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STATISTICAL APPROACH FOR PECTINASE PRODUCTION BY *Bacillus firmus* SDB9 AND EVALUATION OF PECTINO-XYLANOLYTIC ENZYMES FOR PRETREATMENT OF KRAFT PULP

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ABSTRACT

The present study originated with the concomitant production of 1.94 IU.mL^{-1} of pectinase, 0.34 IU.mL^{-1} xylanase and 0.086 IU.mL^{-1} cellulase from a newly isolated *Bacillus firmus* SDB9 in pectin salt media. Plackett-Burman Design (PBD) and Box-Behnken design (BBD) were used for optimization of mass production of pectinase using agro-residues. Statistical optimization of medium enhances the pectinase production to 17.55 IU.mL^{-1}, 17.7% higher activity than that of unoptimized medium. The optimal pectinase activity was found at pH 8.5 and 45°C temperature. The enzyme was alkali-stable over a range of pH 7.0 to 10.0 for 1 h and thermostable at 35 to 70°C for 1h. Out of 12 tested metal cations at 1mM concentration, the enzyme was found to be hindered by the presence of three cations, whereas four cations were reported to augment, and the rest marginally subdued the activity. Pretreatment with 15 IU pectinase and 2.7 IU xylanase per gram of OD pulp reduce kappa number by 7.9%. After bleaching sequence D_0-E_p-D_1, the increment in pulp brightness was 2.7% with acceptable whiteness level. Pretreatment also led to a reduction in ClO_2 consumption by 15% with superior brightness level. The 0.83% increase in pulp viscosity along with 3.2% gain in double fold number indicated the maintenance of relative cellulose content and strength of paper. Minor variations in burst index, tensile index and tear index reflected the conserved properties of the treated pulp. Synergistic application of mixed enzymes preparation produced from raw agro-residue headed by pectinase in preference to xylanase is first time set forth in the present study to help in cost economization and propel the pulp and paper industries towards environmental friendly future.

Keywords: Pectinase; xylanase; optimization; *Bacillus firmus* SDB9; pretreatment; kraft pulp

INTRODUCTION

The pulp and paper industry uses an immense amount of hazardous chemicals to process inordinately high quantities of raw materials for the production of virgin pulp. Pulp and paper industries account for creating extremely high pollution in the environments globally (**Sumathi and Hung, 2006; Thompson *et al.*, 2001**). Therefore, key attention has been to develop cost effective and environmentally benign bleaching technologies for a reduced adsorbable organic halogens (AOX) generation as many countries have now set discharge limits for these compounds in the generated effluents.With the progress in the field of biotechnology; enzymes have found their way into many new industrial processes. Enzymes are already well established in the processing of pulp and paper **(Gavrilescu and Chisti, 2005)**. The earlier reports have shown interesting implications of the enzymes namely xylanase, pectinase, mannanase and laccase (**Lahtinen *et al.*, 2009; Várnai *et al.*, 2011**) in pretreatment and processing of kraft pulp that collectively reduce the toxic discharge of pulp and paper industries.

Xylanase pretreatment of kraft pulp is already being employed across the globe with initial efforts by **Viikari *et al.*, (1986)**. However, the combined use of xylanase with pectinase prove a better option for treatment of Kraft pulp (**Dhiman *et al.*, 2009**). Parenthetically the use of pectinase in the Kraft pulp pretreatment was proposed by **Ahlawat *et al.*, (2007)**. Since then the combination of xylanase with pectinase is being tried (**Kaur *et al.*, 2010**). Nevertheless, Xylanase is the key enzyme in the kraft pulp pretreatment due to its significant role in the kappa number reduction, which reflects the lignin proportion due to its better penetration as a bleaching agent after removing hexenuronic acid from the pulp (Gangwar, Prakash, & Prakash, 2014).In contrast, pectinase depolymerize polymers of galacturonic acids, and subsequently lower the cationic demand of pectin solutions and the filtrate from peroxide bleaching during paper making (**Reid and Ricard, 2000; Viikari and Tenakanen, 2001**). Hence, the combinatorial approach with the reverse proportion of xylanase and pectinase (where pectinase is more than xylanase) may serve as an innovative solution in kraft pulp pretreatment. Nonetheless, this area remains unexplored.

Production of xylanase from *Bacillus* species has been reported by several researchers (**Ersayin *et al.*, 2010; Nagar *et al.*, 2010**). Similarly, pectinase production from *Bacillus* species has also been reported (**Basu *et al.*, 2008; Sharma & Satyanarayana, 2006**). Moreover there is a single report on simultaneous production of xylano-pectinolytic mixture from the same strains of *B. pumilus* (**Kaur *et al.*, 2010**) but there is no report available till date regarding simultaneous production of pectino-xylanolytic mixture from single strain of *Bacillus*.The possibility to use crude enzymes for pretreatment is the first and foremost step in reducing the cost of the process (**Viikari *et al.*,1987; Viikari *et al.*, 1986).** The second step is the cost-effective production of pectino-xylanolytic enzymes to improve the process economics of biobleaching at industrial scale. Pure substrates being highly expensive, it cannot be afforded at the industrial level bulk production of enzymes (**Adhyaru *et al.*, 2014**).This goal can be achieved by employing a huge amount of residual plant biomass considered as waste. In Indian subcontinents, orange peel is massively generated waste agri-residues suitable for pectinase production. However, to use crude and raw substrates for the enzymes production, successful optimization of media is a pre-requisite (Kumar et al., 2014). The traditional practice using OVAT approach is time consuming and cumbersome as against the statistical approach that additionally offers the analysis of interactive effects of various process parameters (**Embaby *et al.*, 2014**).Response surface methodology is a well-accepted statistical approach to enhance the enzyme yield though the magnitude of increase may vary **(Ali *et al.*, 2013)**. However, finally RSM renders the process more feasible at industrial scale.

Assessment of the bleaching potential of crude enzymes in terms of kappa number reduction and improvement in brightness is a well-established strategy (**Choudhury *et al.*, 2006)**. Moreover, the positive inputs in optical and strength properties of pulp and paper support the same. Several studies of the application of crude xylanase with pectinase in pulp bleaching have been reported, but the use of the high content of pectinase than xylanase was not being tried earlier that is presented herewith.

METHODOLOGY

Isolation, Screening and Identification of pectinase and xylanase producer strain

A bacterial strain was isolated from soil samples contaminated with the effluent of an oil refinery, Digboi, Assam, India. The pooled soil samples were homogenized and serially diluted in sterile distilled water followed by plating on pectin agar medium containing pectin 5.0 g.L $^{-1}$ and yeast extract 1.0 g.L^{-1} (pH 8.0). All plates were incubated at 35±2.0ºC for 24 to 72h. Morphologically distinct colony was purified by repeated streaking. Primary evaluation of potent bacterial strains for pectinase production was carried out by plate assay method (**Wood *et al*., 1988**) using nutrient agar supplemented with 0.5% w/v of pectin. The plates were incubated for the growth and then flooded with 1% CTAB. Positive pectinase activity was detected by the clear zone surrounding the colonies. Similarly, the strains were screened for xylanase and cellulase activities using Bushnell-Haas medium containing 0.5% w/v xylan and 1 % w/v carboxymethyl cellulose (CMC) as a substrate. The plates were incubated for growth and stained with 1% w/v Congo red dye and destained with 1.0 M NaCl. Positive xylanase and cellulase activities were detected by the presence of yellow halo against a red background. Potential strainSDB9 was identified based on 16S rRNA gene sequencing. The phylogenetic relationships with some reference strains were determined using the neighbor-joining method using Mega 5.0.

Concurrent production of enzymes and growth profile

Pectino-xylanolytic enzymes were produced under submerged fermentation in 250mL Erlenmeyer flasks containing 100mL of production medium consisting of Yeast extract (0.1 %), $(NH_4)_2SO_4$ (0.2 %), NA_2HPO_4 (0.6 %), KH_2PO_4 (0.3 %), $MgSO_4.7H_2O$ (0.1%),$CaCl_2$ (0.001%) and Pectin (0.5 %) as a sole carbon source (pH 8.0) incubated under shaking (120 rpm) conditions at 40 °C. After 48 h, the fermented broth was centrifuged, and the cell-free supernatant was subjected to purification. The enzyme was partially purified by precipitation with ammonium sulfate and subsequent dialysis from the supernatant obtained by separating the biomass through refrigerated centrifugation at 10,000 g for 20 min and used for further studies.

Enzyme assay

The pectinase activity was assayed by measuring the amount of reducing sugars (xylose equivalent) liberated from polygalacturonic acid using 3, 5-dinitrosalicylicacid (**Miller, 1959**). The xylanase activity was assayed according to the method of **Bailey *et al*., (1992)**. Cellulase activity (carboxymethyl cellulase) was determined as per IUPAC method (**Ghose, 1987**). One unit (IU) of pectinase, xylanase and cellulase activities were defined as the amount of enzyme that catalyzed the release of 1.0 micro mol of reducing sugar as galacturonic acid, xylose and glucose equivalent per minute under the specified assay conditions respectively.

Effect of nitrogen and crude pectin sources on pectinase production

Three inorganic nitrogen sources KNO_3, $NaNO_3$, NH_4Cl and three organic nitrogen sources peptone, yeast extract and casein at 0.5% w/v were studied for optimum enzyme production. A control lacking nitrogen source was run. The pectinase production was also tested by replacing 0.5% w/v pectin with agricultural materials viz. orange peel, hardwood bark, banana peel and jute in the production media. These agricultural waste materials were washed with distilled water and dried in sunlight and powdered. The lignocellulosic substrates were given pretreatment before using it in production medium. For this purpose, the dried powder was treated with 1.0% w/v NaOH for 2 h, washed with distilled water for several times, and allowed to air dry to use as a medium component (**Gharpuray *et al*., 1983**).

Media optimization for pectinase production

The impacts of 10 independent variables on enzyme activity were tested using Plackett-Burman Design (**Plackett and Burman, 1946**). Each variable was tested at two levels: the high level (+) and the low level (-) as shown in Table 1. The factors investigated in the current study included salts $(NH_4)_2SO_4$, Na_2HPO_4, KH_2PO_4, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, yeast extract, orange peel pH, incubation temperature and incubation period.Total 13 runs have been performed and actual experimental and predicted values of pectinase production have been measured.

Table 1 Variables included in the PBD design

Code	Independent variables	Level of variables	
		Low level	High level
A	$(NH_4)_2SO_4$ (% w/v)	0.02	2.0
B	Na_2HPO_4 (% w/v)	0.06	6.0
C	KH_2PO_4 (% w/v)	0.03	3.0
D	$MgSO_4.7H_2O$ (% w/v)	0.01	1.0
E	Yeast extract (% w/v)	0.01	1.0
F	Orange peel (% w/v)	0.5	5.0
G	pH	7.0	9.0
H	Incubation temperature (°C)	50	37
J	Incubation Period (h)	24	72
K	$CaCl_2.2H_2O$ (% w/v)	0.001	0.01

RSM using BBD was applied for optimization of pectinase production that includes full factorial experiment and observation of simultaneous, systematic and efficient variation of significant components on the fermentation process. Three important parameters namely Orange peel concentration (X1), incubation temperature (X2) and initial pH (X3) were selected as the independent variables based on the significant model terms obtained by ANOVA analysis of PB Design and the pectinase activity (IU.mL^{-1}) was the dependent response variable. Each of these independent variables was studied at three different levels as per BBD with a total of 15 experimental runs using statistical software package Design Expert 9.0.5, Stat-Ease, Inc., USA. Pectinase activity (IU.mL^{-1}) corresponding to the combined effects of three variables was studied in their specified ranges as shown in Table 2. All the flasks were analyzed for pectinase activity. Three-dimensional curves were generated with the same software.

Table 2 Experimental range and coded levels of process variables for pectinase production

Coded value	Significant process variables	Range and level of variables		
		-1	0	+1
X1	Orange peel (% w/v)	2.5	3.5	5.0
X2	Incubation temperature (°C)	35	40	45
X3	pH	8.0	8.5	9.0

For statistical calculations the independent variables were coded as:

$$xi = (Xi - X0)/ \delta Xi \quad (1)$$

Where Xi is the experimental value of the variable; X0 is the mid-point of Xi, $^{\delta}$Xi is the step change in Xi and xi is the coded value for Xi, i=1–3.
This response surface methodology allows the modeling of a second order equation that describes the process. Pectinase production data was analyzed and response surface model given by Eq. (2) was fitted with multiple regressions through the least squares method.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_i \quad (2)$$

where β_0, β_i, β_{ii}, and β_{ij} represents, respectively the constant process effect, the linear effect of Xi, quadratic effect of Xi and the interaction effect between Xi and Xj on pectinase activity denoted by Y**(Chapla *et al*., 2010**).

Enzyme characterization

The reaction mixture containing 0.4 mL of 0.1% polygalacturonic acid prepared in 0.1 M Glycine-NaOH buffer (pH 8.5) and 0.1 mL of appropriately diluted enzyme was incubated at 40, 50, 60, 70 and 80˚C temperatures for 10 min for pectinase assay to determine the optimum temperature of the reaction.The pH optima of pectinase at 40°C temperature was determined by measuring activity at various pH values using different buffers, such as sodium citrate (pH 5.5), sodium phosphate (pH 6.0, 6.5, 7.0, 7.5, 8.0), and glycine-NaOH (pH 8.5, 9.0, 9.5) each at 50 mM concentration under standard assay conditions using polygalacturonic acid as substrate.
Rate of polygalacturonic acid hydrolysis was determined, after incubating substrate at various concentrations at optimized conditions, by measuring the enzyme activity at different substrate concentration. The substrate concentration ranged from 2.5 to 25 mg.mL^{-1}. Rate of the reaction was calculated using absorbance at 550nm. Graph of substrate concentration against reaction rate was

plotted. Kinetic parameters such as Km and Vmax were calculated from the Lineweaver-Burk plot.
Effect of various metal salts viz. $AgNO_3$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, $HgCl_2$, KCl, $MgCl_2$, $MnCl_2$, NaCl, $NiCl_2$ and $ZnCl_2$ at a final concentration of 1mM for 5 min was determined under standard assay condition. Thermostability of the pectinase was determined by preincubating the enzyme at 35, 45, 50, 60, 70 and 80°C temperature up to 90 mins. After each interval of 30 min, the enzyme was withdrawn and residual enzyme activity was determined by standard assay procedure.Likewise, the pH stability of pectinase was ascertained by preincubating the enzyme in buffers of different 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 and 10.0 pH. After 30 and 60 min time interval, the enzyme was withdrawn and residual enzyme activity was determined by standard assay procedure.

Pulp sample

Unbleached Kraft pulp used for the present study was a mixture of 82 to 84% mixed hardwood and 16 to 18% bamboo pulp provided by Central Pulp and Paper Research Institute, Saharanpur, U. P., India. The chemical composition of the pulp used was cellulose 75 to 76%, hemicellulose 19 to 20%, lignin 3 to 5% and pectin 0.1%.

Enzymatic pretreatment of Kraft pulp (E_0)

One variable at a time approach (OVAT) was taken to establish the best reaction conditions. Unbleached hardwood pulp was pretreated with the pectino-xylanolytic preparation as per standard laboratory manual of testing procedure of Central Pulp and Paper Research Institute (CPPRI), Saharanpur, U.P. India. Pretreatment of pulp with pectino-xylanolytic enzymes was carried out with different enzyme dosage at 50 °C temperature and pH 8.0 for a retention time of 120 min based on preliminary studies (Table 3). Shredded, screened and unbleached pulp comparable to 100 OD (oven dried) was taken and divided into two parts. One part was kept as control and the second part was treated with a pectino-xylanolytic preparation of crude enzyme in the ratio of pectinase and xylanase of 5.5:1 by adding 5.15 mL and 7.73 mL per gram of OD pulp in set-I and set-II respectively, while maintaining the pulp consistency at 10%. The pulp was thoroughly mixed with enzyme preparation by kneading mechanism and transferred in polyethylene bags to put in a water bath. Control samples were subjected to the same treatment conditions except the enzyme.

Table 3 Pectino-xylanolytic Enzyme Treatment of unbleached pulp (P- stage)

Particulars	Control	Enzyme treated pulp	
Enzyme dose, $IU.gm^{-1}$	-	*Set-I	#**Set-II**
Consistency (%)	10	10	**10**
Temperature (°C)	50	50	**50**
pH	8.0	8.0	**8.0**
Treatment Time (min)	120	120	**120**

*Set-I =P 10.0 +X 1.8, #Set-II = P 15.0 + *X 2.7 where P = Pectinase and X = Xylanase.

Bleaching of Pulp (D_0-E_p-D_1)

The enzyme pretreated pulp was subjected to chemical bleaching through a) D_0-stage (chlorine dioxide treatment - CD), b) E_p-stage (alkali treatment) and c) D_1-stage. Chlorine dioxide dosage was ascertained by estimation of Kappa No. of unbleached pulp. Pulp Bleaching process conditions are briefly summarized in the table 4.

Table 4 Pulp bleaching process conditions

Parameter/stage	D_0	E_p	D_1
Consistency (%)	10%	10%	10%
Temperature (°C)	55	70	80
pH	2-3	>11	3-4
Retention time (min)	45	60	180

a) D_0-stage (chlorine dioxide stage)

In this stage, control pulp was mixed with 6.47 % ClO_2 while the enzyme treated pulp was distributedinto two parts. One part was treated with the same dose of ClO_2 (6.47 %) whereas the other part was treated with a lesser dose of ClO_2 (5.50%). All the three pulp samples (control, same and less dose) were put in the polyethylene bags and placed in the water bath.

b) E_p-stage (alkali extraction stage)

The control and the pulp samples treated with higher and lesser dose of ClO_2 were treated with 2.5% of NaOH and 1.0% of H_2O_2 in different polyethylene bags and put in a water bath. After that, the pulp was washed similarly as done at the end of P-Stage and pulp pad was prepared.

c) D_1-stage (dioxide Stage)

The alkali extracted pulp was treated with Chlorine dioxide in the same way as mentioned in D_0 –Stage. Subsequently the pulp was washed as done after Pretreatment Stage and pulp pad was prepared and tested for kappa number, brightness, whiteness and yellowness as per TAPPI (Technical Association of the Pulp and Paper Industry, Atlanta) protocols.

Analysis of the pulp-free filtrate

Miller's method (1959) and procedure given in Laboratory Manual of Testing Procedure, CPPRI, Saharanpur, U.P. were followed to determine the Total Reducing Sugar (TRS), colour (A_{456}), lignin (A_{280}) and phenolics (A_{237}) of pulp filtrates.Lignin content of the effluents was measured by A_{280}. Samples were diluted if required to acquire the absorbance values within of the range 0.2-0.8. Lignin was calculated using the formula, lignin, $mg.mL^{-1}$ = A_{280} x dilution factor/absorptivity of lignin, where the value 21 is used for hardwood and 20 for agro-based lignin.

Analysis of pulp and paper properties

The enzyme treated pulp was carefully washed and hand sheets were prepared under standardized pressure and air-dried in a room with standardized light, humidity and temperature. The investigation of pulp properties was performed according to TAPPI standard methods. Kappa number, the measure of degree of lignin content of pulp, is estimated by the reaction of pulp samples with acidified potassium permanganate to measure the lignin content **(TM I-D1)/**TAPPI method T236 cm-85). The brightness of the hand sheets was measured as %ISO (International Organization for Standardization, ISO) by reflectance at 457 nm with ISO Colourtech, USA, according to TAPPI protocol (T-452 om-87). The yellowness, whiteness and fluorescence of pretreated pulp were also evaluated by ISO Colourtech, USA at 457 nm (T 1216).

RESULTS AND DISCUSSION

Isolation, Screening and Identification of pectinase and xylanase producer strain

In the present study, 24 bacterial isolates were obtained from the soil contaminated with the effluent of an oil refinery, Digboi, Assam, India. Based on the results of the screening a bacterial strain SDB9 which produced maximum pectinase with concurrent production of mediocre levels of xylanase and negligible cellulase activity was selected for further study. The isolate was identified as ***Bacillus firmus* SDB9** based on 16S rRNA gene sequencing. The sequence was submitted to GenBank with the accession number **KP881618**. The phylogenetic relationship of the isolate SDB9 with other reference taxa is shown in figure 1. The isolate efficiently grew at 30 to 45 °C temperature with an optimum at 40°C (Figure 2a) and optimum pH for the growth was 8.0. *Bacillus firmus* reported from the diverse habitats are well-known for the hydrolases producer. Earlier, *Bacillus firmus* had been reported to be the producer of pectinase and xylanase **(El-Shishtawy *et al*., 2014; Ratanakhanokchai *et al*., 2002; Roosdiana *et al*., 2013**).

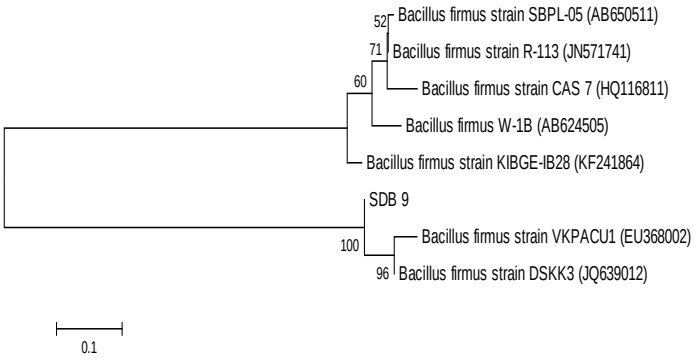


Figure 1 A phylogenetic tree is showing the taxonomic position of culture SDB9 with the GenBank accession numbers in parenthesis. Only bootstrap values greater than N50% are shown at nodes (based on 500 bootstrap resampling).

Concurrent production of enzymes and growth profile

The time course of pectinase production by the isolate was monitored during shake flask fermentation with pectin as a sole source of carbon. Optical density readings at 600nm indicated an active log phase during 6 to 24 h of fermentation. Highest pectinase activity was found 1.94 $IU.mL^{-1}$ during the early stationary

phase at 48 h. The Xylanase activity topped to 0.34 IU.mL^{-1} at 48 h of fermentation with as negligible as 0.086 IU.mL^{-1} cellulase activity at 54 h (Figure 2b). Early production of pectinase was found within 48 h that is desirable for large scale production.Similar rapid pectinase production was reported in *Bacillus licheniformis* (**Rehman *et al*., 2012**), *Bacillus cereus* (**Sanaa *et al*., 2014**) and even in yeast *Saccharomyces cerevisiae* (**Poondla *et al*., 2015**). The use of concurrently produced pectinase and xylanase from the single organism in the pretreatment of kraft pulp can be economically as well as environmentally friendly (**Kaur *et al*., 2010**).

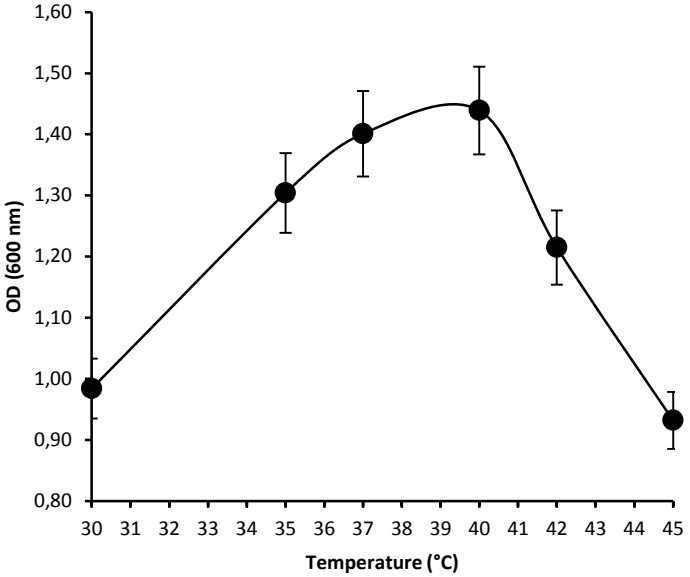


Figure 2a Effect of temperature on growth of isolate SDB9

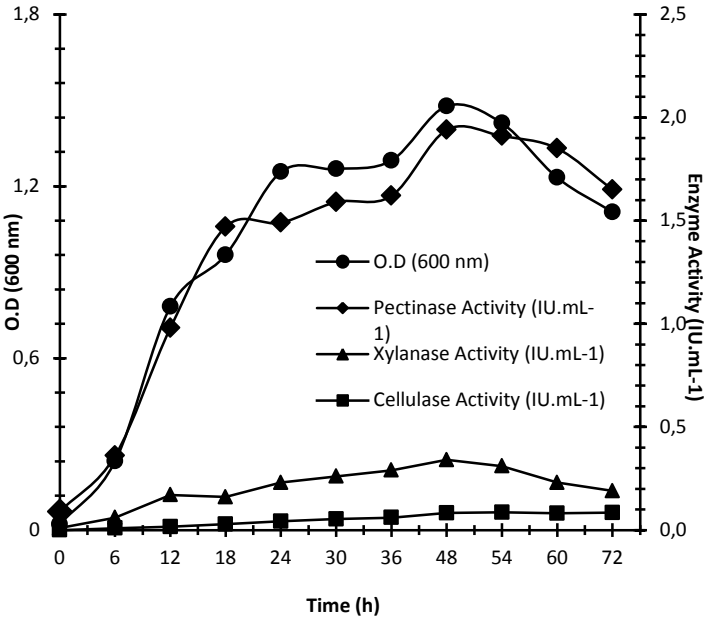


Figure 2b Concurrent production of three enzymes in shake flask condition with growth profile of isolate SDB9

Effect of nitrogen and crude pectin sources on pectinase production

Organic nitrogen sources were superior for the pectinase production than the inorganic nitrogen sources tested in the experiments. A similar pattern is reported in *Bacillus licheniformis* (**Rehman *et al*., 2012**) and *Bacillus* sp. (**Kashyap *et al*., 2003**). Yeast extract was found to be the best source of nitrogen followed by peptone, $NaNO_3$, KNO_3, NH_4Cl and Casein (Figure 3). Yeast extract is more preferentially utilized by the *Bacillus* species for the production of pectinase (**Kashyap *et al*., 2003; Rehman *et al*., 2012**).

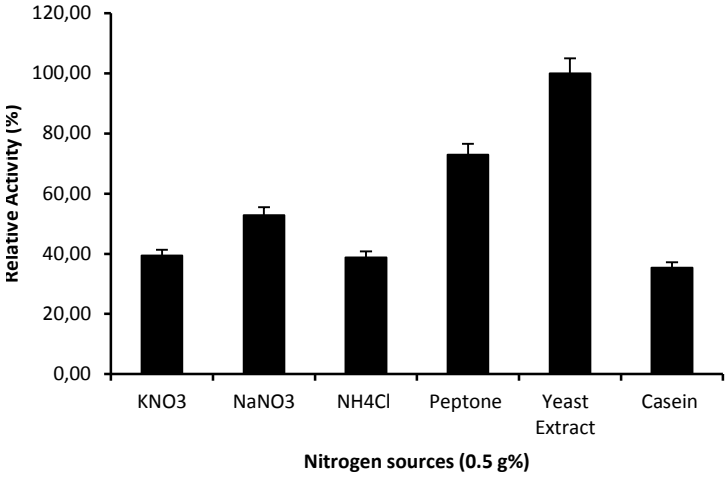


Figure 3 Effect of different Nitrogen sources on pectinase production

The enzyme activity in all the tested crude substrates was found to increase with incubation. On testing different economic waste materials as a substrate for pectinase production, the maximum enzyme output was achieved using an orange peel on 72 h of fermentation. Earlier, orange peel is reported to be the best source for the pectinase production by *Bacillus* sp. NTT33 (**Cao *et al*., 2000**). Though, the initial phase of 48 h did not yield highly differential production data. However after three days the Orange Peel proved to be a most worthy carbon source for the pectinase production followed by hardwood bark, Banana peel, Jute and pectin (Figure 4). The use of agro-residues for the pectinase production is an alternative biotechnological solution for waste valorization (**Martín *et al*., 2013; Rivas *et al*., 2008**) that is highly acceptable for sustainable development.Among the various agricultural waste including oilseed cake, wheat straw, wheat bran, citrus peel/orange peel were well evaluated as a pectin source for the production of pectinase (**Kapoor *et al*., 2001; Sharma and Satyanarayana, 2012**).

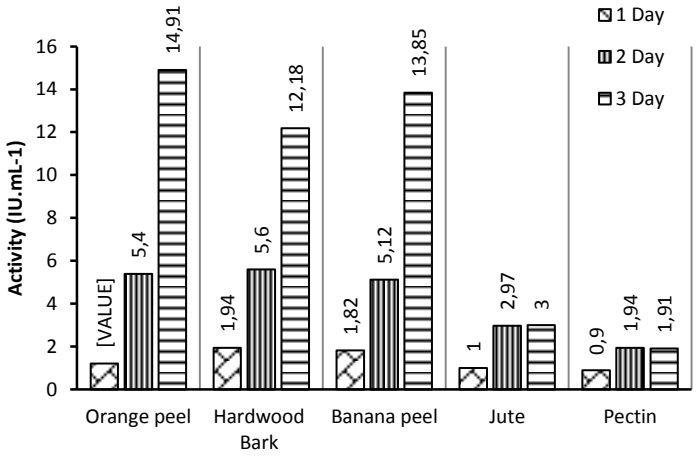


Figure 4 Effect of substrates (Orange peel, Hardwood Bark, Banana peel, Jute and Pectin) on pectinase activity

Media optimization for pectinase production

Pectinase production by SDB9 with media containing 0.5% w/v Pectin was successfully attained with supplementation of 0.5% yeast extract, 0.2% $(NH_4)_2SO_4$, 0.6% Na_2HPO4, 0.3% KH_2PO4 and 0.1% $MgSO_4.7H_2O$. However, in optimization studies pectin was replaced with orange peel that principally consists of as much as 25% to 30% (dry weight) pectins **(Aravantinos-Zafiris *et al*., 1994; Ververis *et al*., 2007**) in order to reduce the cost of production.

Plackett-Burman Design (PBD) for screening significant variables

Manual screening by one variable at-a-time is labor-intensive and time-consuming. Whereas, PBD decreases the number of experiments needed to effectively achieve experimental goals significantly (**Plackett and Burman, 1946**). In all, 13 runs were carried out on ten independent variables and one

dummy variable. The PBD matrix, coded-real values of independent variables studied and the experimental vs. predicted values of pectinase produced are shown in Table 5.

Higher values of studentized effect, the sum of squares and % contribution of orange peel, pH and incubation temperature compared to the rest of the tested variables indicates their pronounced influence on the response (Table 6). Regression analysis and independent variables with significant consequences on pectinase production levels are presented in Table 7. Values of Prob> F less than 0.0500 indicate model terms are significant. In this case, F, G, H are significant model terms. Values greater than 0.1 indicate the model terms are not significant. The Model F-value of 151.69 implies that the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. The "Pred R-Squared" of 0.8887 is in reasonable agreement with the "Adj R-Squared" of 0.8981; i.e. the difference is less than 0.2. The adequate precision, the signal to noise ratio of 15.7, suggests an adequate signal. Coefficient Estimate for orange peel, media pH and incubation temperature turned out to be 5.50, 1.65 and 1.45. The equation in terms of coded factors is pectinase production= +10.18 +5.50*F+1.65*G+1.45*H. The combined effect of (i) orange peel and incubation temperature (ii) orange peel and media pH and (iii) incubation temperature and media pH on pectinase production are depicted in figure 5.

Table 5 PBD design to search the independent variables (Compositions) that affects the pectinase production (response)

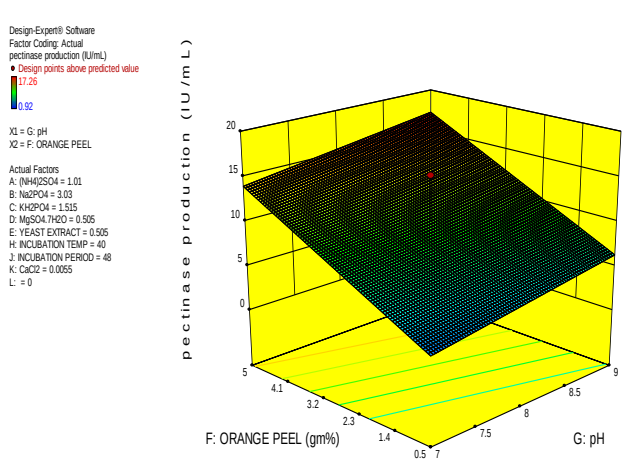
Run	A: $(NH_4)_2SO_4$	B: Na_2PO_4	C: KH_2PO_4	D: $MgSO_4.7H_2O$	E: Yeast Extract	F: Orange Peel	G: pH	H: Incubation Temp.	J: Incubation Period	K: $CaCl_2.2H_2O$	Experimental Pectinase Activity	Predicted Pectinase Activity
	% w/v	% w/v	% w/v	% w/v	% w/v	% w/v		^oC	h	% w/v	$IU.mL^{-1}$	$IU.mL^{-1}$
1	1	1	-1	1	1	1	-1	-1	-1	1	13.5	11.826
2	-1	-1	1	-1	1	1	-1	1	1	1	14.1	14.406
3	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.92	1.163
4	0	0	0	0	0	0	0	0	0	0	15.21	15.21
5	1	-1	-1	-1	1	-1	1	1	-1	1	8.31	7.37
6	-1	-1	-1	1	-1	1	1	-1	1	1	15.1	15.453
7	1	-1	1	1	-1	1	1	1	-1	-1	17.26	18.033
8	-1	1	1	-1	1	1	1	-1	-1	-1	15.42	15.45
9	1	-1	1	1	1	-1	-1	-1	1	-1	1.1	1.163
10	-1	1	-1	1	1	-1	1	1	1	-1	8.57	7.37
11	1	1	-1	-1	-1	1	-1	1	1	-1	14.2	14.4
12	1	1	1	-1	-1	-1	1	-1	1	1	3.81	4.79
13	-1	1	1	1	-1	-1	-1	1	-1	1	2.89	3.743

Table 6 Proportionate effects on independent variables on response (Pectinase production)

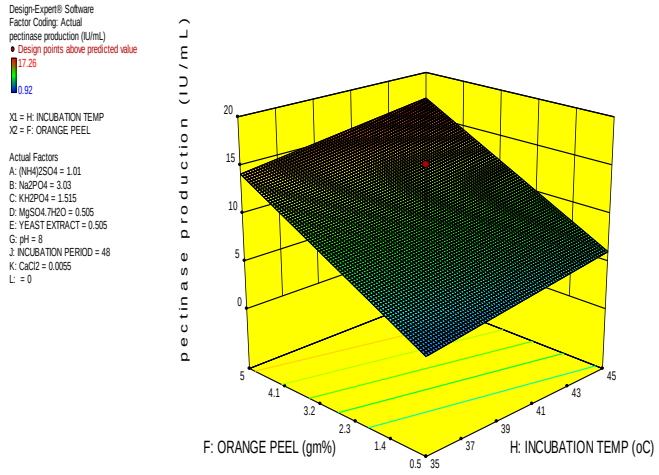
Intercept	Studentized Effect	Sum of Squares	% Contribution
A-$(NH_4)_2SO_4$	0.14	0.062	0.014
B-Na_2PO_4	0.21	0.14	0.030
C-KH_2PO_4	-0.95	2.71	0.59
D-$MgSO_4.7H_2O$	-0.050	7.500E-003	1.648E-003
E-Yeast Extract	1.19	4.25	0.93
F-Orange Peel	10.99	362.34	**79.61**
G-pH	3.30	32.67	**7.18**
H-Incubation Temp.	2.91	25.35	**5.57**
J-Incubation Period	0.090	0.024	5.339E-003
K-$CaCl_2$	0.093	0.026	5.742E-003

Table 7 Regression analysis of PBDesign

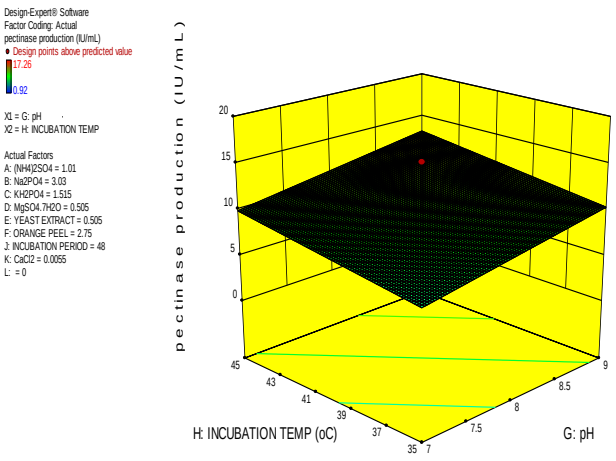
Source	Regression Coefficient Estimate	Sum of Squares	Df
Model		420.36	3
F-Orange Peel	5.50	362.34	1
G-pH	1.65	32.67	1
H-Incubation Temperature	1.45	25.35	1
Curvature	-	27.40	1
Residual	-	7.39	8
Cor Total	-	455.15	12



(i)



(ii)



(iii)

Figure 5 The combined effect of (i) orange peel and incubation temperature (ii) orange peel and media pH and (iii) incubation temperature and media pH on the response (Pectinase production)

Response surface methodology for optimization of pectinase production using Box–Behnken design

Three important parameters namely Orange peel concentration (X1), incubation temperature (X2) and initial pH of media (X3) were selected as the independent variables based on PBDesign to determine the optimal values. The plan of BBD in the coded levels of the three independent variables and the obtained results are shown in Table 8. The enzyme activity (Pectinase IU.mL^{-1}) was the only dependent response variable tested.

Table 8 Box-Benkhen Design with coded levels of three independent process variables and experimental vs. predicted pectinase activity

Run	Orange Peel (X1)	Temperature (X2)	pH (X3)	Experimental Pectinase Activity (IU.mL^{-1})	Predicted Pectinase Activity (IU.mL^{-1})
1	0	-1	1	10.89	10.85
2	-1	0	-1	16.55	16.49
3	0	1	-1	14.69	14.761
4	1	0	-1	17.55	17.587
5	0	0	0	12.87	13.17
6	0	0	0	13.23	13.17
7	-1	-1	0	11.81	11.86
8	-1	1	0	11.37	11.36
9	0	-1	-1	14.17	14.137
10	0	0	0	13.41	13.16
11	1	1	0	12.51	12.45
12	-1	0	1	11.01	11.0
13	1	0	1	12.21	12.24
14	0	1	1	10.05	10.06
15	1	-1	0	12.59	12.6

ANOVA (analysis of variance) was employed for the determination of significant effects of variables for pectinase production. The detected Pectinase production ranged between 10.85 to 17.58 IU.mL^{-1} with the conditions tested. To make the model significant the response transformation from the power family with lambda -1.31 and constant k= 0 was applied based on the recommendation of the Box-Cox plot (Table 9).

Table 9 ANOVA for Response Surface Quadratic model

Source	SS	Df	MS	F Statistics	p-value	
Model	6.639E-004	9	7.377E-005	184.65	< 0.0001	Significant
A-Orange Peel	*2.957E-005*	*1*	*2.957E-005*	*74.02*	*0.0004*	
B-Temperature	*4.116E-006*	*1*	*4.116E-006*	*10.30*	*0.0237*	
C-pH	*5.115E-004*	*1*	*5.115E-004*	*1280.28*	*< 0.0001*	
AB	*7.266E-007*	*1*	*7.266E-007*	*1.82*	*0.2353*	
AC	*3.239E-006*	*1*	*3.239E-006*	*8.11*	*0.0359*	
BC	*9.884E-006*	*1*	*9.884E-006*	*24.74*	*0.0042*	
A^2	*2.479E-006*	*1*	*2.479E-006*	*6.21*	*0.0551*	
B^2	*9.381E-005*	*1*	*9.381E-005*	*234.80*	*< 0.0001*	
C^2	*3.207E-006*	*1*	*3.207E-006*	*8.03*	*0.0365*	
Residual	1.998E-006	5	3.995E-007			
Lack of Fit	*2.248E-007*	*3*	*7.494E-008*	*0.085*	*0.9622*	*not significant*
Pure Error	*1.773E-006*	*2*	*8.864E-007*			
Cor Total	6.659E-004	14				

Df- Degree of Freedom, SS- Sum of Squares, MS- Mean of Square

The Model F-value of 184.65 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. In this case, A, B, C, AC, BC, B^2, C^2 are significant factors or interactions. The Lack of Fit F-value of 0.08 suggests that the model is fit. Analysis of variance for pectinase production shows that quadratic model is significant with F value 184.65 and p-value < 0.0001 as shown in Table 9. The coefficient of determination (R^2) was calculated to be 0.9970 indicating that the model could explain 99% of the variability. The coefficients for the linear effect of Orange Peel, Temperature and pH were highly significant. The interactive effect of Orange Peel and Temperature was less significant than the interactive effect of Orange Peel and pH & Temperature and pH. Also, Temperature and pH were quadratically significant. The fitted Quadratic model for pectinase activity in terms of coded process variables is:

Y= (Enzyme Activity) -1.31 = + 0.034-1.923E-003 * A+7.173E-004* B+7.996E-003* C-4.262E-004* AB-8.999E-004* AC+1.572E-003* BC-8.194E-004 * A2+5.040E-003 * B2 -9.319E-004* C2

The "Adeq Precision" value of 48.759 indicates an adequate signal and therefore, the model is significant for the process. The 3D response surfaces with contour were plotted on the basis of the model equation so as to investigate the interaction of the factors and to measure the optimum values of each factor for maximum pectinase production by *Bacillus* sp. SDB9. The optimum concentration of the respective components is represented by the coordinates of the central point within the highest contour lines in each of the graphics. Contour and 3D plot show the interactions of orange peel and incubation temperature at constant pH 8.0. Pectinase production increases with increasing orange peel concentration (2.5 %, w/v to 5.0%, w/v). The increase in temperature up to 40°C enhanced the production then after it declined (Figure 6a and 6b). Furthermore, moderate increase in pectinase production was seen with increasing orange peel concentration (2.5 %, w/v to 5.0%, w/v) but the rise in pH from pH 8.0 adversely influenced the production (Figure 7a and 7b). Similarly, a modest increase in pectinase production was seen with increasing temperature but the rise in pH from pH 8.0 reduced the production (Figure 8a and 8b).

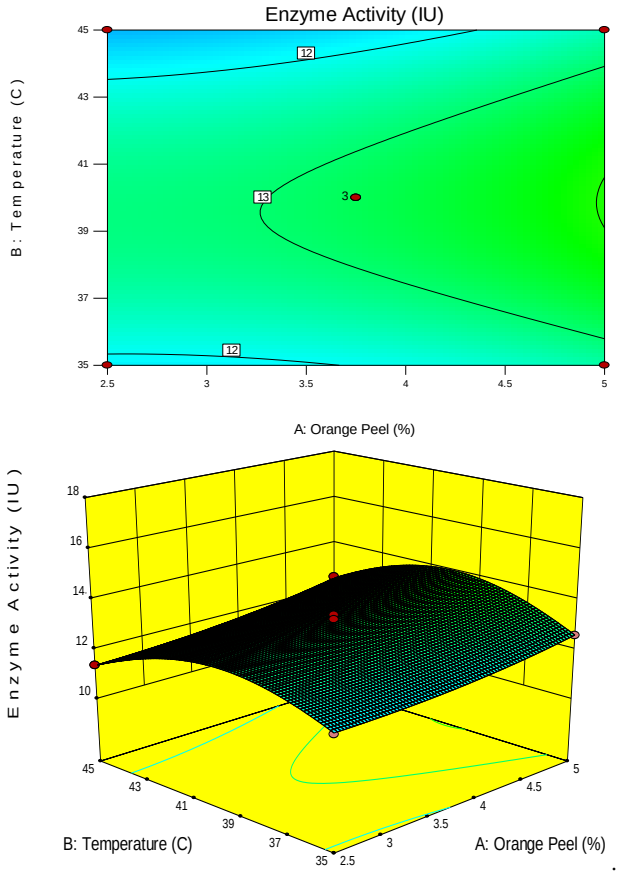


Figure 6 (a) Contour plots and (b) 3D response surface curves shows the interactions of orange peel and incubation temperature on production of pectinase by SDB9 strain at constant pH 8.0

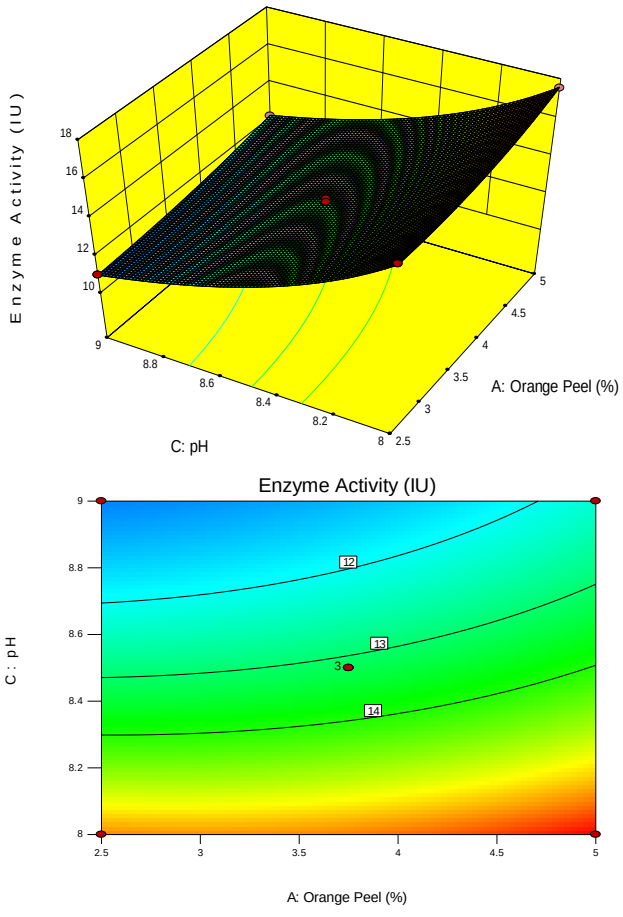


Figure 7(a) Contour plots and (b) 3D response surface curves shows the shows the interactions of orange peel and pH on production of pectinase at constant temperature 40°C

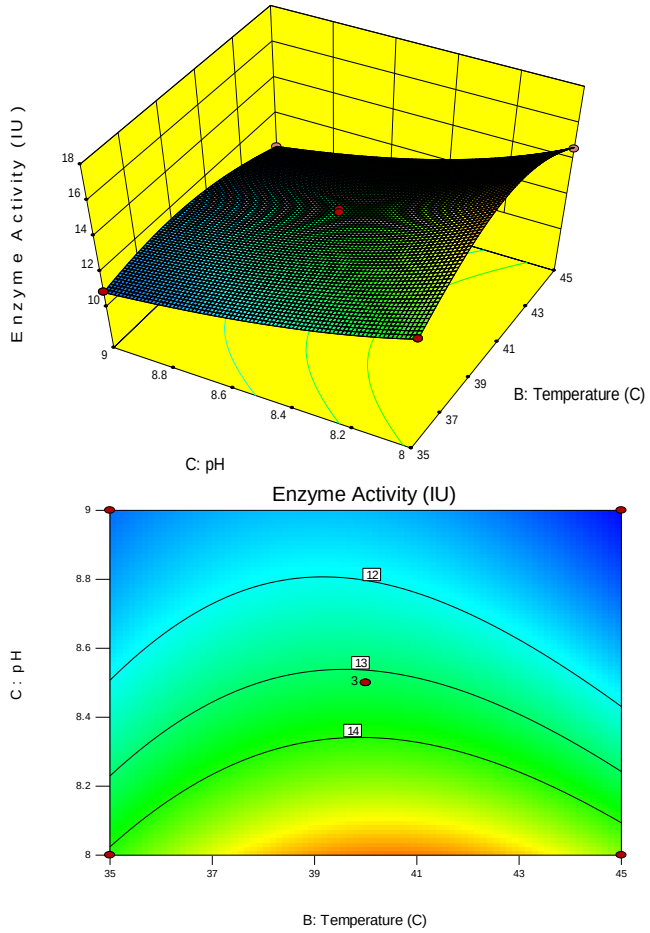


Figure 8 (a) Contour plots and (b) 3D response surface curves shows the interactions of pH and temperature on production of pectinase at constant orange peel concentration (3.75 % w/v)

The maximum predicted pectinase activity was 17.58 IU.mL^{-1}, which was very close to the actual obtained value of 17.55 IU.mL^{-1} corresponding to increasing levels of orange peel (5.0 % w/v), temperature (40°C) and a lower level pH (8.0). (Figure 9). The pectinase yield in the optimized medium was 17% higher than that in the initial medium. The production of pectinase increases with increase in concentration of orange peel, but the incremental production is not proportionate so the lower concentration of 2.5% of orange peel can be considered for practical application and save on the usage of substrate. Production of pectinase from SDB9 at 72 h, 40°C and pH 8.0 on 2.5 % w/v orange peel as agricultural residue material is recommended to achieve higher yield of 16.49 IU.mL^{-1}.Few recent discrete reports on optimization of pectinase production by *Bacillus* species using statistical approach are available in scientific literature that includes the screening by PBDesign and subsequent determination of optimal values of significant model terms C:N ratio, K_2HPO_4 and pH **(Sharma and Satyanarayana, 2006)**.The raw substrate and abiotic parameters dominated the outcome of optimization experiments for pectinase production in *Bacillus licheniformis* SHG10 strains (**Embaby *et al.*, 2014**).

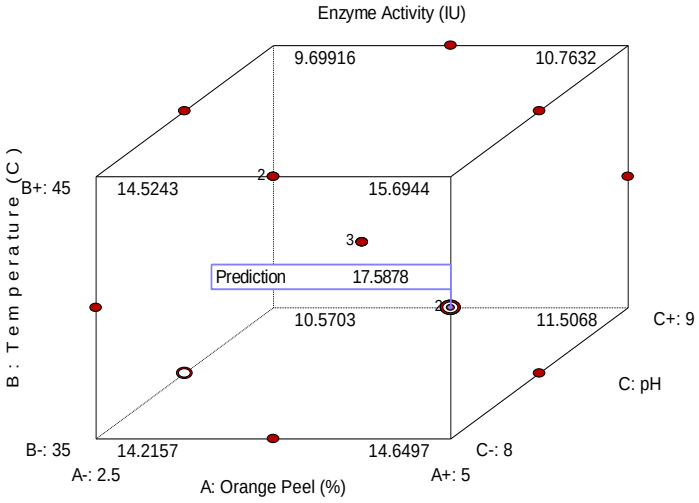


Figure 9 Cube plot showing the influence of factors relevant to the pectinase production A: Orange peel, B: temperature and C: pH

Enzyme characterization

For the precipitation of the enzyme 50% concentration of $(NH_4)_2SO_4$ found to be optimum, followed by dialysis. This partially purified enzyme was used in the characterization.

Using different pH buffers, peak activity of *Bacillus* sp. SDB9 pectinase was observed with assay buffer at pH 8.5 and more than 50% activity was retained at pH 7.5 and 9.5 (Figure 10). The optimum temperature for SDB 9 pectinase activity was found to be 45°C (Figure 11). The catalytic activity of pectinase was reported in neutral to alkaline pH with abroad temperature range that is similar to the pectinase of *Bacillus pumilus* **(Sharma and Satyanarayana, 2006**). The augmented activity of pectinase in the presence of the divalent cations including Mg^{2+}, Ca^{2+}, Mn^{2+} and Co^{2+} suggest the metalloprotein nature. However K^{+}, Zn^{2+} and Fe^{3+} cause the drastic inhibition of the enzyme activity (Figure 12). Positive modulatory effect of Mg^{2+} and Ca^{2+}, on S-I and S-II pectinase of *B. gibsonii* as well as *Bacillus* sp. KSM-P576 supports our investigation **(Kobayashi *et al.*, 2001; Zu-ming *et al.*, 2008**). Zn^{2+} is a well-reported inhibitor of pectinase **(Kusuma and Reddy, 2014; Roosdiana *et al.*, 2013**) which in agreement with the present findings.

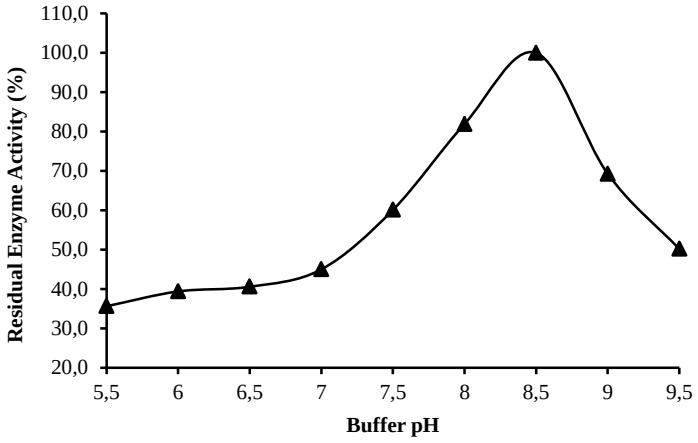


Figure 10 Effect of assay pH on pectinase activity

The enzyme was found to perform optimally at 45°C. However, it works on wide range of temperature ranging from 30 to 50°C. The enzyme was thermo-sensitive beyond 45°C as its activity was decreased drastically (Figure 11).

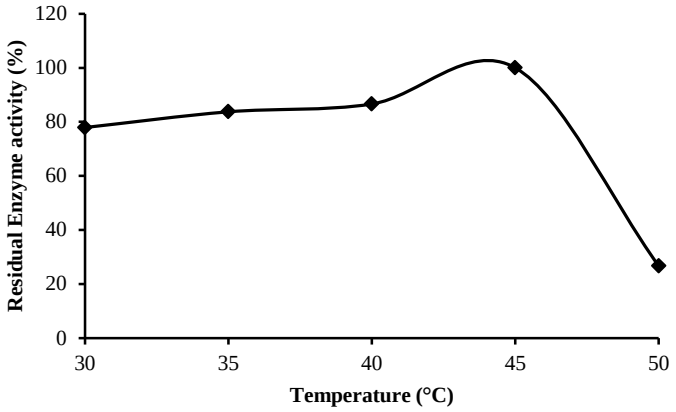


Figure 11 Effect of assay temperature on pectinase activity

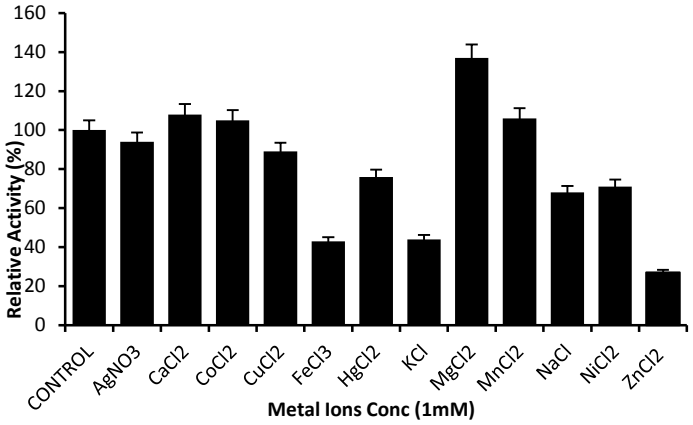


Figure 12 Effect of Metal Ions on SDB9 pectinase activity

The K*m* and V*max* values of the partially purified pectinase from *Bacillus* sp. SDB9 were calculated to be 2.090 $mg.mL^{-1}$ and 1.798 $IU.mL^{-1}$, respectively (Figure 13). The R-value indicates that almost 98% of the variation in v_o^{-1} (y) is due to the variation in S^{-1} (x). The K*m* and V*max* values of the enzyme are harmonious for use at industrial scale.

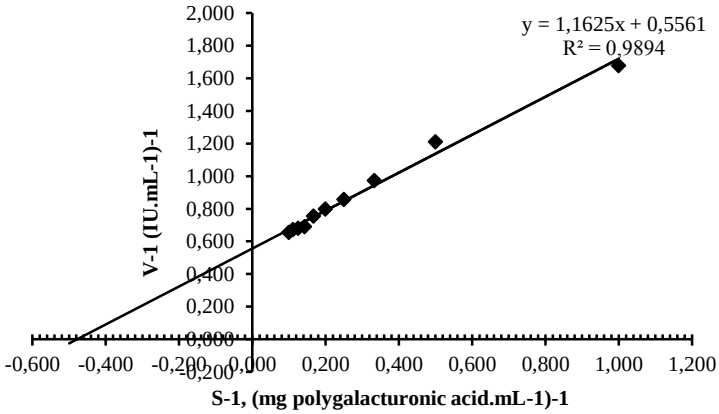


Figure 13 Line Weaver Burk Plot of initial velocity data for pectinase on polygalacturonic acid (2.5 to 25 $mg.mL^{-1}$) measured at 45°C and pH 8.5

An enzyme was found to remain active at elevated temperature, and thus it was thermostable in nature. The enzyme demonstrated highest stability at pH 8.5. The decline in enzyme stability was drastic beyond pH 8.5 whereas it was gradual below this pH. i.e. Pectinase retained only 48.7% and 38.2% activity up to 60 min at pH 7.5 and 9.5 .Furthermore, pectinase from SDB9 retained 50.17% relative activity at pH 9.5 (Figure 14). The enzyme showed thermal stability in a broad range of temperatures. The enzyme retained 86% and 69% activity at 60°C and 70°C for 60 min. Nearly, half activity was conserved up to 90 min at 70°C. Thus, the enzyme is vastly stable thermally up to temperatures as high as 70°C for time up to 90 min. Mere, 12% activity,was recorded at 80°C after 90 min.

(Figure 15). Pectinase of the isolated bacterium is superior in terms of the thermal stability from pectinase of *Bacillus subtilis* CM5 and *Bacillus* sp. MG-cp-2 (**Kapoor *et al*., 2000; Ray, 2010**) ie. 80% at 70 °C for 30 min and 50% for 20 min at 80 °C respectively.

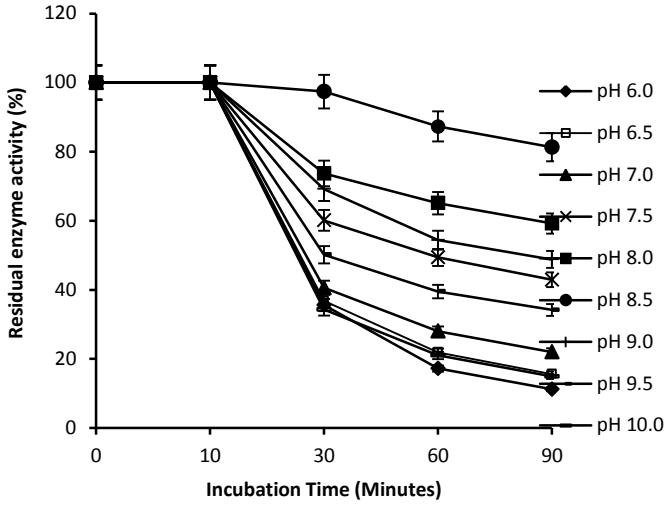


Figure 14 Stability of pectinase in acidic to alkaline pH scale

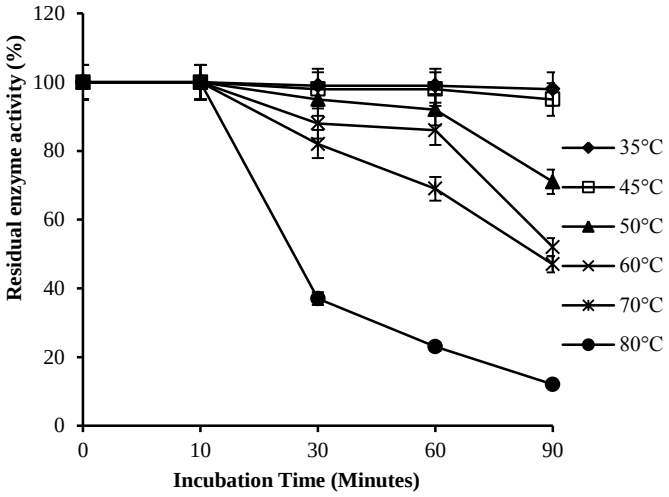


Figure 15 Thermostability of pectinase at various temperature scales

Enzymatic pretreatment of Kraft pulp (E_0)

The drop in kappa number after enzyme pretreatment indicates the inputs of the enzymes in the bleaching of paper pulp. No remarkable changes in kappa number were observed by the treatment with set-I that comprises 10 IU of pectinase and 1.8 IU of xylanase per gram of OD pulp. Whereas, treatment with set-II containing 15 IU of pectinase and 2.7 IU of xylanase per gram of OD pulp reduced the Kappa number by 1.94 unit (Table 10**)** as compared to the control (untreated pulp) and that is nearly double the % reduction than the report of **Thakur *et al*., (2012**). So due to the good result with set-II, it had been channelized for detail study in chemical bleaching process to reduce the load of the chemical bleach agent.

Table 10 Characterization of enzyme (E_0) pretreated and untreated (Control) unbleached pulps

Particulars	Control	Enzyme (E_0) treated pulp	
Enzyme dose (IU.gm^{-1})	Nil	Set-I	Set-II
Kappa no. of pulp	24.55	23.07	22.61
Brightness (% ISO)	24.12	24.24	24.77
Yellowness (%ISO)	40.88	40.91	40.80

Bleaching of Pulp (D_0-Ep-D_1)

The values of improvement in brightness and whiteness of pulp at different sequential stages ranging from ClO_2 stage (D_0) to Alkali Extraction (E_p) Stage and up to ClO_2 stage (D_1) stage of chemical bleaching was determined. Enzyme pretreatment with pectino-xylanolytic preparation showed a reduction in Kappa Number by 1.94 units and increment in % ISO brightness by 1.32 units with set-II at 50˚C temperature and pH 8.0 for 120 min (Table 11). Our result of a reduction in Kappa Number and increment in % ISO brightness is far better than earlier study **(Ahlawat *et al*., 2007**). Moreover, the combined use of pectinase and xylanase are more preferable approach than single enzymes for kraft pulp pretreatment (**Dhiman *et al*., 2009**) because the treatment with the pectinase lowers the cationic demand of the pulp and xylanase predominantly delignify the pulp. Treatment of the kraft pulp with pectino-xylanolytic enzymes extracted from the isolate resulted in 15% less chlorine consumption to obtain the same optical property of the pulp as attained with conventional chemical bleaching. Therefore, this renders the process eco-friendly and sustainable. The outcome of the bleaching after a D_0-E_p-D_1 stage in properties of pulp seemed to be suitable for commercial exploration.

Table 11 D_0-E_p-D_1 bleaching of enzyme treated and untreated pulps

Sr. No.	Particulars	Control	Enzyme treated Set-II	
			Same dose	15% less dose
1	ClO_2 stage (D_0)			
	Applied chlorine, %	6.47	6.47	5.50
	Brightness, % ISO	50.34	50.80	44.91
	Yellowness, %ISO	36.21	32.37	35.18
	Whiteness %ISO	ND	ND	ND
2	Alkali Extraction (E_p) Stage			
	Applied NaOH, %	2.5	2.5	2.5
	Applied Peroxide, %	1.0	1.0	1.0
	Brightness, % ISO	72.46	75.00	69.10
	Yellowness, %ISO	19.41	15.68	19.80
	Whiteness %ISO	38.06	45.00	33.39
3	ClO_2 stage (D_1)			
	Applied chlorine, %	4.31	4.31	4.31
	Brightness, % ISO	88.58	89.90	89.12
	Yellowness, %ISO	7.60	7.77	8.32
	Whiteness %ISO	75.71	76.61	75.18
	Brightness Improvement Unit	-	1.32	0.56

Analysis of treated pulp

The pulp viscosity of enzymes treated Set-II pulp after the D_0-E_p-D_1 stage was 586.67, which was very close to the 581.82 value of enzyme untreated pulp. The viscosity of treated pulp clearly indicated there was no destruction of the cellulosic fibers. Nearly 15 % reduction in post color number (P C Number) of enzyme treated pulp was transpired. Furthermore, after the pulp bleaching process, the chemical analysis of pulp-free filtrate was performed and it divulged the noteworthy enhancement in the release of colour (6.36 Kg.tp^{-1}), phenolics (35.77 Kg.tp^{-1}) and lignin (1.15 Kg.tp^{-1}) as compared to the control. The release of total reducing sugar also supports the investigation **(**Table 12**).** Removal of color, phenolic and lignin with the release of reducing sugars is the indirect assessment to check the efficiency of bleaching using enzymes (**Saleem *et al*., 2009**).

Effects of enzymes treatment on paper quality

Canadian standard freeness enhancement from 280 in untreated to 310 in enzyme treated pulp is the perfect proof of better strength. The gain in Double fold number (3.2%) indicated the improvement in the endurance of paper. Minor variations in Burst index, Tensile index and Tear index of enzyme treated pulp reflected the conservation of pulp properties (Table 13). The result of paper quality testing reflects that the pulp fibrillation, water retention and restoration of fiber bonding are unaffected (**Gupta *et al*., 2000**). Ours is a novel combinatorial approach where the mixture of enzymes is predominated by pectinase rather than xylanase. Nevertheless comparable result was achieved by **Dhiman *et al*., (2009)**.

Table 12 Analysis of pulp filtrates of enzyme treated & untreated pulp samples

Sample Code	TRS Kg.tp^{-1}	Colour at (465nm) Kg.tp^{-1}	Lignin at (280nm) Kg.tp^{-1}	Phenolics at (237 nm) Kg.tp^{-1}
Control	0.15	5.27	0.62	20.88
Enzyme treated (Set-I)	0.17	9.23	1.31	42.75
Enzyme treated (Set-II)	0.20	11.64	1.77	56.65

Table 13 Strength properties of enzyme treated and untreated bleached pulp

Particulars	Control	Enzyme pretreated (Set-II)
Canadian Standard Freeness	280	310
Double Fold	75	77.50
Burst index (Pa.m^2.g^{-1})	3.88	3.44 (11.3%)
Tensile index (Nm.g^{-1})	58.65	56.10 (4.34 %)
Tear index (Nm. m^2.g^{-1})	6.73	6.40

CONCLUSION

The isolated *B. firmus* produced the substantial amount of pectinase and limited xylanase along with negligible cellulase, the enzyme combination worth exploring in pulp pretreatment. Yeast extract and orange peel were screened out as preferred nitrogen source and raw agri-residues respectively using one variable at-a-time approach. More than 17% improvement in pectinase production suggest the successful optimization using RSM and in this way render the process more viable for mass production of pectinase. Enzyme properties concerning the range of optimal pH, temperature and different cationic metals warrant its potential biotechnological practice. The predominant use of pectinase rather than xylanase in combinatorial approach for pulp pretreatment and subsequent analysis of pulp and paper quality yielded an equivalent result to the well-studied approach comprising more xylanase and less pectinase. The lessening of chlorine consumption on account of enzymatic pretreatment eventually leads to a reduction in chlorinated aromatic compounds in the effluent makes it easier to embrace the green technologies in paper industries. Therefore, the bacterial pectinase with thermo-alkalistable nature having the noteworthy ability to reduce the kappa number and increase the brightness of kraft pulp warrants the further investigation for a new horizon.

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