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Optimization of Cultural Conditions for Pectinase Production by *Streptomyces* sp. and Characterization of Partially Purified Enzymes

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Streptomyces sp. · Pectinases · Response surface methodology · Decolorization

Abstract
The cultural parameters of *Streptomyces* sp. for pectinase production were optimized using the Box-Behnken design. The maximum pectinase production was obtained after 58 h at 35°C and pH 7 upon submerged fermentation in yeast extract-containing media. The enzymes were partially purified with acetone precipitation, and the analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and zymogram revealed that *Streptomyces* sp. produced two pectinases protein with molecular weights of about 25 and 75 kDa. The pectinase activity was detected in a wide range of temperatures (30°C–80°C) and pH (3–9) with maximum pectinase activities observed at 70°C and pH 5 and 9. The enzymes retained about 30–40% of their activities even after incubating the enzyme at different temperatures for 120 min. The pectinase activities of *Streptomyces* sp. were enhanced in the media containing 1.5% pectin, 1% casein as a nitrogen source, 0.5 mM $MgSO_4$, and 5 mM NaCl. Further, the addition of Tween-20, amino acids, and vitamins to the media also enhanced the pectinase activity. Moreover, the bacterium illustrated the ability to decolorize crystal violet dye efficiently. The decolorization rate ranged from 39.29 to 53.75%, showing the highest bacterial decolorization in the media containing 2 mg/mL crystal violet at 144 h. Therefore, the bacterium has the potential in treating wastewater produced by industries like textile industries.

Introduction

Pectinases are a group of complex enzymes of the polysaccharidases family that degrade the complex pectin and are leading commercial enzymes [Kavuthodi and Sebastian, 2018; Shrestha et al., 2021b]. Insects, nematodes, plants, and microorganisms are the sources of pectinases, but microorganisms are the leading sources of industrial pectinase production. The microorganisms are easy to grow, can be manipulated genetically, and efficiently study different mechanisms like phytopathogenesis, plant-microorganism symbiosis, and the decomposition of organic matter [Hoondal et al., 2002; Kohli and Gupta, 2015].

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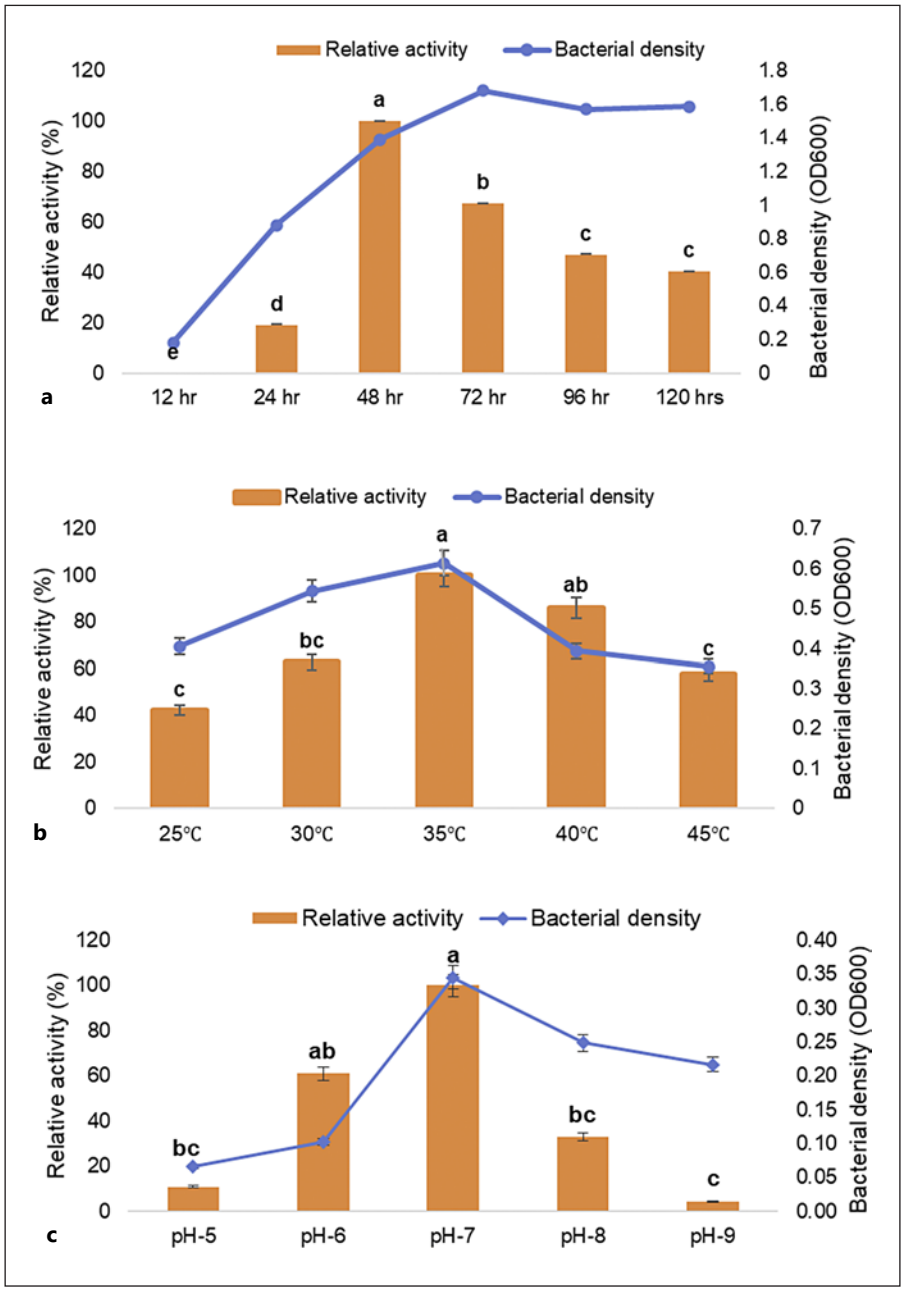

Fig. 1. The effects of incubation period (**a**), temperatures (**b**), and pH (**c**) on pectinase production by *Streptomyces* sp. (different lowercase letters above the bar indicate the significant difference between experimental conditions).

The demand and applications of pectinases are continuously increasing. The pectinases are used in the production of functional foods [Khan et al., 2013], retting and degumming of fibers in the textile industry [Chiliveri et al., 2016], and the production of good quality paper [Ahlawat et al., 2009]. In addition, pectinases are applied in the production of animal feed, liquefaction and saccharification of biomass, bio-scouring of cotton fiber, oil extraction, coffee and tea fermentation [Kashyap et al., 2001; Jayani et al., 2005; Kubra et al., 2017; Shrestha et al., 2021b], treatment of pectic wastewater, and bioethanol production [Kashyap et al., 2001; Wang et al., 2019].

Due to the increasing demands of pectinases, it is necessary to isolate and identify pectinase-producing bacteria and optimize the cultural conditions for maximum pectinase production. The forest soil bacterium, *Streptomyces* sp., showed different enzymatic activities while studying its biochemical characteristics [Shrestha et al.,

Table 1. Experimental results of Box-Behnken design for pectinase activity (U/mL)

Run	Incubation temperature, °C	pH	Incubation time	Pectinase activity, U/mL
1	30	6	48	1.41±0.07
2	40	6	48	1.73±0.09
3	30	8	48	1.65±0.08
4	40	8	48	1.44±0.07
5	30	7	24	0.46±0.02
6	40	7	24	0.42±0.02
7	30	7	72	1.49±0.07
8	40	7	72	2.14±0.11
9	35	6	24	0.41±0.02
10	35	8	24	0.55±0.03
11	35	6	72	1.70±0.09
12	35	8	72	2.30±0.12
13	35	7	48	2.79±0.14
14	35	7	48	2.16±0.11
15	35	7	48	2.72±0.14

Analysis was done using coded units, and the values of pectinase activity are mentioned as mean±standard deviation.

2021a]. Also, many species of *Streptomyces* have the potential to remove dyes via different mechanisms like biosorption and biodegradation [Adenan et al., 2021], and the biological methods of eliminating the dyes are being considered. Crystal violet is a commonly used dye in textile industries, which harms the environment due to its mutagenic and mitotic poisoning properties [Roy et al., 2018a]. The textile industries use different dyes, around 60–70%, and release the effluent into the environment or water or rarely in the treatment system. Such wastewater is harmful, leads to pollution and mutation in the environment, and may cause severe problems in the world [Roy et al., 2018a; Kishor et al., 2021]. Thus, decolorization or degradation of crystal violet is necessary to reduce or eliminate the adverse effect on the environment. Bacteria able to grow in harsh conditions can be helpful for this task. Therefore, this study exploited the soil bacterium that can grow in a wide range of temperatures and pH [Shrestha et al., 2021a] for decolorizing crystal violet and minimizing the negative impact of dyes. The various species of this bacterium are found predominantly in environmental samples [Adenan et al., 2021].

This study aimed to optimize the fermentation conditions of the pectinolytic bacteria isolated from forest soil for maximum pectinase production. Also, the study explored the effects of some additives in pectinase production when added to media and characterized the pectinase in various ranges of temperature and pH. The molecular mass of the enzyme was determined. Eventually, the application of *Streptomyces* sp. in the decolorization of crystal violet was studied to know its efficiency in treating wastewater generated from dye-using industries.

Results

Effects of Incubation Time, Temperature, and pH

The results of incubation time revealed the maximum enzyme activity in 48 h of incubation (Fig. 1a) in a yeast extract pectin (YEP) medium. Similarly, the maximum pectinase activity was observed at 35°C (Fig. 1b), and there was a significant decrease in pectinase activity below or above 35°C.

The present study elicited the maximum enzyme activity at pH 7 (Fig. 1c), followed by pH 6 and 8. However, the pectinase activity was observed in a wide range of pH (5–9).

Optimization of Cultural Conditions Using Response Surface Methodology

Table 1 depicts pectinase activity exhibited by *Streptomyces* sp., when the three factors were selected with three levels. The design included three replicates at the center point to provide a measure of process stability and inherent variability.

The BBD experimental results were fitted with a polynomial regression equation and expressed as $y = -57.0253 + (1.8879 \bullet A) + (6.2229 \bullet B) + (0.1331 \bullet C) - (0.0259 \bullet A$

Table 2. ANOVA of Box-Behnken design for pectinase activity

Source	Sum of squares	Degree of freedom	Mean square	F-value	*p* value	
Regression model	9.69	9	1.08	12.75	0.0060	Significant
Linear	5.0275	3	1.67586	19.83	0.003	Significant
A-temp	0.1597	1	0.1597	1.89	0.2275	
B-pH	0.0583	1	0.0583	0.6901	0.4440	
C-hours	4.81	1	4.81	56.92	0.0006	Significant
Interaction	0.1388	3	0.0463	0.55	0.671	
temp*pH (AB)	0.0673	1	0.0673	0.7970	0.4129	
temp*hours (AC)	0.0181	1	0.0181	0.2142	0.6629	
pH*hours (BC)	0.0534	1	0.0534	0.6316	0.4628	
Square	1.509	3	1.5093	17.86	0.004	Significant
temp*temp (A^2)	1.37	1	1.37	16.20	0.0101	Significant
pH*pH (B^2)	0.5617	1	0.5617	6.65	0.0495	Significant
hours*hours (C^2)	3.16	1	3.16	37.40	0.0017	Significant
Residual	0.4225	5	0.0845			
Lack of fit	0.1854	3	0.0618	0.5213	0.7093	Not significant
Pure error	0.2371	2	0.1185			
Cor total	10.12	14				

R^2 = 95.82%, predicted R^2 = 65.41%, adjusted R^2 = 88.31%.

• B) + (0.0005 • A • C) + (0.0048 • B • C) – (0.0243 • A2) – (0.3900 • B2) – (0.0016 • C2), where y is pectinase activity, A is temperature, B is pH, and C is incubation time. The accuracy and statistical significance of each term in the model were evaluated using analysis of variance (ANOVA) and shown in Table 2. The analysis of factors elucidated F-value for the regression model of pectinase activity as 12.75, which showed that the model of this study was significant.

2D contour and 3D response plots were constructed to illustrate the interactive effect of each independent variable on pectinase activity for maximum pectinase production. Here, the effects of the two variables were shown, and the third variable was fixed (Fig. 2.).

The RSM optimizer found the optimal conditions of 35.62°C, pH 7.15, and 58.40 h for pectinase production (suppl. Fig. S1; see www.karger.com/doi/10.1159/000528257 for all online suppl. material). The bacterium was cultured in the mentioned optimal conditions, and enzyme activity was calculated for verification. The calculation showed that the measured value did not ideally agree with the value predicted by the response model. However, the value (2.37 U/mL) was close to the predicted value (2.74 U/mL). Therefore, the optimal cultural condition for pectinase production by *Streptomyces* sp. was 35°C, pH 7, and 58 h of incubation.

The counterplot Figure 2a, d showed that the pectinase activity increased with an increase in incubation temperature and time to a certain level and decreased afterward. Similar patterns were observed for the effects of temperature and pH and pH and incubation time, as shown in Figure 2b, e, c, f, respectively.

Effects of Different Pectin Concentrations, Nitrogen and Carbon Sources on Pectinase Production

The fermentation media containing 1.5% pectin was observed to show the highest enzyme activity, followed by 2% pectin when used as a carbon source. Although the fermentation medium containing 1.5% observed the highest enzyme activity, there is no significant difference between 1.5% and 2% pectin-containing media when analyzed statistically (Fig. 3a). Thus, 1.5% of pectin was used in further studies. Among the different nitrogen sources studied, casein significantly increased pectinase production (Fig. 3b).

The present study illustrated that the growth of *Streptomyces* sp. and pectinase activity was influenced by the carbon sources added. Sorbitol followed by pectin exhibited the maximum pectinase activity and acted as the best carbon source for pectinase production. The addition of glucose inhibited the pectinase activity. Similarly, fructose, xylose, lactose, and mannitol decreased pectinase production significantly (Fig. 3c).

Effects of Different Metal Ions in Pectinase Production

The different trends in pectinase activities were observed when various concentrations of metal ions in sul-

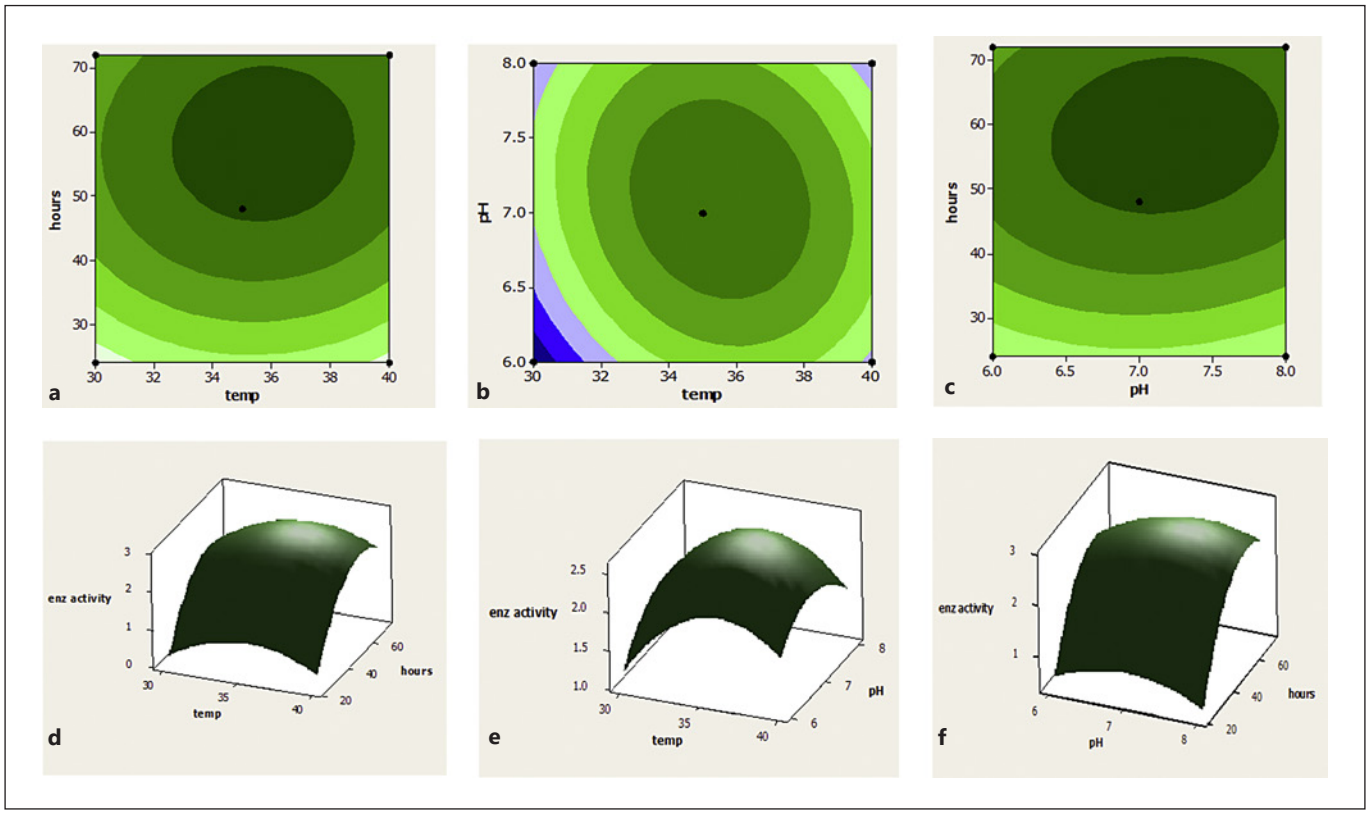

Fig. 2. 2D contour plots and 3D response surface plots showing the effect of incubation temperature and hour (**a**, **d**), temperature and pH (**b**, **e**), and pH and hour (**c**, **f**) for the pectinase activity.

fate and chloride forms were added to the fermentation media (Fig. 4). The result revealed that pectinase production was higher with 0.5 mM of various metal ions in sulfate forms (Fig. 4a). Similarly, NaCl exhibited the higher pectinase activity than control in various concentrations among the chlorides of varying metal ions supplemented in basal media. $CaCl_2$ exhibited significantly higher activity in 1 mM but lower in other concentrations (Fig. 4b).

Effects of Different Surfactants and Chemicals on Pectinase Production

Different surfactants and additives may affect pectinase production. All the surfactants and chemicals added to this research inhibited the pectinase production except Tween-20 (Fig. 5.).

Effects of Amino Acids and Vitamins on Pectinase Production

The different amino acids and their analogs showed the pectinase activity as in Figure 5a; arginine and alanine inhibited the activity compared to control. However, asparagine, methionine, and cysteine slightly increased the activity, but glycine stimulated the activity significantly (Fig. 6a).

The pectinase production by *Streptomyces* sp. was enhanced by adding vitamins to the fermentation media (Fig. 6b). Ascorbic acid significantly increased pectinase production, and there was a slight increase in pectinase activity by thiamine, biotin, nalidixic acid, and riboflavin.

Partial Purification and Characterization of Pectinase

The cell-free supernatant was partially purified using ammonium sulfate, acetone, or ethanol precipitation. The acetone-precipitated enzyme illustrated maximum enzyme activity followed by ethanol and ammonium sulfate precipitation (Fig. 7). Also, the result revealed that a higher concentration of solvents decreased the enzyme activity. Indeed, the enzyme was inhibited in the presence of acetone or ethanol at a concentration higher than 70%

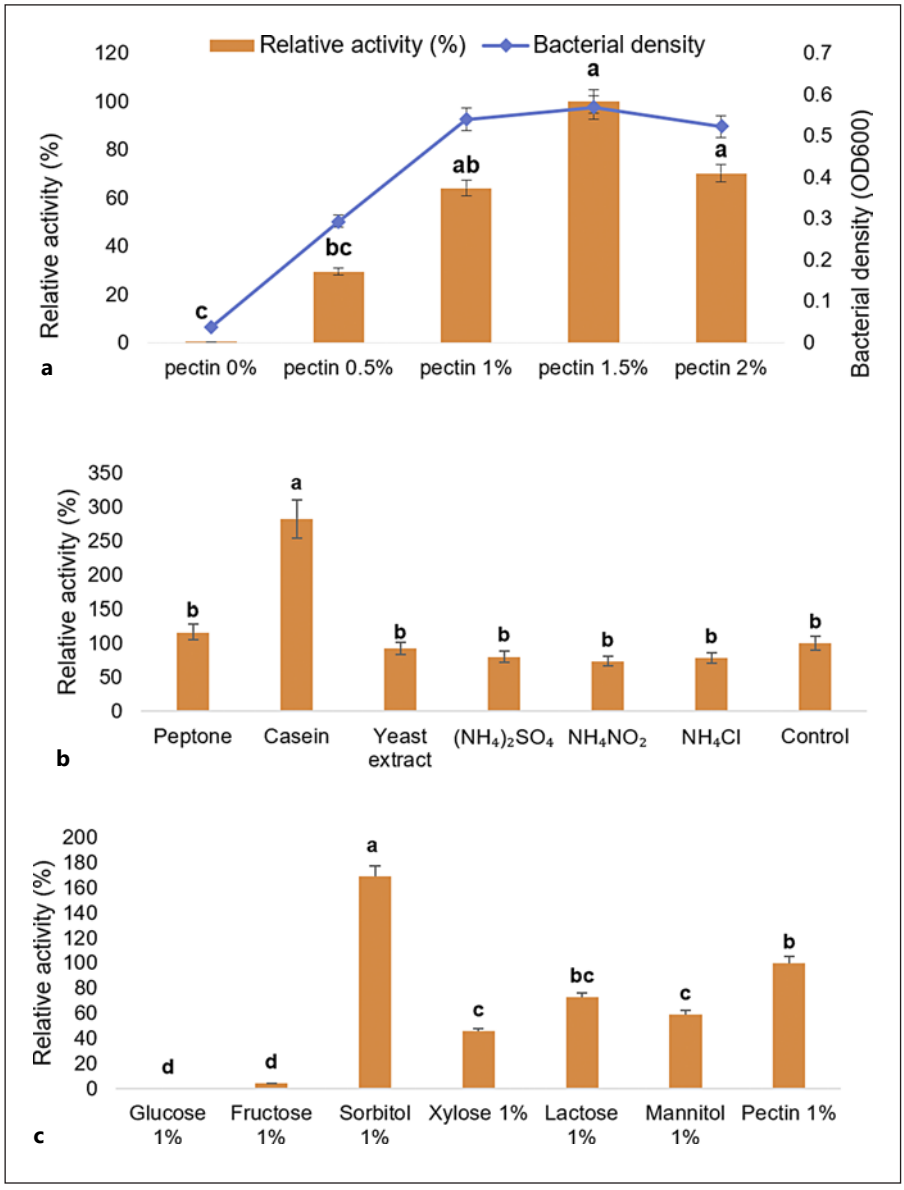

Fig. 3. Effects of different pectin concentrations (**a**), nitrogen (**b**), and carbon sources (**c**) on pectinase production (different lowercase letters above the bar indicate the significant difference between experiments).

and in the presence of ammonium sulfate at a concentration higher than 60%.

The activity of partially purified pectinase continuously increased till 70°C, showing its maximum activity at 70°C, and declined at a higher temperature above 70°C (Fig. 8a). However, the enzyme showed a different trend when the enzyme extract reacted at a different pH. The activity increased sharply from pH 3–5, decreased from pH 5–7, and again increased continuously from pH 7–9. The pH above 9 decreased the activity, showing maximum activity at pH 5 and 9 (Fig. 8b). The pectinase from *Streptomyces* sp. was observed to have residual activity of approximately 30–40% after 120 min of exposure to different temperatures (Fig. 8c).

SDS and Zymogram Analysis

The partially purified enzyme was electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The multiple protein bands were observed around molecular weight 20–70 kDa (Fig. 9.). The zymogram revealed two hydrolyzed bands at approximately 25 and 70 kDa, which indicated the presence of two pectinases produced by *Streptomyces* sp.

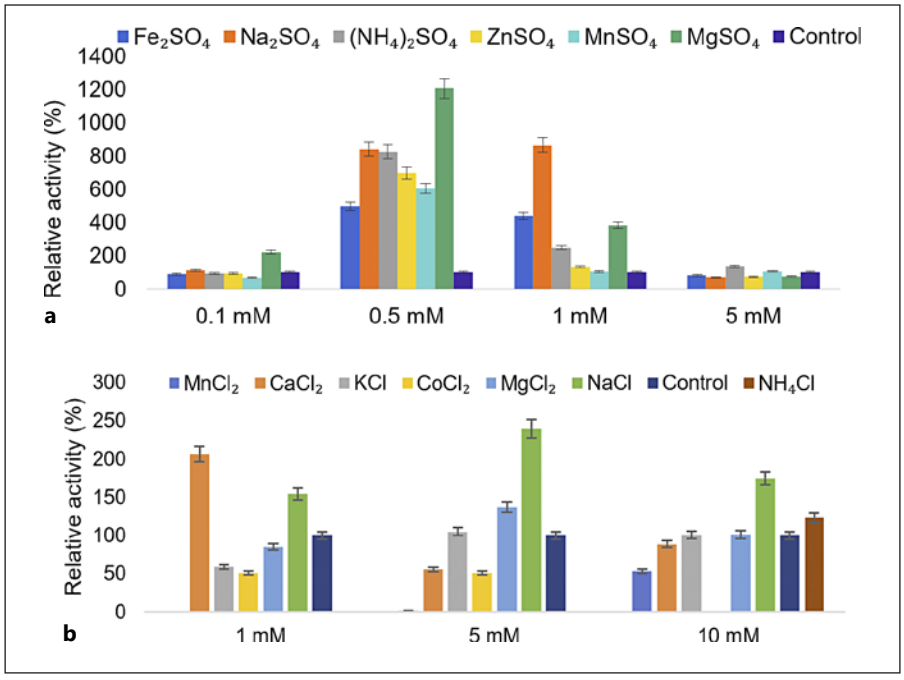

Fig. 4. Effects of different metal ions in sulfate (**a**) and chloride forms (**b**) for pectinase production by *Streptomyces* sp.

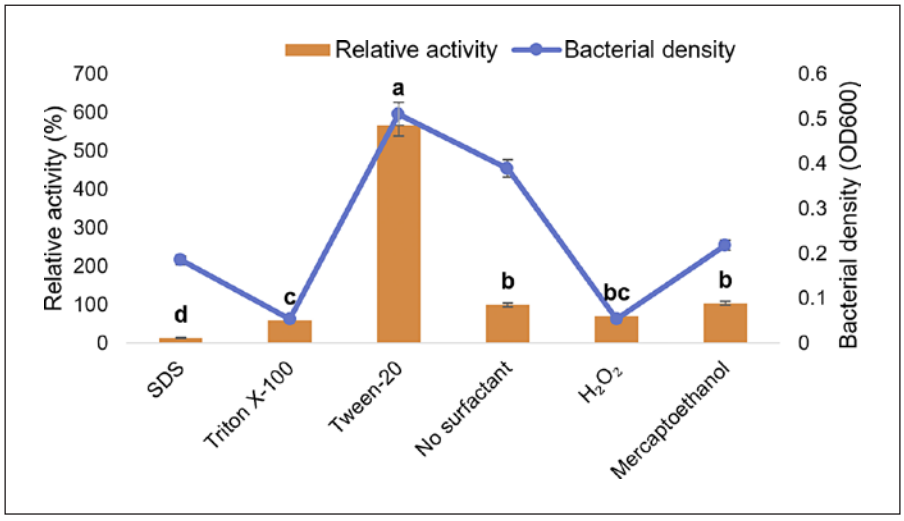

Fig. 5. Effects of different surfactants and chemicals on pectinase production by *Streptomyces* sp. (different lowercase letters above the bar indicate the significant difference between experiments).

Decolorization of Crystal Violet Dye

The experimental result revealed the decolorization of crystal violet dye by *Streptomyces* sp. ranged from 39.29 to 53.75%. The highest decolorization rates of crystal violet dye (53.75%) and the lowest (39.29%) were observed in the medium containing 2 mg/mL and 1 mg/mL of crystal violet at 144 and 24 h, respectively (Table 3.).

Discussion

The present study exploited *Streptomyces* sp. that was isolated from forest soil [Shrestha et al., 2021a] to determine the optimum fermentation conditions for maximum pectinase production and to characterize the enzyme produced. The bacterium *Streptomyces* sp. illustrated significantly higher enzyme activity (suppl. Fig. S2) in YEP, so this medium was used as the pectinase production media in this study. Another study supported the

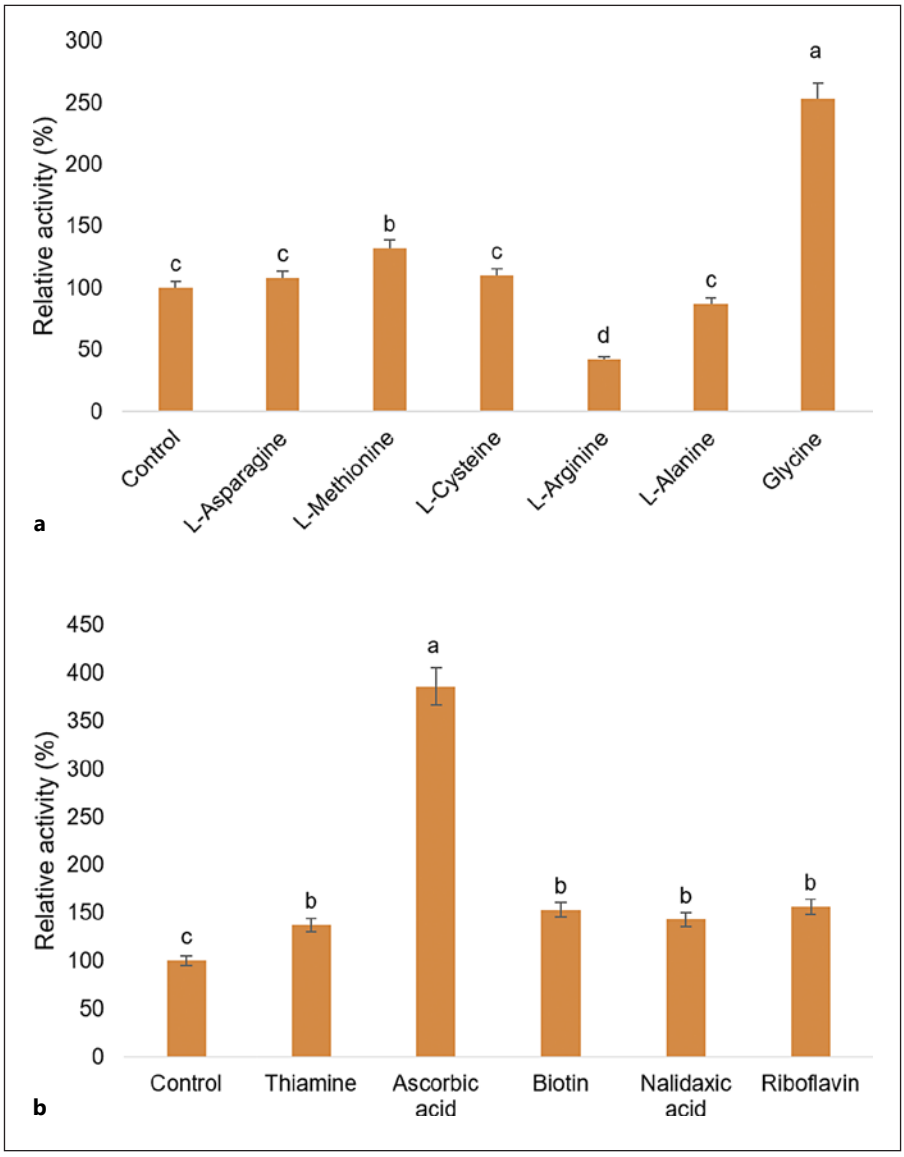

Fig. 6. Effects of different amino acids (**a**) and vitamins (**b**) on pectinase production by *Streptomyces* sp. (different lowercase letters above the bar indicate the significant difference between experiments).

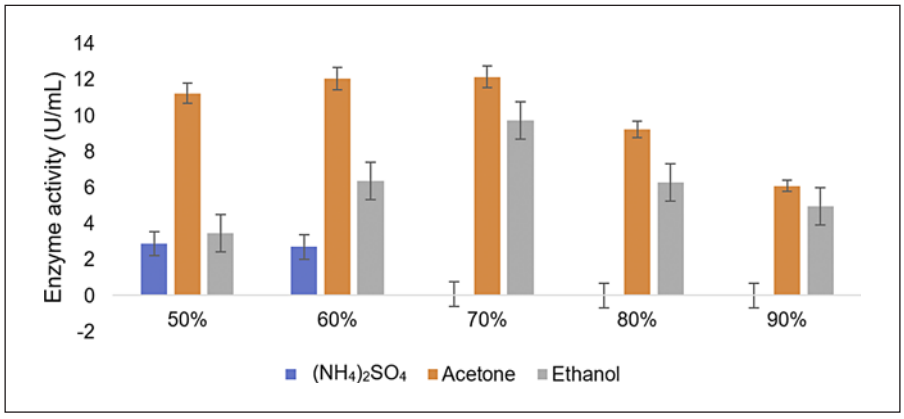

Fig. 7. Partial purification by different solvents in different concentrations.

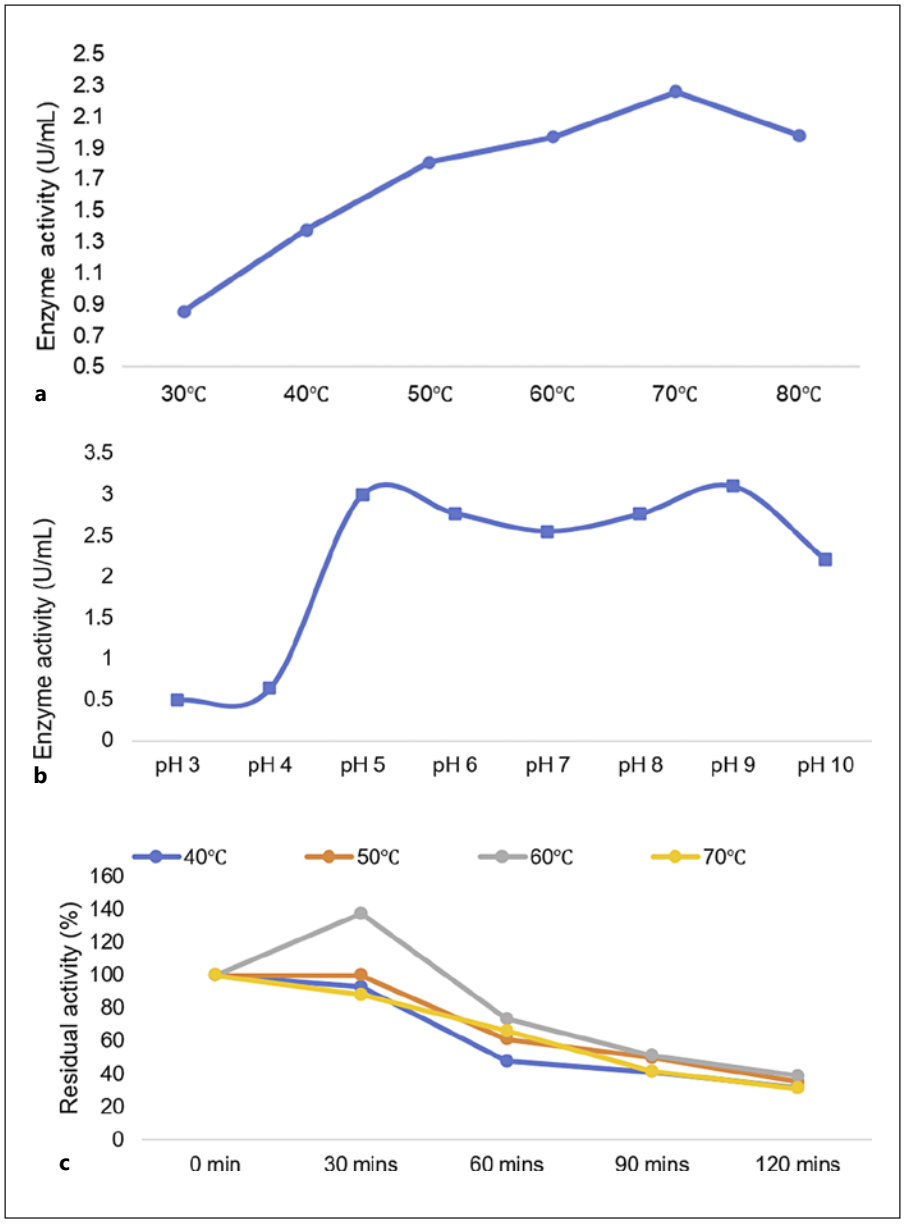

Fig. 8. Effects of different temperatures (**a**), and pH (**b**) on enzyme activity, and stability at different temperatures of partially precipitated enzyme.

result, as yeast extract is a good source of essential nutrients and organic nitrogen [Kaur et al., 2016]. Temperature is one of the influencing factors in bacterial growth and metabolic and enzymatic activities. The maximum pectinase activity was observed at 35°C, so it was considered the optimum temperature for pectinase production by this bacterium. Similar findings were reported for *Chryseobacterium indologenes* strain SD [Roy et al., 2018b] and *Bacillus* species [Aaisha and Barate, 2016]. However, *Saccharomyces cerevisiae* illustrated the maximum enzyme activity at 30°C for PG and PL [Poondla et al., 2015]. The decrease in activity at low temperatures may be due to insufficient energy. In contrast, the low activity at high temperatures may be due to denaturation or some modification in enzyme structure [Rehman et al., 2016; Ibrahim et al., 2021].

The pH of the fermentation media plays a vital role in enzyme production. The maximum microbial growth and enzyme activity in our study were observed at pH 7. Previous studies reported that the *Bacillus* sp. produced a high amount of pectinase between pH 7.5 and 8 [Aaisha and Barate, 2016; Roy et al., 2018b; Oumer and Abate,

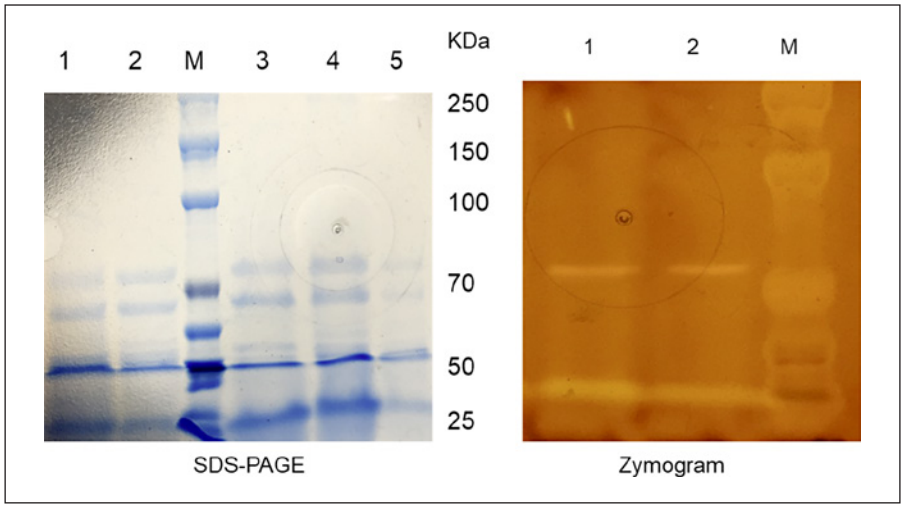

Fig. 9. SDS-PAGE (1 and 5: crude enzyme extract (supernatant before precipitation), 2: ammonium sulfate precipitated, 3: ethanol precipitated, 4: acetone precipitated pectinase, M: marker) and zymogram (1 and 2: acetone precipitated, M: marker) of pectinase.

Table 3. Decolorization of crystal violet by *Streptomyces* sp. at different concentrations and incubation hours

Crystal violet	Decolorization rate, %					
	24 h	48 h	72 h	96 h	120 h	144 h
1 mg/mL	39.29	43.26	43.32	44.39	44.06	44.09
2 mg/mL	43.52	46.12	48.11	51.33	52.51	53.75
3 mg/mL	41.52	43.12	44.01	45.06	45.30	46.13

2018]. The result from this study inferred that very low and very high pH conditions are not favorable for the growth of the organism, which might decrease the growth and enzyme activity of the organism.

The *p* values from the analysis of BBD were used to ensure the significance of each coefficient and interaction strength between each independent variable. The *p* value <0.05 ($p < 0.05$) observed in the analysis indicated the model term is significant. The quality of fit of the second-order model equation was expressed by the coefficient of determination (R^2), and the F-test determined its statistical significance [Gonçalves et al., 2012; Handa et al., 2016]. In this analysis, C, A^2, B^2, and C^2 were significant model terms as the *p*- values <0.05. Also, the coefficient of determination (R^2) was calculated as 0.984, indicating that this model could explain 98.4% of the variability in the response. The R^2 value closer to 1.0 predicts a better and stronger model. Similarly, the lack of fit F-value of 0.52 and *p*- value of 0.7093 implied the lack of fit was not significant relative to the pure error, and there was a 70.93% chance that a large *p*- value could occur due to noise. Nonsignificant lack of fit was what we wanted, and this indicated the model was well fitted to the experimental data [Gonçalves et al., 2012; Handa et al., 2016].

In the present study, pectin was observed as a stimulator for pectinase activity because in the absence of pectin, there was no pectinase activity (Fig. 7a). Ramírez-Tapias et al. [2015] demonstrated that 1% pectin gave the maximum polygalacturonase activity [Ramírez-Tapias et al., 2015] which was similar to the results reported by [Ketipally and Ram, 2018; Ketipally and Ram, 2018]. Our study result illustrated the maximum activity at 1.5% pectin, although the activity was not significantly different between 1%, 1.5%, or 2% pectin. The result indicated that a higher concentration of citrus pectin in the fermentation media could have an antagonistic effect on pectinase production [Ramírez-Tapias et al., 2015].

Nitrogen is required for microbial growth because it is an essential protein constituent, and different nitrogen sources influence the growth of microorganisms and metabolite production. The inorganic nitrogen sources inhibited enzyme production compared to the control. Hence, the organic nitrogen source is more beneficial than the inorganic source. Furthermore, our study results supported that organic nitrogen sources increased pectinase activity.

The carbon in the fermentation media plays a crucial role in providing energy for bacterial growth and enzyme

production. *Streptomyces* sp. produced maximum pectinases when sole pectin and sorbitol were added to the media because they may act as a suitable carbon source, whereas other carbon sources decreased pectinase activity when added to the basal media similar to other studies [Kaur and Gupta, 2017; Oumer and Abate, 2018]. The pectinase production decreased slightly when glucose, lactose, and xylose were added to the media [Kaur and Gupta, 2017]. Furthermore, other studies illustrated that adding glucose inhibited the PG production by *Aspergillus* sp., and maximum PG was observed in the absence of glucose [Runco et al., 2001]. This may be because glucose has a repressive impact on the catabolism of enzymes or a negative effect of glucose on enzyme production. In contrast to our result [Kuhad et al., 2004] found higher pectinase activity in glucose-supplemented media and the highest pectinase yield in the media containing pectin and glucose [Kuhad et al., 2004]. *Bacillus subtilis* produced maximum activity when lactose was added, whereas maltose acted as the best carbon source for *Bacillus amyloliquefaciens* [Arekemase et al., 2020]. The inconsistency in the maximum enzyme activities may be due to the genetic differences between microorganisms. In addition, the study illustrated that the addition of glucose or sucrose did not reduce pectinase activity in solid-state fermentation but strongly decreased in submerged-state fermentation [Solís-Pereira et al., 1993]. Thus, the nature and concentration of carbon sources play a significant role in enzyme production. The pectinase production media containing glucose or sucrose like carbon depends on the types of fermentation too.

The high concentration of metal ions inhibited enzyme production, possibly due to the blockage of protein secretion into the external medium [Ahlawat et al., 2009]. The presence of metal ions affects the enzyme's catalytic properties by inhibiting or activating the enzyme activity that affect the enzyme's active site and the protein molecules' stability. The lower concentration of Mg^{2+} (2 mM or 4 mM) acts as the activator, but the higher concentration (6 mM, 8 mM, or 10 mM) inhibited the enzyme activity [Anggraini et al., 2020]. Similar can be the reason for $CaCl_2$, Fe_2SO_4, $MgSO_4$, $MnSO_4$, and $ZnSO_4$ in this study.

The surfactants may affect cell membrane permeability, leading to blockage in the pectinase secretion, or the surfactants may denature the enzyme [Ahlawat et al., 2009]. The surfactants generally interact with proteins and alter particular regions affecting the structure of proteins and enzyme activities [Zohdi and Amid, 2013]. Another study demonstrated that the addition of Tween-20 significantly enhanced the pectinase yield by *Bacillus subtilis* [Ahlawat et al., 2009]. In contrast, pectinase secretion by *Aspergillus* sp. was inhibited by Tween-20, Tween-80, and Triton X-100 and enhanced significantly by polyethylene glycol [Li et al., 2015]. In this study, Tween-20 enhanced the enzyme activity, which might be due to this surfactant providing a suitable membrane structure for enzymes to bind with the substrate and increase the activity.

Amino acids and vitamins also affect pectinase activity. In a prior study, DL-isoleucine, DL-norleucine, L-lysine monohydrochloride, L-leucine, casamino acid, and DL-b-phenylalanine showed a stimulatory effect on pectinase production by *Streptomyces* sp. QC-11–3 [Beg et al., 2000]. Proline, L-tyrosine, L-cysteine, DL-aspartic acid, L-cystine monohydrochloride, and DL-threonine inhibited pectinase yield, but DL-alanine, L-histidine monohydrochloride, DL-b-phenylalanine, and L-lysine monohydrochloride stimulated the pectinase production by *Streptomyces* sp. RCK-SC [Kuhad et al., 2004]. *Aspergillus terrus* increased polygalacturonase production when leucine, tyrosine, and methionine were added to the production media [Runco et al., 2001]. Another study showed vitamins like ascorbic acid, riboflavin, nicotinic acid, and biotin enhanced the pectinase production by *Streptomyces* sp. RCK-SC and thiamine reduced pectinase production [Kuhad et al., 2004]. In our study, glycine and ascorbic acid may act as modifiers for fitting the pectinase precisely in the active sites, changing the configurations. Also, ascorbic acid might function as a coenzyme, and the glycine charges play critical roles in the catalytic reactions, increasing enzymatic activities.

The acetone precipitated enzyme illustrated maximum enzyme activity which possibly may be because the enzyme favors the organic compound rather than the inorganic compound (ammonium sulfate). The partially precipitated pectinase was explored to study the effect of temperature and pH on its activity and stability at different temperatures. These findings revealed that pectinase produced by *Streptomyces* sp. was thermostable and not neutral (can resist both acidic and alkaline pH but not very low and very high pH). This characteristic of the enzyme was unique and beneficial for various industrial applications. The decrease in activity after 70°C and in very high and low pH may be due to the denaturation of enzymes and changes in the native structure of pectinases. The higher temperature increases the collision between the reactant molecules and kinetic energy. The higher kinetic energy after a certain limit may denature the enzyme and change the structure of the enzyme [Rehman et al., 2015]. The study conducted by [Ramírez-Tapias et al.,

2015] observed the optimum catalytic conditions of PG produced by *Streptomyces halstedii* at 50°C and pH 12. They stated that the alkaline PG is applicable in the depectinization of wastewater from the pulping mill and paper-making industries [Ramírez-Tapias et al., 2015]. In another study, the maximum activity of pectin lyase (PNL) was observed at 60°C and pH 8 [Demir et al., 2014].

The result of the zymogram showed that *Streptomyces* sp. produced enzymes able to degrade citrus pectin and have molecular weights of around 25 kDa and 70 kDa. This result is in harmony with another study which revealed two bands of ethanol precipitated pectinases with a molecular weight of 60 and 64 kDa [Takcı and Turkmen, 2016] and PG with molecular weights of ~36 kDa and ~72 kDa [Paudel et al., 2015]. In addition, Poondla et al. [2015] found 2 bands of PG isoenzymes having molecular weights around 47 kDa and 50 kDa [Poondla et al., 2015] and ammonium sulfate-precipitated pectinase from *Aspergillus foetidus* illustrated 2 bands at 34 and 44 kDa [Kumar et al., 2012]. Ramírez-Tapias et al. [2015] revealed that the molecular weight of alkaline PG produced by *Streptomyces halstedii* was approximately 48 kDa [Ramírez-Tapias et al., 2015], and Demir et al. [2014] observed the molecular weight of PNL of about 36 kDa [Demir et al., 2014]. The molecular weight of pectinases varies with different microorganisms; for example, PG from *Bacillus licheniformis* was observed to be 153 kDa [Rehman et al., 2015], PNL of 25 kDa in *Bacillus pumilus* [Güllüce and Demir, 2010], and polygalacturonase of 153 kDa in *Bacillus licheniformis* [Rehman et al., 2015]. The polygalacturonase from *Rhizopus pusillus* was purified by two chromatographic steps (Sephadex G-200 and Sephacryl S-100) gave a band around 32 kDa in SDS-PAGE [Siddiqui et al., 2012].

The degradation of the dye depends on different factors such as pH, temperature, concentrations of dye, incubation time, and inoculum size. Roy et al. [2018a] illustrated the complete (100%) crystal violet degradation after optimizing the environmental parameters, and *Enterobacter* sp. effectively decolorized the dye of textile effluents [Roy et al., 2018a]. Similarly, *Pseudomonas aeruginosa* isolated from textile industry wastewater was exploited to decolorize methyl orange dye. *P. aeruginosa* decolorized 99% of methyl orange in 100 mg/L and showed *P. aeruginosa* could be effectively used to detoxicate industrial wastewater [Kishor et al., 2021]. Our study illustrated that the highest decolorization rate was ~54% which is lower than other studies which might be because this study was performed without optimization of environmental parameters.

Thus, the bacterium (*Streptomyces* sp.) isolated from forest soil revealed to have the potential for pectinase production and crystal violet decolorization. Further, a detailed study on the purification of pectinase enzyme, stability, and different applications, and the oxidizing enzyme from *Streptomyces* sp. able to decolorize dye are recommended. Moreover, the pectinase production in solid-state fermentation needs to be compared with submerged fermentation, and differences in pectinase characteristics comparison are needed to be explored.

Materials and Methods

Bacterium Strain and Inoculum Preparation

The bacterium exploited in this study was isolated, screened, and identified as *Streptomyces* sp. [Shrestha et al., 2021a] and stored at −80°C in the laboratory as a bacterium stock. The bacterium was revived by inoculating 1% v/v (40 µL) of preserved bacterium stock into 4 mL of LB broth and incubated at 35°C for 18 h. For further studies, this overnight cultured broth was taken and proceeded.

Optimization of Fermentation Conditions

The overnight cultured *Streptomyce*s sp. of 0.5 mL was inoculated in an Erlenmeyer flask containing 50 mL of YEP media composed of 0.3% w/v yeast extract, 1% w/v pectin, 0.2% w/v KH_2PO_4, and 0.2% w/v K_2HPO_4 in distilled water. The inoculated flasks were incubated at 35°C, 200 rpm for 4 days. Samples from inoculated flasks were collected at regular intervals of 24 h, and enzyme activities were assayed and compared. Pectinase activity was calculated by measuring the reducing sugar content released from the substrate following the 3,5-dinitrosalicylic acid (DNS) method [Miller, 1959], as mentioned in a previous study by Shrestha et al. [Shrestha et al., 2021a]. Each enzymatic activity was expressed as the amount of enzyme that releases 1 µmol of galacturonic acid in 1 min under the mentioned conditions.

The various parameters that affect enzyme production were optimized by one variable at a time, while all other factors were kept constant. The experiments carried out in this study were performed in triplicates.

Effect of Incubation Period, Temperature, and pH in Pectinase Production

The *Streptomyces* sp. was cultured in pectinase production media (YEP) for 120 h. At different incubation periods, the cultured broth was aseptically taken out, and the cell-free supernatant was used for enzyme activity assay. The bacterial growth was monitored by measuring the optical density (OD) at 600 nm using the pellet resuspended in distilled water. The pectinase activities at different incubation temperatures (30°C, 35°C, 40°C, and 45°C) and pH (5, 6, 7, 8, 9, and 10) were also studied.

RSM Design for Optimization of Pectinases Production by Streptomyces sp.

Three factors and three-level Box-Behnken design was employed to produce maximum pectinase and understand the interactions of different variables. The study considered three variables: incubation

time, temperature, and pH of cultural conditions, and the range of variables was determined from optimization one variable at a time method. The designation of the experiment (15 runs with three replicates of center points) and analysis of results were performed using Minitab software. The response variable (pectinase activity) was fitted by a second-order model to relate the response variable to the independent variables. The maximum pectinase activity was obtained from optimizing these cultural conditions, and the statistical analysis of the model was performed in the form of ANOVA.

Factors Affecting Pectinase Production

Effects of Different Pectin Concentrations, Carbon, and Nitrogen Sources for Pectinase Production

Different concentrations of pectin (0.5%, 1%, 1.5%, and 2% w/v), carbon sources (glucose, fructose, xylose, lactose, mannitol, sorbitol, and pectin), and nitrogen sources (peptone, casein, yeast extract, ammonium sulfate, ammonium nitrate, and ammonium chloride) were supplemented as individual components to the basal media to know their effects on pectinase production.

Effects of Different Metal Ions in Chloride and Sulfate Forms for Pectinase Production

The effect of various metal ions was also studied for pectinase enzyme activity by using 0.1 mM, 0.5 mM, 1 mM, or 5 mM of each metal ion, such as Fe_2SO_4, Na_2SO_4, $(NH_4)_2SO_4$, $ZnSO_4$, $MnSO_4$, and $MgSO_4$ in sulfate forms. Similarly, in chloride forms, $MnCl_2$, $CaCl_2$, KCl, $CoCl_2$, $MgCl_2$, NaCl, and NH_4Cl were studied to know their effects on pectinase production.

Effects of Different Surfactants and Chemicals for Pectinase Production

Different surfactants such as SDS, Triton X-100, Tween-20, and chemicals like mercaptoethanol and H_2O_2 were added to the culture media in 1%, and their effect on pectinase activity was studied.

Partial Purification and Characterization of Pectinase

Precipitation and Molecular Weight Determination of Pectinase

The crude enzyme extract obtained after culturing the bacterium in the optimized cultural conditions and centrifugation was precipitated by 50–70% of saturated ammonium sulfate, acetone, or ethanol. All the mixtures were kept at 4°C overnight and centrifuged at 12,000 rpm for 20 min. After centrifugation, each residue was suspended in a minimal amount of 0.1 M phosphate buffer at pH 7 and kept at 4°C for future use. The pectinase activity of the partially purified enzyme was assayed by the method described earlier.

The molecular weight of the produced pectinase was decided by running SDS-PAGE, which was carried out in a 3-mm slab using 5% (w/v) stacking and 12% (w/v) separating gels. For this, samples were heated for 5 min at 95°C in the sample loading buffer before loading on wells. After running the gel for a specific time, it was stained with Coomassie brilliant blue and destained by keeping it in a destaining solution. The standard board range protein marker was used to compare the molecular weight of the enzyme sample.

For zymogram, the gels after electrophoresis were soaked in 2.5% (w/v) Triton X-100 for 30 min, washed in phosphate buffer (pH 7.4) for 30 min, flooded with 2% pectin, and kept at 45°C for 60–90 min. Then the gel was stained with 0.05% (w/v) potassium iodide solution for 10 min and washed with water until bands became visible.

Effects of Temperature and pH on Pectinase

The reaction mixture of the partially purified enzyme and substrate was incubated at different temperatures (25°C, 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C) for 10 min to determine the effects of temperature on pectinase activity. Similarly, the effect of pH on pectinase activity was accessed using different pH buffers such as citrate buffer (pH 3–4), potassium-phosphate buffer (pH 5–8), and glycine-NaOH buffer (pH 9) and incubating for 10 min at 50°C.

Ability of the Bacterium to Decolorize Crystal Violet

The bacterium was positive for catalase, DNase, cellulase, xylanase, the amylase screening test, and spore former. Further, the bacterium was grown in a wide range of temperatures and pH [Shrestha et al., 2021a], so it was exploited to determine its ability to decolorize crystal violet. The overnight grown bacterium was inoculated in YEP media supplemented with crystal violet (1 mg/mL, 2 mg/mL, or 3 mg/mL) and incubated at 35°C. The uninoculated medium with crystal violet was used as a control. Every 24 h, the decolorization rate was determined by observing the absorbance at 680 nm. The decolorization efficiency was evaluated as the percentage ratio of change in OD by initial OD based on the equation [Roy et al., 2018a]:

$$\text{Decolorization } (\%) = \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \times 100$$

Statistical Analysis

All the experiments in this study were performed in triplicates, and the data were analyzed using ANOVA. The level of significance of results was determined by Tukey's post hoc test with a 95% confidence level and considered significant if $p < 0.05$.

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Statement of Ethics

Ethical approval is not required for this study in accordance with local or national guidelines.

Conflicts of Interest Statement

The authors have no conflict of interest to declare.

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Author Contributions

Sarita Shrestha carried out conceptualization, methodology, investigation, formal analysis, and writing of original manuscript; Dr. Wensheng Qin contributed to resources, supervision, reviewing, and editing. Chonlong Chio, Janak Raj Khatiwada, Aristide Laurel Mokale Kognou, and Xuantong Chen coordinated and helped in reviewing. All authors read the final draft of the manuscript and approved it for publication.

Data Availability Statement

The data generated and analyzed are included in this article. Further inquiries of this study can be directed to corresponding author.

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