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BIOTECHNOLOGICAL UTILIZATION OF AGRICULTURE RESIDUES FOR SUSTAINABLE DEVELOPMENT

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ABSTRACT

Lignocellulosic biomass is the most abundant organic raw material in the world. Lignocelluloses constitute a major portion of agricultural and forest wastes. They are the most promising feed stock for the production of enzymes. The bioconversion of agro wastes into xylanase has received considerable interest during the recent years, because the production process is eco-friendly and use cheap biomass resources as substrates, which help to reduce enzyme prices. To investigate the production of xylanase from *Bacillus species* isolated from the soil sample collected from the vicinity of paper industry, submerged fermentation was performed, using wheat straw, wheat bran, rice straw, rice bran and bagasse as substrates. Wheat bran supported xylanase production with maximum activity (0.196 units/mL) in the medium with 1% wheat bran on 3rd day of fermentation. Optimum temperature and pH for maximum xylanase production reported were 37^{o}C and 7, respectively. Further, the inoculum size, 0.4% v/v, gave the maximum yield. L.B. plot for reaction of xylanase revealed that V_{max} of the reaction was 3.1 µmoles/min. and M.M. constant (Km) was 0.0028 µg/mL. In addition, it was observed that activity of xylanase produced was 0.32 units/mL at pH 7 and temperature 50^{o}C.

Key words: Biotechnological, Agricultural residue.

INTRODUCTION

India has abundant agricultural wastes. There is urgent need to manage bulk wastes effectively and economically. Agricultural waste can be utilized for the production of value added chemicals. Utilization of waste will lesson undesirable impact of the agricultural waste on the environment. At the same time, it is also necessary to generate value added products from these wastes. Submerged fermentation involving micro-organisms has been used for production of chemicals of commercial value from microbial sources. We are particularly interested in xylanase enzyme with many industrial applications mainly for the bioconversion of lignocellulosics to sugars for manufacturing of many products, clarification

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of fruit juices, deinking of waste papers etc.[1] Most researchers have used submerged cultures for xylanase production, which allows control over the parameters for optimum enzyme production. Extracellular enzymes are considered important from the industrial view point as they ease the extraction procedure. The use of purified xylan as the substrate to induce xylan synthesis increases the cost of enzyme production.[2] This work describes the selection of *Bacillus sp. (*S4) as potential extracellular xylanase producer via submerged fermentation, optimization of the cultivation system, for enhancing the production of enzyme and enzyme kinetics as well as production of xylanase from inexpensive substrates[2].

EXPERIMENTAL

Isolation of microorganisms and screening for the xylanolytic activities

The isolation of xylanase producing bacteria was carried out on xylan containing medium using the samples collected from the vicinity of paper industry of Gondia. One g of each of the sample was suspended in 10 mL of sterile distilled water and shaken vigorously for 10 min. Later, 1.0 mL of resulting liquid was spread on the surface of malt extract agar containing 0.5 % w/v xylan using an L-shaped glass rod and then inoculated plate was incubated at 37^{o}C for 2 days. Xylanolytic isolates were detected by observing the zones of hydrolysis. The isolates were subcultured and maintained on nutrient agar slants. The slants were stored at 4^{o}C prior to use.[3]

Identification of bacterial isolate S4, the potential producer of xylanase.

Identification of the potential isolate was carried out based on morphological, biochemical and cultural characteristics given by Bergey's Manual of Determinative Bacteriology. Potential isolate was subcultured and maintained on nutrient agar slants. The slants were stored at 4^{o}C with regular subculturing.

Inoculum preparation

To prepare inoculum for fermentation studies, 24 hrs 1% w/v bacterial suspension of potential isolate washed out from nutrient agar slant using 10 mL sterile distilled water was used.

Fermentation of cultivation system

Cultivation of bacteria was performed in 250 mL Erlenmeyer's flask containing xylan broth with the composition (g/l) 10.0 g xylan, 20 g peptone, 2.5 g yeast extract, 2 g NH_4NO_3, 2 g KH_2PO_4, 1 g $MgSO_4$ and 0.05 g $MnSO_4$.[4], under static condition.

Optimization of medium composition and cultural conditions

The optimization of medium composition and cultural conditions was carried out based on the stepwise modifications of the governing parameters for xylanase production. The parameters included in study to know their impact on production of xylanase were incubation period, pH, volume of inoculum, different crude carbon sources, different concentrations of selected carbon source, effect of additional nitrogen sources, different concentrations of selected additional nitrogen source and temperature.

Enzyme extraction

The biomass of the production medium was separated from the suspension by filtration through Whatmann's filter paper No. 1. The cell free supernatant was used as the source of crude enzyme.

Analysis

Xylanase activity was assayed by using the method of Takashi[4]. To perform xylanase assay; 1% w/v xylan solution, which was prepared in 0.1 M acetate buffer (6.5) was used. In assay procedure, 1 mL of substrate solution was mixed with 1 mL enzyme solution and the mixtures was incubated at 55^{o}C for 30 min. The reducing sugars released were quantified by dinitrosalicyclic acid method according to Miller[5], using xylose as standard. One unit of xylanase activity was defined as the enzyme amount that releases 1 micromole of reducing sugar per minute under assay conditions.

Characterization of xylanase

Optimum pH for maximum xylanase activity was determined by assaying xylanase over the pH range 5.5-8.5 in 0.1 M acetate buffer at 50^{o}C for 30 min.

The optimum temperature for maximum xylanase activity was determined by assaying xylanase over temperature range 45-65^{o}C in 0.1 M acetate buffer (pH 7) for 30 min. The optimum enzyme volume for maximum xylanase activity was determined by assaying xylanase at 55^{o}C in 0.1 M acetate buffer of pH 7 for 30 min. The volume of crude enzyme used ranges from 0.25-2 mL. The optimum substrate concentration for maximum xylanase activity was determined by assaying xylanase at 55^{o}C in 0.1 M acetate buffer of pH 7 with substrate concentration of xylan ranging from 0.5.-2.5% w/v.

The Km value was determined for the hydrolysis of xylan by xylanase by using Lineweaver-Burk and Michaelis–Menten plot.

RESULTS AND DISCUSSION

Selection and identification of isolate S4

Based on the screening programme, a total of 4 bacterial isolates were capable of exhibiting xylanolytic activities on xylan agar with the diameter of clear zones ranging from 9 to 14 mm. The isolate S4 showing highest zone of hydrolysis was selected as the potential producer of xylanase and identified to be *Bacillus sp. S4.*

Table 1: Screening for best xylanolytic bacterial isolate

Bacterial isolate number	Zone of hydrolysis (mm)
S1	9 mm
S2	11 mm
S3	10 mm
S4	14 mm

Optimization of cultural conditions and medium composition for maximum production of xylanase by *Bacillus sp.* S4

Effect of time course study

Time course study revealed a significance increase in enzyme production with increase in time, which was presumed to be due to rapid hydrolysis of xylan in the medium. Increase in incubation period after 72 hrs resulted in decreased enzyme production.

Effect of initial pH

Effect of initial pH of medium, on xylanolytic enzyme production by *Bacillus Sp.* S4 was studied using xylan broth, keeping various pH levels ranging from pH 4 to 8. pH is the important environmental parameter that determine the growth rates of microorganisms and significantly affect the level of xylanase produced. The influence of pH on xylanase production during *Bacillus* sp. S4 cultivation is shown in the Fig. 2. Xylanase activity was detected in all pH evaluated. The isolate favored slightly acidic medium for xylanase production. When the pH was increased or decreased to values other than 6.0 to 6.5, the production gradually decreased, this might be due to the inhibition of growth by lowering or increasing the pH[6].

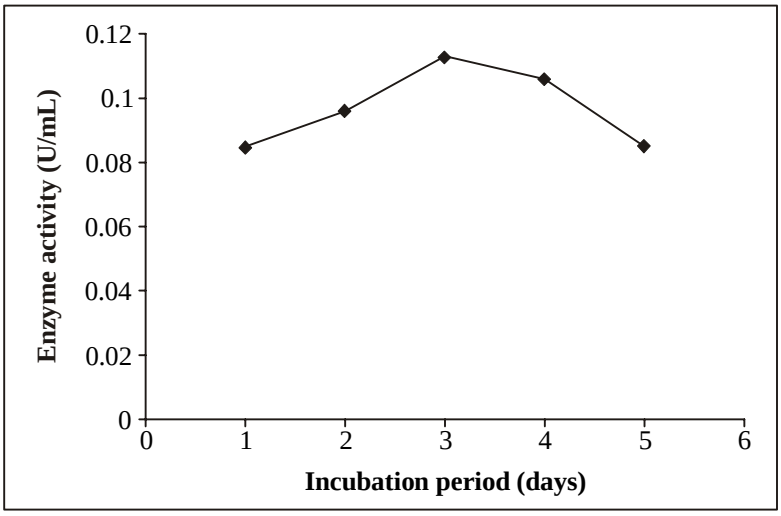

Fig. 1: Effect of time course on xylanase production by *Bacillus sp.* S4

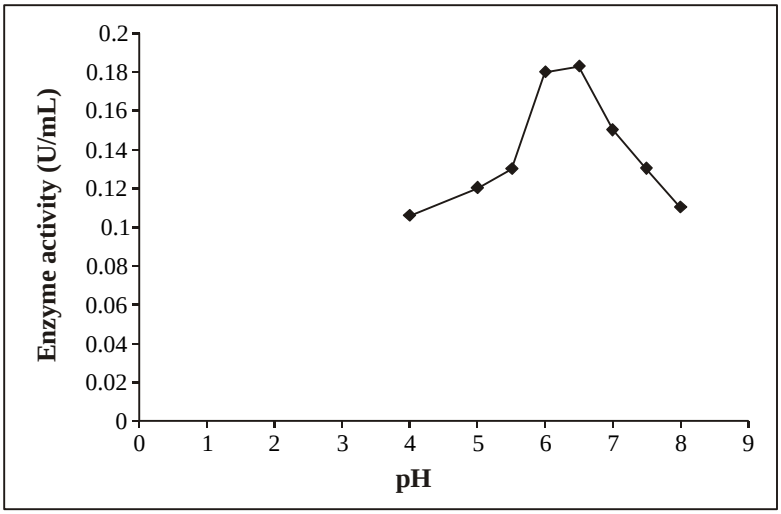

Fig. 2: Effect of initial pH on xylanase production by *Bacillus sp.* S4

Effect of inoculum size

It was found that the increase in inoculum size resulted in rapid increase is xylanase production. The inoculum size required to achieve maximum xylanase production was 0.4% v/v on this day of fermentation. With increase in volume of inoculum above optimum, xylanase production decreases. Similar observation was reported by Raimbault and Alazard[7], who showed that maximum enzyme production and declination was achieved much

faster due to rapid degradation of substrate as a consequence of rapid growth.[11]

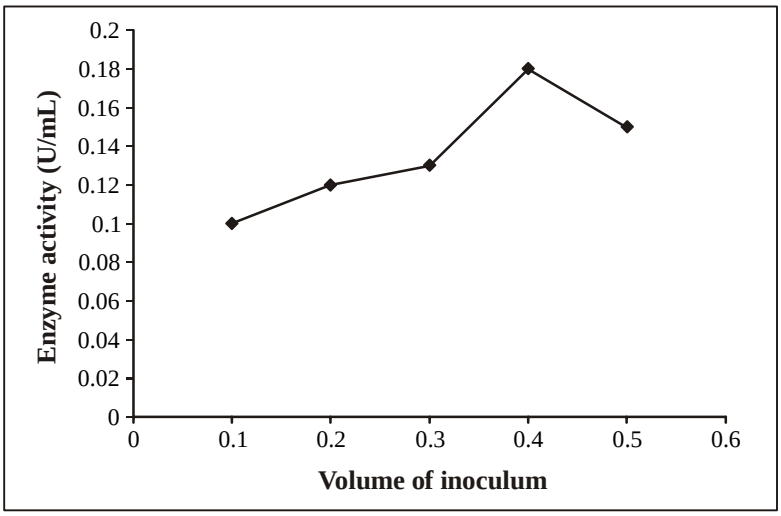

Fig. 3: Effect of inoculum size on xylanase production by *Bacillus sp.* S4

Effect of crude carbon sources

Xylan broth was supplemented with various carbon sources at concentration of 1% w/v by replacing the xylan. The inoculated flasks were incubated for 72 hrs at 37°C under stationary condition. Among the different substrates tested, xylan was found best inducer.

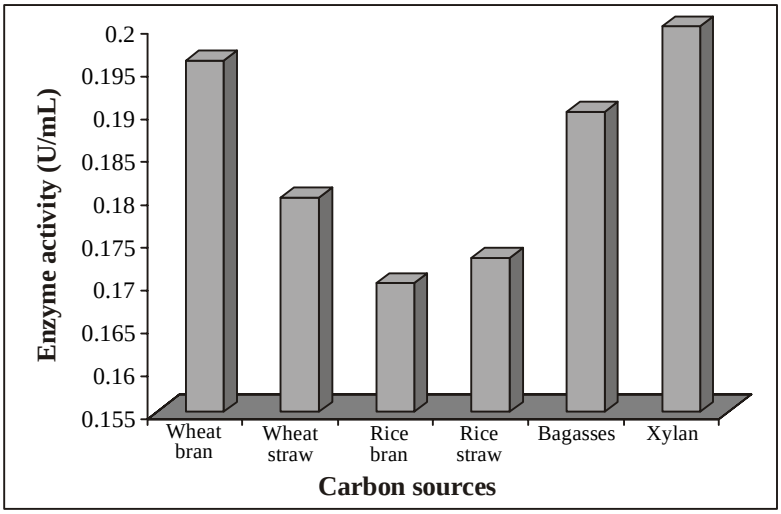

Fig. 4: Effect of crude carbon sources on xylanase production by *Bacillus sp.* S4

Maximum xylanase activity was obtained in medium containing 1% w/v xylan after 72 hrs of incubation in comparison with the wheat bran, wheat straw, rice bran, rice straw and sugarcane bagasse. According to Kulkarni and Rao[8], xylanase activity is inducible and substrates with xylan play an important role in xylanase induction. In many species purified, xylan was found best inducer for xylanase production. According to Gosh et al.[9], agricultural residues efficiently induced the production of xylanases. Wheat bran has been known for being ideally suitable for xylanase production.[10]

In agreement with this, it could be noted that the results obtained with *B. subtilis* S4 reflected high potential of wheat bran as an external stimulus on production of xylanase.[8] Bacterial and actinomycetes xylonolytic enzymes are generally induced by xylan and by lignocellulosic residues that contain xylan[11]

Effect of different concentrations of xylan as carbon source

In order to determine the best amount of xylan for xylanase production, different concentrations of xylan were tested. The results showed highest yield of xylanase with 2.0% w/v xylan.

The data presented in Fig. 5 showed that biosynthesis of xylanase decreased, when xylan concentrations was increased above optimum in production medium. This inhibition of xylanase synthesis could be interpreted in terms of catabolic repression likewise described by other enzymes.[12] Similar results were obtained by other researchers by using high concentrations of lignocellulosic materials as substrates for enzyme production[13].

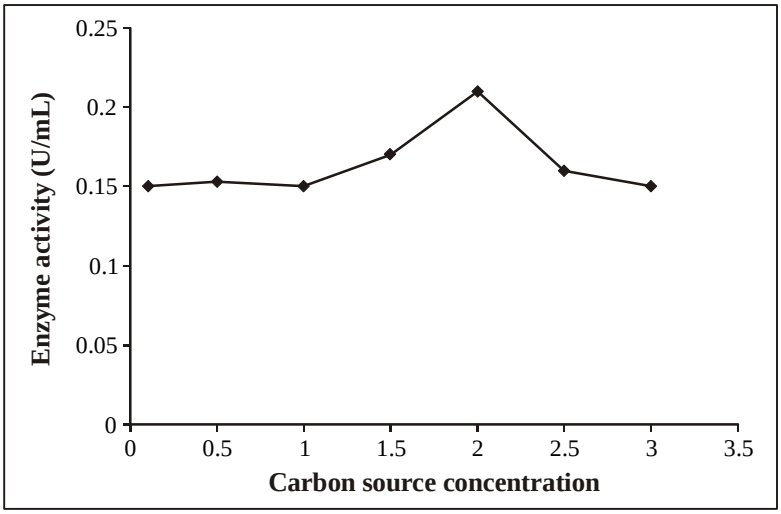

Fig 5: Effect of different concentrations of xylan on xylanase production by *Bacillus sp.* S4

The phenomenon of repression was also reported by Gessesse and Mama[14], who showed that xylose strongly repressed xylanase production by *Bacillus sp.* at the concentration of higher than 1% w/w, The extent of induction of xylanase synthesis is dependent upon the nature and concentration of these carbon sources. The increase in substrate concentration is usually favorable to product synthesis; however, many fermentation products are subjected to catabolic repression, that is repression of enzyme synthesis by easily metabolisable sugar.[15]

Effect of additional nitrogen sources

Effect of different nitrogen sources on production of xylanase was studied and the results obtained are shown in Fig. 6. Among the nitrogen sources tested, $(NH_4)_2HPO_4$ was found best nitrogen source for xylanase production at concentration 0.2 %w/v. This nitrogen source was added to medium in addition to yeast extract and in place of ammonium nitrate.

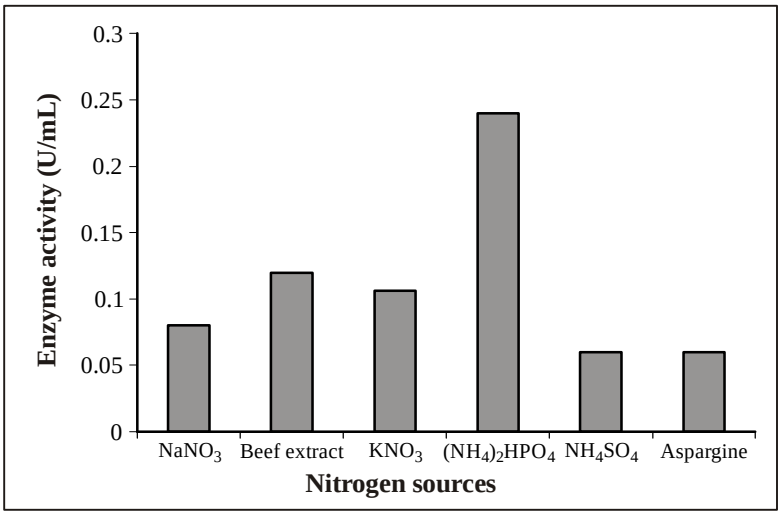

Fig. 6: Effect of additional nitrogen sources on xylanase production by *Bacillus sp.* S4

Effect of different concentrations of $(NH_4)_2HPO_4$

The effect of different concentrations of $(NH_4)_2HPO_4$ between 0.1-0.5%w/v on enzyme production was examined. Optimum concentration of $(NH_4)_2HPO_4$ reported for maximum xylanase production was 0.3% w/v.

Effect of cultivation temperature

Temperature is one of the important parameter that determines success of ferment-

ation system. Therefore, the effect of temperature on xylanase production by *Bacillus sp.* S4 was examined and the results are presented in Fig. 8. The higher xylanase activity per unit volume was verified at 37°C. Lower activities were obtained with cultivation temperature lower or above the ambient temperature. According to Aiba et al.[16] at lower temperature, the transport of substrate across cells is decreased and lower yield of products are obtained.

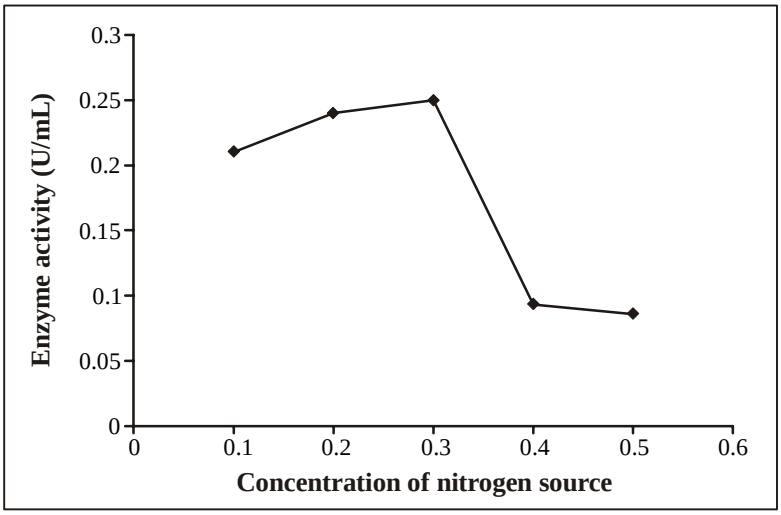

Fig. 7: Effect of different concentrations $(NH_4)_2HPO_4$ on xylanase production by *Bacillus sp.* S4

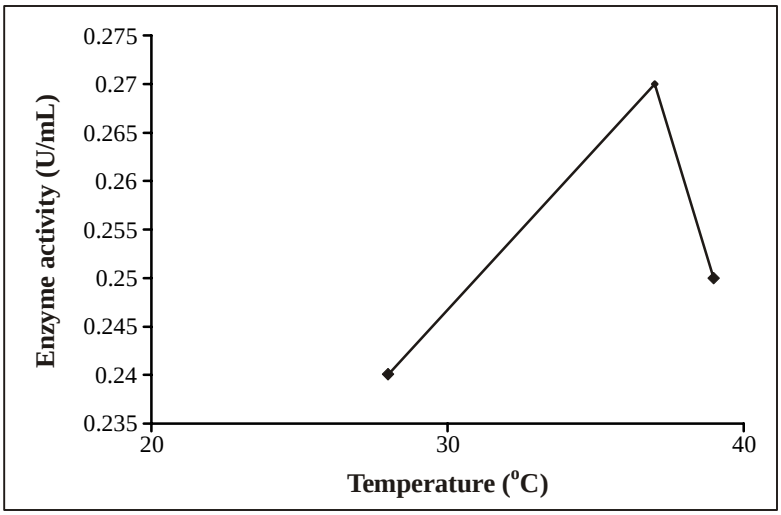

Fig. 8: Effect of cultivation temperature on xylanase production by *Bacillus sp.* S4

Enzyme characterization

Effect of pH

The initial pH showed profound influence on xylanase production. This study revealed that the best pH for xylanase activity was around 7.

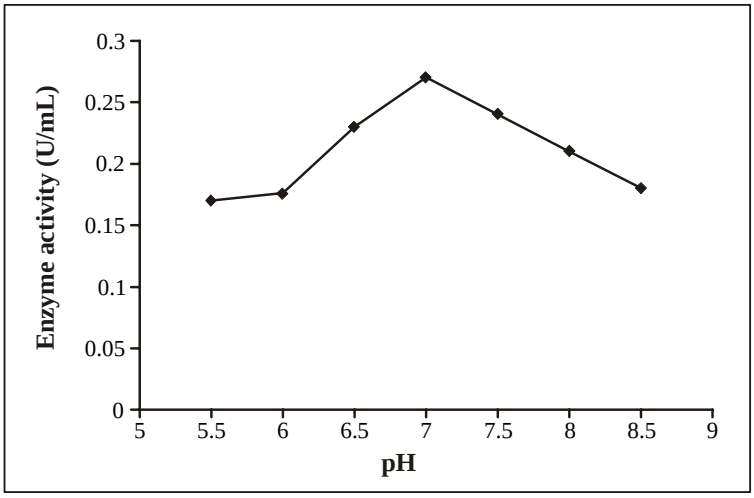

Fig. 9: Effect of pH on activity of crude xylanase from *Bacillus sp*. S4

Effect of temperature

The optimum temperature for xylanase activity was 55^{o}C.

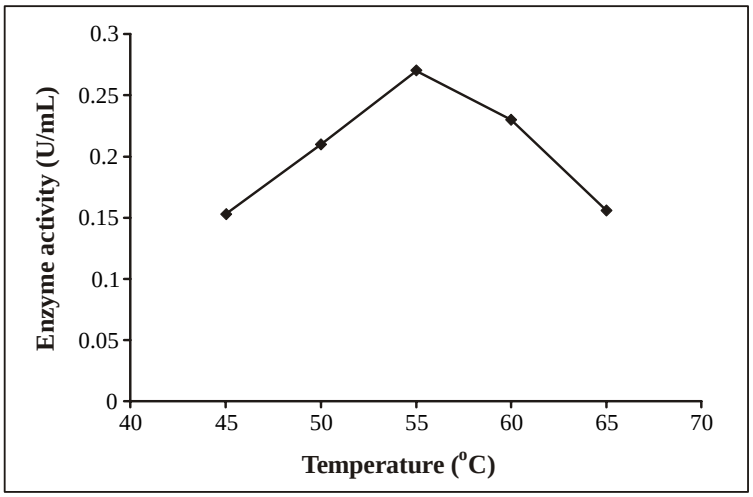

Fig. 10: Effect of temperature on activity of crude xylanase from *Bacillus sp*. S4

Effect of enzyme concentration

Xylanase activity was reported to be optimum at 1.5 mililiter

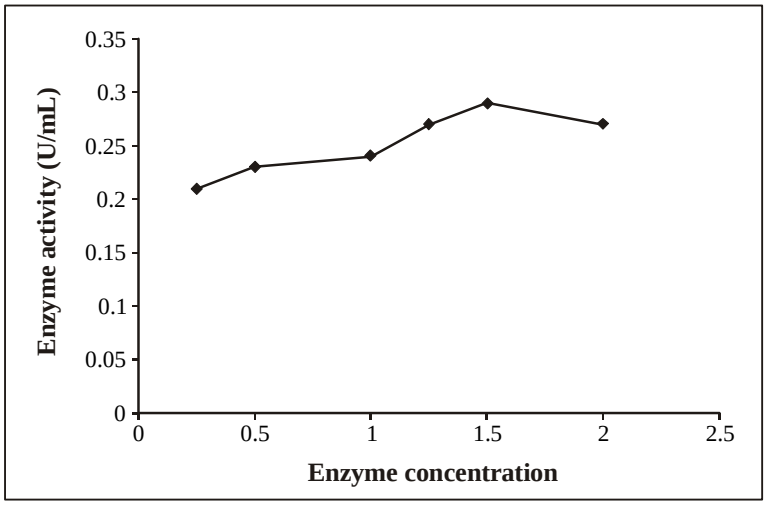

Fig. 11: Effect of enzyme concentration on activity of crude xylanase from *Bacillus sp*. S4

Effect of substrate concentration

Substrate saturation kinetics was assayed for extracellular xylanase produced by

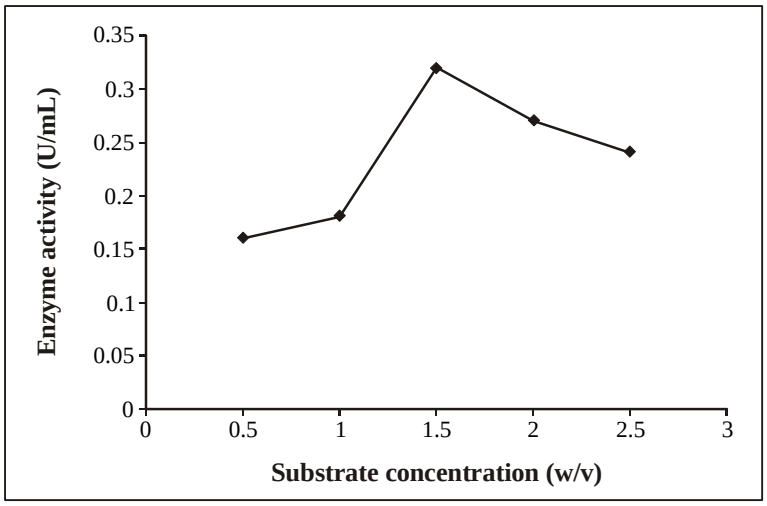

Fig. 12: Effect of substrate concentration on activity of crude xylanase from *Bacillus sp*. S4

Bacillus Sp. S4. After reaching to maximal, the substrate inhibitory effect started due to higher substrate concentration and no further increasing xylanase activity was detected. The effect of substrate concentration on the velocity of the reaction with extracellular xylanase is shown in Fig. 12 the values for Km and Vmax were computed from Linewever – Burk plot (Fig. 13.).

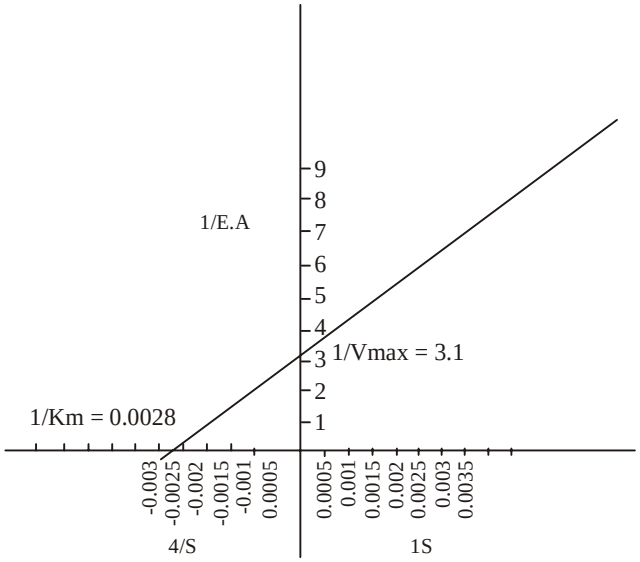

Fig. 13: Lineweaver- Burk plot

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