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Factors and Correlations Controlling Cellulase-Free Xylanase Production by *Streptomyces Halstedii* NRRL B-1238 in Submerged Culture

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Abstract: Cellulase-free xylanase production of *Streptomyces halstedii* NRRL B-1238 was optimized in submerged fermentation during which the environmental and nutritional factors controlling enzyme production were investigated. Xylanase was maximally produced at the 120th h of fermentation at initial pH 7.5 and incubation temperature of 32 °C with medium volume to flask volume of 25/250 (ml/ml). The optimum nutritional conditions showed xylan hydrolysate at 20 gL^{-1} and peptone as the most effective nutrients for xylanase production. Maximum production of cellulase-free xylanase was recorded at 5% inoculum ratio, the overall specific activity reached up to 45.15 U/mg protein. The maximum partial purification of the cellulase-free xylanase was achieved with ammonium sulphate at 60% saturation. Aappreciable amounts of cellulose-free xylanase could be produced on the selected moderately low cost medium.

Key words: *Streptomyces halstedii*, xylanase, xylan hydrolysate, submerged fermentation

INTRODUCTION

Xylan, the hemicelluloses containing heteropolysaccharides, constitutes from 20 to 40 % of the dry weight of some higher plants and agricultural wastes, as it is widely distributed in plant cell walls and forms a main part of the hemicellulose fraction (Joseleau *et al*., 1992). Xylan consists of a backbone of β-1,4-linked D-xylopyranose residues with substitutions of *O*-acetyl, arabinosyl and methylglucuronosyl as side chains (Chang *et al*., 2004; Collins *et al*., 2005; Rawashdeh *et al*., 2005).

The complete hydrolysis of xylan requires the combined action of various xylanases. Endo-1,4-*β*-xylanase (1, 4-*β*-D-xylan xylanohydrolase; EC 3.2.1.8) cleaves the internal glycosidic bonds in the xylan backbone in a random mechanism. This in turn, leads to accumulation of short-chain xylooligomers of *β*-D-xylopyranosyl, which may inhibit the endoxylanase. Another enzyme, *β*-D-xylosidases (1,4-*β*-D-xylan xylohydrolase; EC 3.2.1.37) hydrolyzes the resulted short oligomers by endwise attack of xylooligosaccharide, removing the cause of inhibition, and increasing the efficiency of xylan hydrolysis which finally releases D-xylose from short-chain xylooligomers (Wong and Saddler, 1992; Coughlan, 1993; Zanoelo *et al.*, 2004). Finally, the *O*-acetyl groups from positions 2 and/or 3 on the β-D-xylopyranosyl residues is removed by the action of acetylxylan esterase (EC 3.1.1.6) (Caufrier *et al*., 2003).

Nature is abundant with many microorganisms that produce different types of xylanases and efficiently degrade xylan. Several *Streptomyces* spp. were reported to produce xylanase such as *S. cyaneus* (Ninawe *et al*. 2008) and *S. actuosus* A 151 (Wang *et al*. 2003). The thermophilic *S. thermovulgaris* TISTR1948 was also reported to secret high levels of cellulase-free xylanases (Thanongsak *et al*., 2011).

There are potential applications of xylanases include; 1) replacing chlorine with xylanase in the pulp and paper industry to increase the extractability of lignin for production of high-quality paper (Ali and Sreekrishnan, 2001; Techapun *et al*., 2003), 2) reducing the viscosity of the raw material in animal feed by break down arabinoxylans in the ingredients of the feed (Twomey *et al*., 2003), 3) xylanase plays a crucial role in textile industry during process plant fibers like linen, for this purpose xylanase enzyme should be free from cellulases (Csiszár *et al*., 2001), finally, 4) there is an increasing trend to use xylanases in the baking industry (Butt *et al*., 2008).

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The present paper deals with the production of xylanase enzyme from xylan assimilating *Streptomyces halstedii* NRRL B-1238 strain as potent producer of xylanase. The optimum nutritional requirements and fermentation conditions for maximum yield of xylanase have been studied. The correlation among different parameters was also estimated.

MATERIALS AND METHODS

Streptomyces Strain:

Streptomyces halstedii NRRL B-1238 was provided by the Agricultural Research Service (ARS) culture collection, Northern Regional Research Laboratory (NRRL), Peoria, Illinois, USA.

Culture Media and Inoculum Preparation:

Streptomyces halstedii NRRL B-1238 strain was maintained and sub cultured on slants of International *Streptomyces* Project (ISP) medium 2 consisting of (gL^{-1}): glucose, 4; malt extract, 10; yeast extract, 4 and agar 20. Slants of 10 days old containing the well-grown experimental microorganism was scratching with sterile distilled water to form a spore suspension. For inoculum preparation, 50 ml ISP-2 broth medium was inoculated with the spore suspension of *S. halstedii*. The resulted 48-h-old vegetative inoculum (2×10^7 spores ml^{-1}) was used as the standard inoculum. The batch fermentation for xylanase production was carried out in 250-ml flasks containing 50 ml of the basal medium contains (gL^{-1}): Xylan hydrolysates from sugar cane bagasse, 20; sodium nitrate, 2; K_2HPO_4 anhydrous, 1; $MgSO_4.7H_2O$, 0.5; $CaCO_3$, 3; adjusted to pH 7.0-7.2 and sterilized at 1.5 atmospheric pressure for 20 min. Inoculated flasks were incubated at 30°C under shaking conditions in a reciprocating incubator shaker (New Brunswick Scientifics Co., New Brunswick, NJ.USA) at 200 rpm. The culture medium was inoculated with 4% from the freshly prepared inoculum.

The Tested Parameters and Fermentation Procedure:

For optimization, the following criteria were investigated; fermentation period (24 - 240 h), pH (5.5 - 8.5), incubation temperature (28 - 45 °C), medium volume to flask volume (25/250 to 100/250ml/ml), carbon source (xylose, xylan hydrolysate, glucose, fructose, galactose, lactose and sucrose), concentration of xylan hydrolysate (5 - 30 gL^{-1}), nitrogen source (peptone, yeast extract, sodium nitrate, potassium nitrate, ammonium sulfate, ammonium nitrate and ammonium oxalate) which was added to the fermentation medium on the basis of nitrogen equivalent and finally inoculum ratio (1 - 6 ml 100^{-1} medium). After the proper time of fermentation, the final culture pH was determined using pH radiometer pH M 62 Copenhagen, Denmark. Cells of *S. halstedii* were separated by filtration using Whatman filter paper No.1. The clear filtrate was used as the crude extracellular enzyme source. The mycelia were then washed with distilled water to remove all traces of medium. Drying was carried out by using a hot oven at 80 °C for a constant weight for biomass determination.

Simple correlation coefficient (r) was performed to examine the relationships between individual criteria using the statistical analysis software; CoStat v6.4.

Cellulase Assay:

The cellulase activity was performed using carboxymethyl cellulose (CMC) (Sigma, low viscosity) as a substrate and D-glucose as the standard. A 0.6 ml cell-free extract was added to 0.6 ml of CMC solution (final concentration 0.1%) of pH 5 (0.2M acetate buffer), and incubated at 50 °C (Pointing, 1999). After 20 min, 2 ml of 3, 5-dinitrosalicylic acid reagent was added, and the amount of reducing sugars released in the reaction was estimated by measuring absorbance at 535 nm (Miller, 1959).

Assay of Xylanase:

Xylanase was assayed according to Bailey *et al*. (1992) using 1% oat splet xylan (Sigma) in 0.1 M potassium phosphate buffer pH 7.0 as substrate for enzyme reaction, the assay mixture containing 0.5 ml substrate and 0.5 ml diluted enzyme solution in the buffer was incubated at 40 °C for 10 min. The reducing sugars released were determined by the dinitrosalycylic acid method (Miller, 1959) using xylose (Sigma) as a standard. One unit of xylanase was defined as the amount of enzyme that produces 1 µml of xylose per minute under the assay conditions.

Protein Determination:

According to the method of Lowry *et al*. (1951), the extracellular protein was determined at 280 nm using bovine serum albumin as standard.

Partial Purification:

Ammonium sulphate was added to 100 ml of the culture filtrate at different concentrations to obtain various fractions at 20, 40, 60 and 80% saturation levels. The precipitated protein was obtained by centrifugation for 15 min at 5000 rpm under cooling conditions. Each fraction was dissolved in a definite volume of 0.1 M citrate phosphate buffer of pH 5 and dialyzed against distilled water in a refrigerator overnight. This dissolved fractional precipitate was tested for both xylanase activity and protein content.

RESULTS AND DISCUSSION

Before optimization process for xylanase production, the supernatant of *S. halstedii* was assayed for the possible activity of cellulases. The test confirmed the hypothesis of the absence of such enzymes. This in turn, supposed that the present enzyme is cellulase-free xylanase, thus the optimization of fermentation conditions was carried out on the base of cellulase-free xylanase.

Optimization of Environmental Conditions:

In regard to the influence of fermentation period on xylanase production that lasted for ten days, the profile of the final culture pH of the growth medium followed the nature growth curve of *S. halstedii*, in which after 24 h of fermentation the pH recorded 7.1 and continuously increased to reach its maximum after 120 to 144, and decreased afterwards to reach 8.1 at the end of the time course. The results are similar to those of Techapun *et al*. (2001) who found that 6 days is the optimum time for maximum production of xylanase. The biosynthesis of cellulase-free xylanase activity was detected at the beginning of the fermentation period (24 h) showing specific activity of 10.98 U/mg (Fig. 1). Xylanase was maximally produced at the 120th h of fermentation. It is obvious to note that xylanase production followed the growth curve of *Streptomyces* strain. Maximum enzyme activity was observed at the end of the logarithmic growth phase and start of the stationary phase of growth followed by decrease in activity with lag phase. The results are on line with those reported by Rifaat *et al*. (2005). Based on the time course profile, 120 h was selected in the next trials.

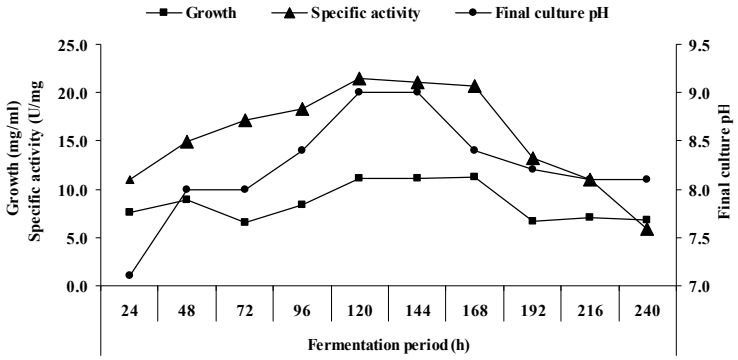

Fig. 1: Growth and xylanase production by *S. halstedii* as a response of fermentation period.

Xylanase production greatly depends on initial culture pH. Fig. 2 clearly indicates that whatever the initial pH was, the final culture pH turned into the alkaline side. The specific activity of cellulase-free xylanase started with 11.44 U/mg at initial pH 5.5 and reached the maximum at initial pH 7.5 (22.9 U/mg) followed by gradual decrease with the increasing of pH up to 8.5 (12.67 U/mg). The correlation between different tested criteria is introduced in Table 1, only the growth of the tested *S. halstedii* strain was positively correlated with the initial culture pH ($r = 0.856$ at $P \leq 0.05$), and the growth in turn, was positively correlated with total protein although the latter did not reach to the significant level. However, the total protein recorded positive significant correlation with xylanase activity reflecting the importance of protein content during optimum pH determination.

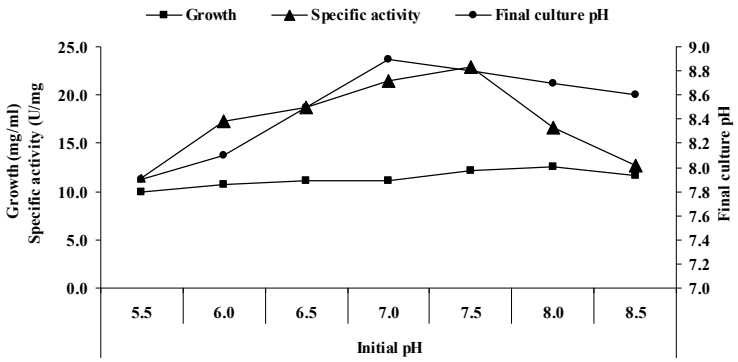

Fig. 2: Growth and xylanase production by *S. halstedii* at different initial pHs.

Table 1: Correlations between different parameters during determination of the optimum pH.

	Initial pH	Final pH	Growth	Total protein	Xylanase activity
Final pH	0.751 ^{ns}				
Growth	0.856*	0.763*			
Total protein	0.595 ^{ns}	0.843*	0.643 ^{ns}		
Xylanase activity	0.377 ^{ns}	0.784*	0.538 ^{ns}	0.953*	
Specific activity	0.119 ^{ns}	0.668 ^{ns}	0.404 ^{ns}	0.699 ^{ns}	0.866*

** significant at $P \leq 0.01$, * significant at $P \leq 0.05$, ^{ns} not significant

Temperature is an important factor controlling xylanase production. *S. halstedii* showed high sensitivity in xylanase production in response to variation of temperature. As illustrated in Fig. 3, the maximum xylanase production was at 32 °C with specific activity of 30.7 U/mg. However, higher temperature led to significant reduction in xylanase productivity. At 45 °C, more than 50 % reduction in the production could be easily observed. This finding was confirmed by the statistical analysis (Table, 2) in which negative correlation coefficient could be observed between temperature from one side and all tested criteria except total protein from the other side. So, the data recorded here are evidence for the mesosphilic nature of the present *Streptomyces* strain. No dramatically changes in the final culture pH could be detected, which means that *S. halstedii* possesses the same biological activity and metabolites under the different temperature, but the only change is the quantity of the secreted enzyme.

Generally, most *Streptomyces* sp. strains producing cellulase-free xylanase are mesophilic alkalophilic and reaches to the maximum production after 5 day; such as *Streptomyces* sp. QG113 on wheat bran medium at 37 °C at pH 8.0 after 5 days (Beg *et al*., 2000), *Streptomyces albus* at 30 °C at pH 7.5 after 5 days (Antanopoulos *et al*., 2000) and *Streptomyces cuspidosporous* in a xylan medium at 37 °C at pH 7.5 after 5 days (Maheswari and Chandra, 2000). Thanongsak *et al*. (2011) suggested that the production of xylanase tends to occur under relatively high temperature levels and neutral conditions, and they deduced that the pH and temperature are interacted and this interaction influences xylanase production. On the other hand, Techapun *et al*. (2002) demonstrated that *Streptomyces* sp. Ab106 tends to produce xylanase at a relatively high temperature and in neutral or alkaline conditions but interactions between temperature and pH had a low significance and did not interact with each other i.e., pH did not change when the temperature changed.

The ratio of medium volume to flask volume was investigated; this criterion is generally tightly related to the efficiency of aeration during the fermentation process. As shown in Table 3, cellulase-free xylanase production was affected by changes in the ratio of medium volume to flask volume. The highest productivity was attained at medium volume to flask volume ratio of 25/250 (ml/ml), yielding 40.2 U/mg protein compared with the other ratios. The gradual increase in this ratio led to remarkable reduction in xylanase production recording 15.0 U/mg at 100 /250 (ml/ml). Moreover, the growth of *Streptomyces* strain is reversely correlated with both xylanase specific activity and final culture pH, but is directionally related with the medium volume to flask volume ratio. However all these relations did not reach the level of significancy except for the positive correlation between final pH and xylanase activity where the value of r reached 0.971 at $P \leq 0.05$, also, between medium volume to flask volume ratio and growth (0.979 at $P \leq 0.05$). On the other hand, negative significant correlation was detected between medium volume to flask volume ratio and specific activity (r = -0.986 at $P \leq 0.05$)

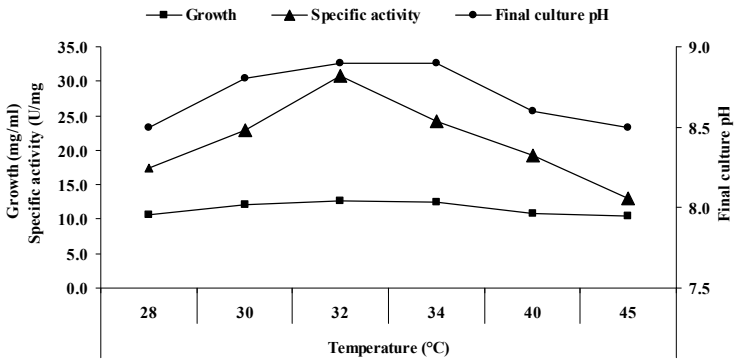

Fig. 3: Effect of incubation temperature on growth and xylanolytic activity of *S. halstedii.*

Table 2: Correlations between different parameters during the investigation of optimum temperature.

	Temperature	Final pH	Growth	Total protein	Xylanase activity
Final pH	-0.391^{ns}				
Growth	-0.477^{ns}	0.991**			
Total protein	0.649^{ns}	0.299^{ns}	0.198^{ns}		
Xylanase activity	-0.371^{ns}	0.908*	0.914*	0.382^{ns}	
Specific activity	-0.529^{ns}	0.906*	0.934**	0.183^{ns}	0.978**

** significant at $P \leq 0.01$, * significant at $P \leq 0.05$, ^{ns} not significant

Table 3: Effect of the ratio of medium volume to flask volume on the growth and xylanase production by *S. halstedii.*

Medium volume to flask volume ratio (ml/ml)	Final culture pH	Growth (mg/ml)	Total protein (mg/ml)	Xylanase activity (U/ml)	Specific activity (U/mg)
25/250 (0.1)	8.9	5.900	0.300	12.066	40.220
50/250 (0.2)	8.9	12.686	0.380	11.670	30.711
75/250 (0.3)	8.5	15.200	0.201	3.890	19.353
100/250 (0.4)	8.3	24.200	0.230	3.450	15.000

Optimization of Nutritional Conditions:

Carbon source is one of the most critical nutritional conditions for the biosynthesis of different bio-agents such as biocatalysts. Among different carbon sources (Table 4), xylan hydrolysate was the most effective source for xylanase production (40.2 U/mg), in spite of the moderate growth of *S. halstedii* on xylan hydrolysate compared with other carbon sources i.e., fructose and galactose. However, xylose > galactose > lactose > glucose > fructose > sucrose occupied the next order of xylanase specific activity. Anyhow, all the tested carbon sources had stimulation effect but with various extend, except sucrose that did not show any stimulation for xylanase productivity. This in turn led to the simple conclusion of the induced nature of the enzyme, with little constitutive ability. It is also clear that the growth of *S. halstedii* was not on line with the enzyme production, e.g., the highest growth was observed on galactose (7.3 mg/ml) with specific activity of 8.3 U/mg. No touched variation in the final culture pH among the different carbon sources could be observed.

Accordingly, xylan hydrolysate, the best inducer for production of cellulase-free xylanase, was investigated at different concentrations (Fig. 4). The minimum productivity was attained at a concentration of 5 gL^{-1} (14.9 U/mg). Increasing of xylanase production was recorded up to 20 gL^{-1} of xylan hydrolysate being 40.2 U/mg followed by gradual reduction in the specific activity down to 15.2 U/mg at 30 gL^{-1}. The pH curve followed the same trend of xylanase production. However, the maximum growth of *S. halstedii* was at 20 gL^{-1}.

Techapun *et al*. (2001) reported maximum cellulase-free xylanase activities by growing *Streptomyces* sp. on 1% (w/v) corn hull, corn cob, bagasse and oat spelt xylan, whereas, Rifaat *et al*. (2005) found that xylan at 2 % is the highest stimulator for xylanase production by the different *Streptomyces* strains. However, Rawashdeh *et al*. (2005) found that when glucose plus xylan were used as a carbon source, the activity was reduced by 67% of that with xylan alone suggesting catabolite repression of glucose. Correlation coefficients presented in Table 5 clearly indicate that most of the relationships of tested criteria showed non-significant ones, the few significant correlations were between xylan hydrolysate concentration and final pH ($r = 0.890$ at $P \leq 0.05$) also, between total protein and both of xylanase activity

(r = 0.917 at $P \leq 0.01$) and specific activity (r = 0.902 at $P \leq 0.05$) which means that most of the produced protein is xylanolytic active.

Table 4: Biosynthesis of xylanase in the presence of different carbon sources.

Carbon source	Final culture pH	Growth (mg/ml)	Total protein (mg/ml)	Xylanase activity (U/ml)	Specific activity (U/mg)
Xylose	8.4	5.037	0.397	4.432	11.164
Xylan hydrolysate	8.9	5.900	0.300	12.066	40.220
Glucose	8.5	2.521	0.199	0.212	1.065
Fructose	8.5	2.521	2.450	2.523	1.030
Galactose	8.9	7.300	0.199	1.667	8.377
Lactose	8.9	6.393	0.885	5.433	6.139
Sucrose	8.9	3.653	0.395	0.000	0.000

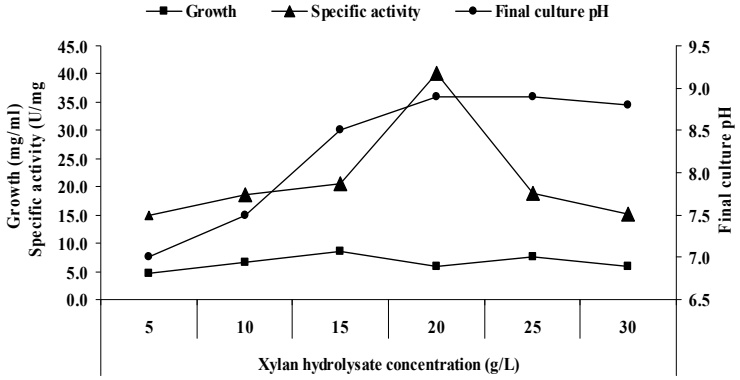

Fig. 4: Production of xylanase by the tested actinomycete strain at different concentrations of xylan Hydrolysate.

Table 5: Correlations between different parameters during the investigation of optimum xylan hydrolysate concentration.

	Xylan hydrolysate concentration	Final pH	Growth	Total protein	Xylanase activity
Final pH	0.890*				
Growth	0.210ns	0.460 ns			
Total protein	0.272 ns	0.643 ns	0.308 ns		
Xylanase activity	0.146 ns	0.467 ns	0.012 ns	0.917**	
Specific activity	0.127 ns	0.446 ns	0.002 ns	0.902*	0.999**

** significant at $P \leq 0.01$, * significant at $P \leq 0.05$, ns not significant

The next step in optimization process dealt with the nitrogen source, sodium nitrate was substituted with different nitrogen sources in the fermentation medium to study their impact on the production of cellulase-free xylanase (Fig 5). In this respect, peptone was the best-utilized nitrogen source by *S. halstedii* NRRL B-1238 followed by sodium nitrate, potassium nitrate, ammonium sulfate, ammonium nitrate, yeast extract and ammonium oxalate, in descending order. Rifaat *et al*. (2005) on *S. chromofuscus* and *S. albus* and Thanongsak *et al*. (2011) on thermophilic *S. thermovulgaris* reported yeast extract as the determinant nitrogen sources for cellulase-free xylanase production.

Xylanase Production at Different Inoculum Ratios:

Inoculum ratio is one of the important factors affecting cellulase-free xylanase production. The pattern of xylanase production with respect to inoculum ratio indicates that, with the gradual increase in inoculum ratio, *S. halstedii* showed positive improvement in the enzyme productivity and reached the maximum specific activity (45.15 U/mg) at 5% inoculum ratio (Fig. 6), xylanase production was slightly decreased when inoculum ratios were out of this point. Larger inoculum ratio has been shown to affect adversely the yield of enzymes (Suresh and Chandrasekaran, 1999). Data of the analysis of correlation coefficient continue providing evidence for the significant positive correlation between inoculum ratio and specific activity of xylanase (r = 0.948 at $P \leq 0.01$). On the other side the correlation coefficient between xylanase production and inoculum ratio (r= 0.701, ns) did not reach to any level of significancy.

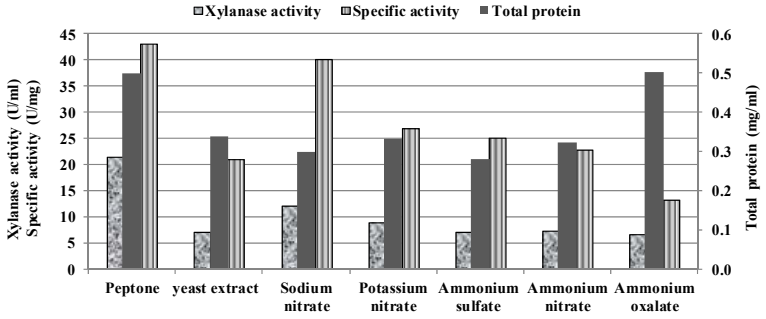

Fig. 5: The efficacy of the tested actinomycete strain to produce xylanase on some common sources of nitrogen

*The nitrogen sources were added to the fermentation medium on the base of nitrogen equivalent

Partial Purification of Xylanase:

The crude cellulase-free xylanase of *S. halstedii* NRRL B-1238 was produced on the previous optimized medium; pH 7.5, temperature at 32 °C, medium volume 25ml/250ml flask, xylan hydrolysate at 20 gL^{-1}, peptone as nitrogen source and inoculum ratio of 5 %. The partial purification of the obtained crude xylanase was performed by precipitation of crude enzyme using different concentrations of ammonium sulphate (Table 6). The results revealed that specific activity; recovery and purification fold in the precipitate increased with the increasing of ammonium sulphate saturation reaching their maximal values at 60% saturation being 71.743 U/mg protein, 61.60% and 1.78 recovery (%) and purification fold, respectively, these parameters decreased thereafter.

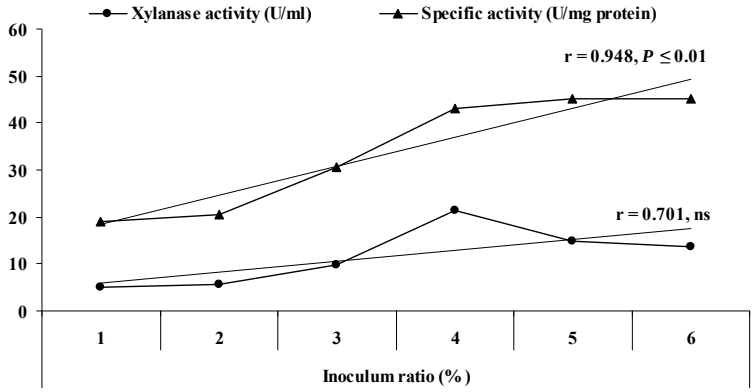

Fig. 6: Influence of inoculum ratio on the production of xylanase by *S. halstedii.*

Table 6: Fractional precipitation of *S. halstedii* xylanase with ammonium sulphate and activity and specific activity of the partial purified enzyme.

Ammonium sulphate saturation (%)	Xylanase activity (U)	Total protein (mg/ml)	Specific activity (U/mg protein)	Recovery (%)	Purification fold
Crude supernatant	1125.00	27.892	40.334	100.0	0.00
20	260.00	4.953	52.493	23.1	1.30
40	480.00	8.878	54.066	42.7	1.34
60	693.04	9.660	71.743	61.6	1.78
80	410.00	7.500	54.667	36.4	1.36

Based on the previous optimization trials, appreciable amounts of cellulase-free xylanase could be produced on the selected moderately low cost medium, the resulted enzyme will expected to find its way in much food, industrial and pharmaceutical aspects.

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