catalyzes pectin substance degradation through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions. **Method:** The present investigation is envisaged to study the production of alkaline and thermostable polygalacturonase using isolated soil *Bacillus subtilis*(C4) by submerged fermentation. The potential of the isolate was compared with that of procured ones. Effect of fermentation factors like incubation time, temperature, pH, inoculum age, inoculum volume, agitation speed, carbon and nitrogen sources were optimized. Effect of crude substrates like corn, bajra, white jower, yellow jower, wheat bran, lemon peel, lemon pulp, papaya peel, mango peel, apple pomace and orange peel were tested. **Result:** Process parameters like incubation time (19.65U/mL), temperature (28.08 U/mL), pH 10.0, inoculum volume(1mL), inoculum age (24h) and agitation (150rpm) were studied for maximum production of polygalacturonase. To the optimized medium, different carbon, nitrogen and amino acids supplements were used for the enhancement of enzyme activity. Polygalacturonase production significantly improved when yellow jower was used as a carbon source(42.64U/mL) and casein (45.69 U/mL) as nitrogen sources. Cysteine, glycine, arginine and histidine also stimulated polygalacturonase activity. **Conclusion:** The present study suggests that the new soil isolate *Bacillus subtilis* C4 is a good producer of polygalacturonase. Yellow jower as a carbon source significantly influenced enzyme activity. Considering the process advantages of thermostable enzyme and wide applications of alkaline polygalacturonase, the new soil isolate *Bacillus subtilis* C4 is a potential candidate for commercial exploitations.

KEYWORDS::Polygalacturonase, *Bacillus subtilis*,yellow jower etc.

1. INTRODUCTION

Enzymes are proteins that catalyze the chemical reaction both biosynthetic and degradative occurring in living cells. Pectic substances are high molecular mass glycosidic macromolecules found in higher plants. They are present in the primary cell wall and are the major components of the middle lamellae, a thin extracellular adhesive layer formed between the walls of adjacent young cells. In short, they are largely responsible for the structural integrity and cohesion of plant tissues[1]. Pectinases are an enzyme group that catalyzes pectin substance degradation through depolymerization (hydrolases and lyases) and deesterification (esterases) reactions. One of the most important and widely used commercial pectinase is polygalacturonase. Polygalacturonase catalyse hydrolysis of a-1, 4-glycosidic linkage in poly galacturonic acid producing D-galacturonate[2]. Pectinolytic enzymes are naturally produced by many organisms like bacteria, fungi, yeasts, nematodes, protozoan and plants. Alkaline pectinases are generally produced by bacteria particularly species of *Bacillus*, but are also made by some filamentous fungi and yeasts[3] and acidic pectinases are produced by fungi. Selection of the microbial source for polygalacturonase production depends on several features, such as the type of culture (solid-state or submerged fermentation), number and type of the produced pectinases (esterases, hydrolytic depolymerases and eliminative depolymerases), pH and thermal stability of the enzymes, and genotypic characteristic of the strain[4].

Submerged fermentation and solid state fermentation have been successfully used in pectinase production by fungi[5] and bacteria[6]. Pectinase production occupies about 10% of the overall manufacturing of enzyme preparations. These enzymes are widely used in the food industry in the production of juices, fruit drinks and wines[7].

They may be used in the pretreatment of waste water from vegetable food processing that contains pectin residues, the processing of textile fibers such as flax, jute and hemp, coffee and tea fermentation, vegetable-oil extraction and the treatment of paper pulp[8].

***Corresponding author.**
M.P.Kusuma
3-2-800, 3rd floor,
Anand Swaroop Nivas,
Kachiguda, Hyderabad,A.P.

2 MATERIALS AND METHODS

2.1 Microorganisms

Streptomyces lydicus (MTCC 4067), *Bacillus pumilus* (MTCC 1456), *Aspergillus niger* (*M*TCC 281), *Bacillus subtilis* C4 (isolated) were

used in this study. First three cultures were procured from Microbial Type Culture Collection, MTCC),Chandigarh, India and the fourth one was isolated from the soil, identified, maintained in nutrient agar and subcultured monthly. All the chemicals and reagents used were of analytical grade and obtained from reputed chemical manufacturers. The isolated pure cultures were screened for extracellular polygalacturonase using agar media containing pectin as a substrate(plate assay). Three replicates were prepared for each sample.

2.2 Polygalacturonase production by submerged fermentation

Erlenmayer flasks(250mL) containing media composed of 2g of KH_2PO_4, 1g of $(NH_4)_2SO_4$, 1g of $MgSO_4$, 0.95g of $Na_2HPO_4 2H_2O$, 1g of yeast extract, 5g of pectin were taken in 100ml of distilled water. The medium was inoculated with 1mL of a spore suspension of isolated *Bacillus subtilis* and cultivated at 50^0 C for 48h. After 2days of incubation the medium was centrifuged at 5000rpm for 15min and the supernatant was taken for the assay of amount of galacturonic acid produced.

2.3 Polygalacturonase assay

Polygalacturonase activity was assayed by the colorimetric method of Miller[9]. 0.5mL of supernatant was incubated with 1.0%w/v of pectin at 40 ^{0}C for 10min under static conditions. After adding 3mL of di nitro salicylic acid, the mixture was boiled for 15min and finally diluted to 5mL with distilled water. The absorbance was measured at 540nm. One unit (U) of polygalacturonase was defined as 1µmole of galacturonic acid released per mL-min.

2.4 Polygalacturonase production by submerged fermentation

The fermentation medium was prepared by using following ingredients (Table 2.1). The flasks were sterilized at 121^0C at 15lb/sq inch pressure. The sterilized medium was inoculated with 1mL of 12h culture of *Bacillus subtilis* maintaining a pH of 7.2. The flasks were incubated at 37^0C for 2 days.

Table 2.1 Composition of fermentation medium[6]

S.No	Medium Components	Quantity g/mL
1	KH_2PO_4	2
2	$(NH_4)_2SO_4$	1
3	$MgSO_4$	1
4	$Na_2HPO_4 2H_2O$	0.95
5	Yeast Extract	1
6	Pectin	5
7	Distilled water	100
8	pH	7.2

2.5 Optimization of process parameters

Fermentative factors like incubation time, incubation temperature, pH, inoculum age, inoculum volume, agitation speed, effect of carbon source, organic nitrogen and inorganic nitrogen were optimized. The strategy adopted for standardization of fermentation parameters was to evaluate the effect of an individual parameter and incorporate it at standard level before standardizing the next parameter.

2.5.1 Effect of Incubation time

In order to determine the optimum fermentation time, the fermentative medium was prepared, sterilized and inoculated with 1mL of 12h culture of *Bacillus subtilis*, incubated at 37^0C for 12,24,36,48,60,72 and 84h and samples were drawn aseptically at12h intervals and estimated for polygalacturonase activity.

2.5.2 Effect of Incubation temperature

To determine the effect of incubation temperature on the production of polygalacturonase the fermentation medium was prepared in 250mL, each flask was inoculated and incubated at a range of 5 ^{0}C - 60^0C temperatures with 5^0C increments.

2.5.3 Effect of pH

To determine the effect of pH on the production of polygalacturonase, the fermentation medium was adjusted to various pH ranging from 4-12 with pH interval of 1.0 and each flask was sterilized, inoculated and incubated.

2.5.4 Effect of Inoculum volume

To determine the effect of inoculum volume on the production of polygalacturonase, each flask was sterilized, inoculated with 0.8×10^2CFU/mL different inoculum volumes of 0.75,1.0,2.5, 5.0,7.5,10,12.5 and 15mL of 12h inoculum and incubated.

2.5.5 Effect of Inoculum age

In order to determine the optimum inoculum age, each flask was inoculated with inoculum of varying inoculum age ranging from 12h to 48h with 12h interval and assayed for enzyme activity.

2.5.6 Effect of Agitation speed

In order to determine the optimum agitation speed, each flask with fermentative medium was inoculated and subjected to various rpm viz. 120,150,180 and 210, incubated and assayed for the presence of enzyme.

2.5.7 Effect of Carbon source

To determine the effect of carbon source on enzyme activity, the fermentative medium in each flask was added with 1%w/v concentration of various natural materials like corn, bajra, white jower, yellow jower, wheat bran, lemon peel, lemon pulp, papaya peel, mango peel, apple pomace, orange peel and carbohydrate sources like starch, cellulose, maltose, sucrose and glucose assayed for enzyme activity.

2.5.8 Effect of Nitrogen source

To determine the effect of nitrogen source on enzyme activity, the fermentative medium in each flask was added with 1%w/v concentration of organic nitrogen source which include casein, peptone and gelatin and inorganic nitrogen sources of 0.1%w/v like ammonium citrate, ammonium chloride, ammonium oxalate, urea, ammonium sulphate, ammonium nitrate and ammonium molybdate inoculated, incubated and assayed for enzyme activity.

2.5.9 Effect of Amino acid

To determine the effect of amino acid on enzyme activity, the fermentative medium in each flask was added with 1mM conc. of various

amino acids like cysteine, glycine, arginine, leucine, glutamic acid, phenylalanine, ornithine, tryptophan, histidine and alanine sterilized, inoculated, incubated and assayed for enzyme activity.

3 RESULTS AND DISCUSSION

3.1 Isolation of polygalacturonase producing bacteria

A total of 66 colonies were isolated from four soil samples, on the basis of macroscopic characters and microscopic observations, eliminating those that appeared close to each other. All 66 isolated colonies were tested for their polygalacturonase potential using plate assay.

Of the 66 isolated colonies tested, only eight bacterial strains shown the polygalacturonase potential. Of the 8 positive isolates, C4 was showing relatively high activity. (Table 3.1 a and b)

Table 3.1(a) Screening of microorganisms having polygalacturonase activity

Sl.No	Source	No. of bacterial colonies isolates	No. of isolates shown polygalacturonase activity
1	Vegetable market place(MP1)	8	2
2	Compost (C)	20	3
3	Area surrounding the Musi river (MR)	14	2
4	Garden(G)	9	1
5	Soil near the laboratory	10	0
6	Soil in the parking area	5	0

Table 3.1 (b): Polygalacturonase activity of the isolates by plate assay

S.No	Source	Isolate No.	Polygalacturonase Activity (mm)
1	Vegetable Market	MP1	4
2	Vegetable Market	MP2	2
3	Compost	C2	4
4	Compost	C3	2
5	Compost	C4	14
6	Musi river	MR1	3
7	Musi river	MR2	5
8	Garden	G1	1

3.1.1 Screening of microorganisms for high polygalacturonase production

Plate assay was adopted for screening the polygalacturonase[10]. Pure culture was inoculated into the pectin agar medium and incubated at 37ºC for 48h. After the colonies reached around 3 mm, iodine-potassium iodide solution (1.0g iodine, 5.0g potassium iodide and 330mL H_2O) was added to detect clear zones.

Four cultures were used for the assay, out of which *Streptomyces lydicus* (MTCC 4067), *Bacillus pumilus* (MTCC 1456), *Aspergillus niger* (MTCC 281) were procured from MTCC, Chandigarh and isolate C4 isolated from soil. *Bacillus pumilus* (MTCC 1456), *Aspergillus niger* (MTCC 281) and isolate C4 shown maximum clear zone of 14mm and *Streptomyces lydicus (*MTCC 4067) has shown only 10mm zone (Table 3.2 and Fig.3.1).

Table 3.2 Comparison of polygalacturonase production of the new isolate with procured species

S.No.	Microorganism	Zone(mm)
1.	*Streptomyces lydicus* (MTCC 4067)	10
2.	*Bacillus pumilus* (MTCC 1456)	14
3.	*Aspergillus niger* (MTCC 281)	14
4.	Isolate C4	14

Fig 3.1 Plate assay of isolate C4 showing clear zone

3.2 Optimization of polygalacturonase production by one factor at a time approach

A wide range of fermentation conditions like time, temperature, pH, inoculum volume, inoculum age, agitation speed, effect of carbon source, nitrogen source and aminoacids were studied for the production of polygalacturonase of *Bacillus subtilis* C4 under submerged fermentation.

3.2.1 Effect of Incubation time

The effect of incubation period on enzyme activity was tested by incubating the medium for various time intervals ranging from 12h to 84h. Enzyme activity increased gradually and high titer (19.65U/mL) was observed at 48h of incubation and the activity of enzyme is almost constant till 72h thereafter, the activity decreased slightly either due to decrease in nutrient availability in the medium or catabolic repression of the enzyme[11] (Fig 3.2). The reduction in pectinase production after 72h might be the result of change in pH during fermentation [12] or due to the denaturation or decomposition of enzyme due to interaction with other components of medium[13].

Similar findings also reported 73h to be optimum incubation time for maximum polygalacturonase activity by a diploid construct from two *Aspergillus niger* over producing mutants[15]. It was reported that there was gradual increase in production from 24th h and highest production occurred at 36thh and stated that bacteria should have maintained its log phase from 24 to 36h[16].

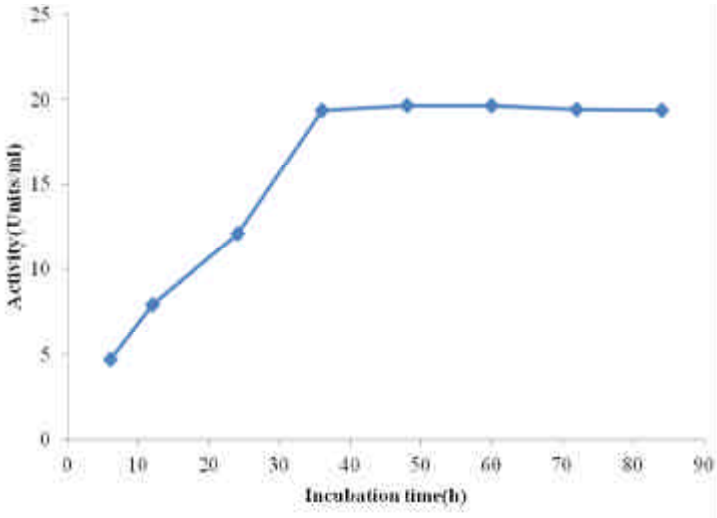

Fig. 3.2. Effect of Incubation time

3.2.2 Effect of Incubation temperature

Effect of incubation temperature on polygalacturonase production was tested at different temperatures ranging from 5^{0}C - 60^{0}C. The enzyme activity gradually increased with increasing temperature and reached maximum (28.08 U/mL) at 50^{0}C. Thereafter, the enzyme production decreased which may be due to enzyme denaturation (Fig. 3.3).

At higher temperature, the maintenance energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway results in minimum amount of product formation. At low temperatures the transport of substrate across the cells is suppressed and lower yield of product is attained [17].

Polygalacturonase production using *Bacillus subtilis* was stable in neutral to alkaline pH range at 70^{0}C[18]. Similar findings reported optimum temperature for PG and PME activities at 50^{0}C when *Bacillus licheniformis* was used[20].

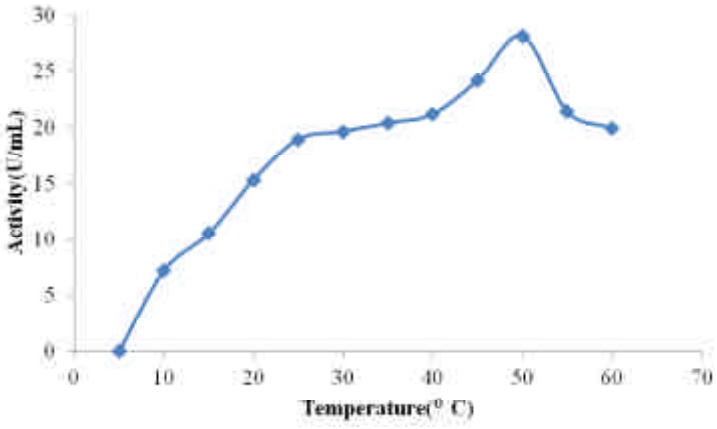

Fig. 3.3. Effect of Incubation temperature

3.2.3 Effect of pH

A gradual increase in polygalacturonase production was observed till pH7.0 thereafter it decreased and again attained a maximum value at pH10. The reduction in pectinase production might be due to denaturation or decomposition of enzyme due to interaction with other components of the medium[13] (Table 3.3).

Similar findings reported that most of the *Bacillus* sp. produce high amount of pectinase between pH 7-9[21]. It was reported that medium pH declined from 7.5 to 7.2 within 6h and thereafter, started to rise again till it reached 9.3 and this might be due to the fact that organic nitrogen was consumed when polygalacturonase was produced by *Bacillus* sp[22].

Table: 3.3 Effect of pH

pH	Activity (U/mL)
4	13.83
5	21.32
6	25.90
7	28.06
8	22.84
9	23.40
10	30.18
11	29.94
12	25.96

3.2.4 Effect of Inoculum volume

Different inoculum volumes of 0.75,1.0,2.5,5.0,7.5,10.0,12.5 and 15.0 (%v/v) (0.8×10^{2}CFU/mL) were tested to determine the optimum inoculum volume for maximum polygalacturonase production.

The optimum inoculum volume needed to produce the highest yield of enzyme was 1.0mL (30.10U/mL).Optimum inoculum size of 1mL was able to provide enough biomass with optimal length of log phase leading to high levels of enzyme production. Further increase in inoculum decreased the enzyme production. This condition could be due to competition for the nutrients among the bacterial cells. Adequate nutrient supply could be the reason of higher enzyme production with optimum inoculum volume[24]. When the inoculum volume was increased more than 1mL, the activity decreased (Table 3.4). This may be due to the amount of pectin which was limited in 250mL flask and any increasing inoculum volume does not produce any increase in the yield of polygalacturonase [25].

High inoculum levels are inhibitory in nature and high inoculum density leads to population overcrowding, high nutrient competition and rapid exhaustion of nutrients. The lower inoculum density, may give insufficient biomass causing reducing product formation[26]

Table 3.4 Effect of Inoculum volume

Volume(mL)	Activity (U/mL)
0.75	25.78
1.0	30.10
2.5	18.78
5.0	6.89
7.5	4.71
10.0	3.92
12.5	3.88
15.0	3.84

3.2.5 Effect of Inoculum age

Effect of inoculum age was tested by inoculating the fermentative medium with 1mL inoculum of varying age 12h, 24h, 36h and 48h and the filtered broth was assayed for enzyme activity.Maximum activity(30.17U/mL) was observed when 24h inoculum was used. Thereafter the activity decreased which might be due to the fact that the younger cultures being nonsporulating, entered the growth phase very soon, while the older culture produced spores which took longer time to germinate, and therefore, the enzyme production was low[27] (Table 3.5).

Table 3.5 Effect of Inoculum age

Age of the Inoculum(h)	Activity (U/mL)
12	29.20
24	30.17
36	27.48
48	21.59

3.2.6 Effect of Agitation speed

The effect of agitation speed was studied by subjecting the fermentation medium to various rpm viz. 120,150,170 and 200.The optimum agitation speed required to produce greater polygalacturonase production was 150rpm(30.19U/mL) (Table 3.6). Speed greater or lesser than this produced less polygalacturonic acid. At this speed sufficient aeration must have been present which will provide sufficient dissolved oxygen to the medium[29].

It was observed that increase in mixing help the microbial synthesis of enzyme which consequently increases the assimilation of sugars[30]. The cell growth started to decline after achieving the maximal production. This condition could be due to shear forces derived from higher agitation speed where collision among the cells occurred and damaged them[24]. At lower agitation speed, the inadequate mixing of the broth towards the later stages of growth affected the enzyme synthesis, while the drastic dropped in enzyme activity at higher agitation speeds was due to shearing effect on the cells. Changes in morphology of microorganisms caused by agitation speed also influenced enzyme production and growth of the microorganisms[31].

Table 3.6 Effect of agitation speed

Agitation speed(rpm)	Activity (U/mL)
120	28.27
150	31.19
180	29.48
210	26.70

3.2.7 Effect of Carbon source

To determine the effect of carbon sources on enzyme production, different carbon sources were tested which include natural materials like husks of corn, bajra, white jower, yellow jower, wheat bran, lemon peel, lemon pulp, papaya peel, mango peel, apple pomace and orange peel and various carbohydrates like cellulose, starch, maltose, glucose and sucrose. Each carbon source were incorporated at 1%w/v concentration into the fermentation medium in place of pectin. Polygalacturonase production was significantly increased about 33% when yellow jower was used as a carbon source (42.64U/mL), while wheat bran also increased the production (36.64U/ml) (Table 3.7). Whereas sucrose, starch, cellulose have repressive effect. Similar results were observed where all the tested carbon sources failed to induce polygalacturonase production by growing *Bacillus firmus*-I-10104 on *Solanum tuberosum* peels under SSF conditons[25].

1%w/v apple pectin showed maximum polygalacturonase production at 37^0 C after 48h incubation when carbon sources were tested in the production of pectin depolymerising enzyme using *Bacillus licheniformis* KIBGE IB-21[33]. Glucose, sucrose, starch, cellulose did not have any affect which might be due to catabolite repression[16].

Table 3.7 Effect of Carbon source on polygalacturonase

Carbon source(1.0%w/v)	Activity (U/mL)
Control	31.19
Corn	26.42
Bajra	23.85
White Jower	30.92
Yellow Jower	42.64
Wheat bran	36.64
Lemon peel	29.98
Lemon pulp	20.90
Papaya peel	27.39
Mango peel	28.35
Apple pomace	30.85
Orange peel	26.05
Carbohydrates	
Cellulose	21.88
Starch	24.13
Maltose	26.91
Glucose	29.38
Sucrose	25.49

3.2.8 Effect of Nitrogen source

The effect of different nitrogen sources was studied by introducing 1%w/v concentration of organic nitrogen sources and 0.1%w/v of inorganic nitrogen sources.

The polygalacturonase production marginally improved in the presence of casein (45.69 U/mL) followed by ammonium sulphate (43.95 U/mL) whereas other sources tested have repressing effect on enzyme activity (Table 3.8). Similar reports states that casein hydrosylate was found to enhance the polygalacturonase activity up to 20% and ammonium chloride decrease the enzyme activity up to 10%[14] and yeast extract was found to the best nitrogen source for bacterial isolate to produce pectinase enzyme compared to other nitrogen sources like ammonium sulphate, tryptone, peptone and potassium nitrate[16].

Table: 3.8 Effect of Nitrogen source on polygalacturonase production

Nitrogen source	Activity (U/mL)
Organic nitrogen	
Casein	45.69
Peptone	29.78
Gelatin	23.95
Inorganic nitrogen	
Ammonium citrate	29.05
Ammonium chloride	28.66
Ammonium oxalate	25.52
Urea	24.34
Ammonium sulphate	43.95
Ammonium nitrate	24.34
Ammonium molybdate	28.66
Control	42.64

3.2.9 Effect of Amino acid

The influence of different amino acids on polygalacturonase activity was studied by supplementing the aminoacid at 10mM.

Cysteine, glycine, arginine and histidine stimulated polygalacturonase activity. Whereas other aminoacids had an inhibitory effect (Table 3.9). The results indicate that enzyme production is not a direct function of cell growth because polygalacturonase production was having stimulative effect against various aminoacids [32].

The addition of aminoacids and their analogues such as DL-nor Leucine, L-leucine, DL-isoleucine and L-lysine monohydrochloride to the growth medium stimulated pectinase upto 2.78 fold when *Streptomyces sp*.QG-11-3 was used [34]. Production of an alkaline polygalcturonase from *Bacillus sp* MG-CP-2 was also influenced in the presence of aminoacids, vitamins and surfactants in both SMF and SSF[33] .

Table 3.9 Effect of amino acid

Conc of Amino acid (1mM)	Activity (U/mL)
Control	45.69
Cysteine	51.24
Glycine	49.10
Arginine	48.89
Leucine	29.37
Glutamic acid	29.48
Phenylalanine	29.09
Ornithine	15.31
Tryptophan	26.31
Histidine	35.50
Alanine	36.23

4. CONCLUSION

The present study suggests that the new soil isolate *Bacillus subtilis* C4 is a good producer of polygalacturonase. Yellow jower as a carbon source significantly influenced enzyme activity. Considering the process advantages of thermostable enzyme and wide applications of alkaline polygalacturonase, the new soil isolate *Bacillus subtilis* C4 is a potential candidate for commercial exploitations.

ACKNOWLEDGEMENTS

The author thank the Principal of RBVRR College of Pharmacy for providing laboratories.

REFERENCES

1. Rambouts FM and Pilnik W. Pectic enzymes. In: Economic Microbiology, Bioconversions. (Ed.Rose AH) Academic Press, London, 1980; pp.227-72.
2. Coutinho PM and Henrissat B. Carbohydrate active enzymes: an integrated database approach. In “Recent advances in carbohydrate bioengineering”, Gilbert H J,Davis M, Henrissat B, Svensson B.Eds. Cambridge. The Royal Society of Chemistry, 1990; pp. 3-12
3. Kashyap DR.,Chandra S,.Kaul A and Rupinder T. Production, purification and characterization of pectinase from a *Bacillus* sp.DT7. *World J. Microb Biotechnol.,* 2000;16: 277-282.
4. Torres EF, Sepulveda TV and Gonzalez GV. Production of hydrolytic depolymerising pectinases, *Food Tech Biotech.,* 2006; 44:221-227.
5. Dinu D,Marina TN,Gheorge S,Marieta C and Anea D. Enzymes with new biochemical properties in the pectinolytic complex produced by *Aspergillus niger* MIUG 16. *J Biotechnol.*,2001;131: 128-237.
6. Jacob N,Poorna AC and Prema P. Purification and partial characterization of polygalacturonase from *Streptomyces lydicus. Bioresour Technol.*,2008;99:6697-6701.
7. Semenova MV, Grishutin SG, Gusakov AV,Okunev ON and Sinitsyn AP. Isolation and properties of pectinases from the fungus *Aspergillus japonicus. Biochem.*,2003;68:559-569.
8. Kashyap DR,Vohra PK,Sanjeev Kumar S and Rupinder T. Degumming of buel (*Grewia optiva*)bast fibres by pectinolytic enzyme from *Bacillus* sp DT7. *Biotechnol lett*.,2001 23:1297-1301.
9. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem.,* 1959 31: 426-428.
10. Chesson A and Codner RC. The maceration of a vegetable tissue by a strain of *Bacillus subtilis. J Appl. Microbiol*, 1978; 44: 347-364.
11. Saber WIA and Noura E. El-Naggar**.** Optimization of fermentation conditions for the biosynthesis of inulinase by the new source; *Aspergillus tamarii* and hydrolysis of some inulin containing agro-wastes. *Biotechnology,* 2009; 8:425-433.
12. Samriya S, Rishi PM and Jitendu S. Utilization of agroindustrial residues for pectinase production by the novel strain *Pseduozyma* sp SPJ under solid state cultivation. *Ann.Microb.* 2012;62:169-176.
13. Soares MMCN, Da Silva R, Carmma E.C and Gomes E.

Pectinolytic enzyme production by *Bacillus* sp and their potential application in juice extraction. *World J. Microb Biotechnol.*, 2001; 17:79-82.

14. Jayani RS, Surender Kumar S and Gupta R. Screening of bacterial strains for poly galacturonase activity; Its production by *Bacillus sphaericus* (MTCC7542) *Enzyme res.*,2010;10:1-18.
15. Loera O ,Aguirre J and Gonz´alez GV. Pectinase production by a diploid construct from two *Aspergillus niger* overproducing mutants, *Enzyme and Microb. Technol.*, 1999; 25: 103–108.
16. Namasivayam E, Ravindar DJ, Mariappan K, Akil J, Mukesh Kumar and Jayaraj L. Production of extracellular pectinase by *Bacillus cereus i*solated from market solid waste. *J Bioanal. Biomed.*,2011; 3: 70-75.
17. Alba S, Humphrey AE and Miller NF. *Biochemical Engg.* 2nd Edition, New York, Academic Press.1973; pp 92-127.
18. Ahlawat S, Bindu B, Sudha SD, Jitender Sharma and Mandhan RP. Production of thermostable pectinase and xylanase for their potential application in bleaching of kraft pulp. *J Ind. Microbiol.* 2008; 34:763-770.
19. Qureshi AS, Bhutto MA, Chisti Y, Khushk I, Dahot MU and Safia Bano. Production of pectinase by *Bacillus subtilis* EFRL 01 in a date syrup medium. *Afr J Biotechnol.*, 2012;11:12563-12570.
20. Rehman HU,Qader SAU and Aman A. Polygalacturonase : Production of pectin depolymerising enzyme from *Bacillus licheniformis* KIBGE IB-21. *Carbohyd polym,* 2012;90:387-391.
21. Kobayashi T, Hatada Y, Higaki N, Ozawa DDLT, Koike K, Kawai S and Ito S. Enzymatic properties and deduced amino acid sequence of a high-alkaline pectate lyase from an alkaliphilic *Bacillus* isolate. *Biochimica et Biophysica Acta (BBA) - General Subjects,* 1999; 1427:145-154.
22. Andrade MVV, Delatorre AB, Silvania Alves Ladeira and Martins MLL. Production and partial characterization of alkaline polygalacturonase secreted by thermophilic *Bacillus* sp. SMIA-2 under submerged culture using pectin and corn steep liquor.*Cienc. T 206 ecnol. Aliment., Campinas,* 2011; 31: 204-208.
23. Kashyap DR, Vohra PK, Chopra S and Rupinder T. Applications of pectinases in the commercial sector. A review. *Bioresour Technol.,* 2001; 77: 215-227.
24. Darah I, Nisha M and Lim SH. Enhancement of polygalacturonase production from *Enterobacter aerogenes* NB02 by submerged fermentation. *Adv Studies in Bio.* 2013; 5: 173-189.
25. Reda AB, Hesham M, Yassin HM, Swelim MA and Abdel EZ. Production of bacterial pectinases from agro-industrial wastes under solid state fermentation conditions. *J. Appl Sci. Res.*, 2008, 4:1708-1721.
26. Mudgett, A.E.1986. Solid state fermentation in A.L.Demain and N.A. Solomon, eds. Manual of Industrial Microbiology and Biotechnology, *Amercian Society for Microbiology*. Washington,D.C.1980. pp. 66-83.
27. Krishna C, "Solid-state fermentation systems—an overview," *Crit.Rev. Biotech.,* 2005; 25: 1–30.
28. Jayani RS, Surender Kumar S and Gupta R. Screening of bacterial strains for poly galacturonase activity; Its production by *Bacillus sphaericus* (MTCC7542) *Enzyme res.*,2010;10:1-18.
29. Heydarian and Seyed. Mohammed. Polygalacturonase production by *Aspergillus* sp.in Airlift and agitator fermenter. *Iran J Chem. Chem. Eng.,* 2009; 28:103-107.
30. Potumarthi LR and Jelly A. Alkaline protease production by submerged fermentation in stirred tank using *Bacillus licheniformis NCIM 2042*, Effect of aeration and agitation regimes, *Biochem. Engg. J.* 2007;34:185-192.
31. Darah I and Ibrahim CO. Comparison of lignin degrading enzyme productivity by free and immobilized cells of *Phanerochaete chrysosporium* in agitated cultivation vessel. *Malays.Appl.Bio.*,1998;27:91-99.
32. Kapoor M, Beg QK, Bhushan B, Singh K, Dadhich KS and Hoondal GS. Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp. Mg-CP-2 in degumming of ramie (*Boehmedria nivea*) and sun hemp(*Crotolaria juncea*) bast fibers. *Process Biochem.*, 2001; 36 : 803-807.
33. Kapoor M and Kuhad RC. Improved polygalacturonase production from *Bacillus* sp.MG-CP-2 under submerged (SmF) and solid state (SSF) fermentation. *Lett. Appl. Microbiol.*, 2002; 34: 317-322.
34. Beg QK, Bhushan B, Kapoor M and Hoondal GS. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp.QG-11-3. *J Ind. Microbiol.* 2000; 24:396-402.
35. Rehman HU,Qader SAU and Aman A. Polygalacturonase : Production of pectin depolymerising enzyme from *Bacillus licheniformis* KIBGE IB-21. *Carbohyd polym,* 2012;90:387-391.

Source of support: Nil, Conflict of interest: None Declared