FERMENTATION OF WHEAT STRAW FOR THE EXTRACTION ANDCHARACTERIZATION OF INDUSTRIALLY IMPORTANT ENDOGLUCONASE BY *PHAEOLUSSPADICEUS*

Amna Gull[1], Raja TahirMahmood*[1], Maleeha Masood[1], Anosha Safder[1], Noshaba Zia[1],Aimen Maqsood[2], Majid Mehmood[3] and Dawood Ahmed[4]

*Corresponding Author: raja.tahir@must.edu.pk

ABSTRACT

Pakistan is an agricultural country but the burning of agricultural waste presents a major threat to the environment and human beings by producing smog. On the other hand, industrial enzymes are widely used in many industries including food, chemical, medicine and drug *etc*. But the high cost of industrial enzymes limits their uses. In this study, microbial source of enzymes was used to degrade substrate and the agricultural waste was utilized as a substrate for fermentation process. Agricultural waste, also known as lignocellulosic biomass, is widely available and hence, it provides a cheap substrate for industries. Endoglucanase enzyme was produced and extracted from *Phaeolusspadiceus* using wheat straw as the substrate. The study reported that maximum activity of endoglucanase was observed after 96 hours (4 days), at 25-30˚C, at 50% moisture level and pH 4.5. Moreover, addition of carbon source (5% sucrose) and nitrogen source (2.5% urea) enhances the production of endoglucanase. After description to find out its optimum temperature, pH, effect of different metal ions and Km and Vmax. Endoglucanase activity at different conditions presented that the optimum temperature was 30˚C and pH was 4.5. And values of Km and Vmax showed that endoglucanase has high empathy for its substrate.

Keywords: Endoglucanase, Enzyme Kinetics, Fermentation, *Phaeolusspadiceus*

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[1]Department of Biotechnology, Mirpur University of Science and Technology (MUST), Mirpur AJK Pakistan, [2]MohtaramaBenezir Bhutto Shaheed Medical College, Mirpur AJK Pakistan

3 Department of Zoology, University of Poonch, Rawalakot AJK Pakistan, 4 Department of Medical Laboratory Technology, University of Haripur, Haripur KP Pakistan

Introduction

Pakistan is an agricultural country and one of the major producers of important crops like wheat, maize, rice, cotton *etc*. Agriculture serves as the backbone of the country's economy. However, as a result of cutting crops, a huge amount of agricultural waste is produced(Rabbani and Fatmi, 2018). This agricultural waste is called *lignocellulosic biomass*. Due to the unawareness among farmers, they burn this agricultural waste. Burning of agricultural waste may lead to the environmental pollution such as production of smog. Smog leads to many health problems related to respiratory system and also leads to depletion of ozone layer(Katongole, 2009;Azam *et al*., 2015).

On the other hand, for the agriculture sector, there is an increased demand of industrial enzymes for the production of fertilizers, insecticides, pesticides etc. As Pakistan's economy relies mainly on agriculture, the country needs to import industrial enzymes for agricultural industries(Gurung*et al*., 2013). Besides agriculture, industrial enzymes have also many applications in other sectors including food, medicine and drugs, textile, cosmetics *etc*. Industrial enzymes provide many benefits such as high specificity and sensitivity, reusability and biodegradability. But at the same time, they are expensive to produce (Robinson, 2015).

From the above discussion, we come to know about two problems:

1. Environmental pollution due to the burning of agricultural waste
2. High cost of industrial enzymes

As enzymes can be extracted from the cells of plants, animals and microbes, we had used the microbial source of enzymes. Microbial enzymes are preferred to use in industries on large scale as the microbes are easy to produce. Microbes can easily be produced on large scale in the form of large colonies and thus, a large amount of amount of enzymes can be extracted from their cells. Microbial enzymes are easy to use and handle and they provide the reasonable, biodegradable

and cheap source of enzymes. Bacteria and fungi are mainly used for the production of microbial enzymes (Lynd *et al*., 2002).

Furthermore, the agricultural waste, also known aslignocellulosic biomass, serves as the cheap and easily available substrate in industries. It is the rich source of carbon and has high percentage of cellulose in its composition.It is being used as the substrate for many industrial processes including solid state fermentation (Ahmed *et al*., 2009). Microbes can use this lignocellulosic biomass as a substrate to convert it into simple sugars by the action of cellulytic enzymes (Singh *et al.,* 2016). Examples of lignocellulosic biomass are wheat straw, sugarcane bagasse *etc*.

In our research work, solid state fermentation (SSF) was conducted in which *Phaeolusspadiceus* was used for the extraction of endoglucanase and wheat straw was used as substrate. *P. spadiceus* degraded wheat straw by the action of extracellular enzymes including endoglucanase and exoglucanase. So, these extracellular enzymes could easily be extracted from the medium after centrifugation.

MATERIALS AND METHODS

Substrate

Wheat straw was used as a substrate for the expansion of*Phaeolusspadiceus* to provide endoglucanase.Wheat straw was elected because it's low-cost, contain high share of polysaccharide (30-40%), simply accessible in West Pakistan and sensible for the expansion of cellulolytic microorganisms (Ahmed *et al*., 2009).

Substrate Collection

Wheat straw was collected from the rural area of Mirpur (AJK) and then air dried for 10 days. It was then ground to powder and packed in air tight jars for successive use in fermentation method.

Fermentative Organism

The collection of *P.spadiceus* was obtained from Sozo Adventure Park near lower topa, Murree, used for the assembly of Endoglucanase. First take the loop packed with spores of P.

spadiceus and then shifted on Malt Extract Agar media (pH=5.0) aseptically. Place the media plates in incubator for 3-4 days for growth. After incubation, store the plates at 4^{o} C for further use.

Inoculum Preparation

From the culture plates of *P. spadiceus*, a portion of species growth is inoculated aseptically to the Malt Extract broth media (pH=5.0) in flask. Place the flask in shaking incubator at 30^{o} C and 180 rpm for 2-3 days.

Fermentation Process

Solid state fermentation (SSF) method was used for the assembly of Endoglucanse by *P.spadiceus* victimization wheat straw as a carbon supply. Flasks having 5g of powdered wheat straw were moisten with 2.5ml (30%) of distilled water having pH 5.5 (maintained with the assistance of 1M HCL and 1M NaOH). Every flask once autoclaved was inoculated aseptically with 1ml of *P. spadiceus*. These flasks then incubate at 30℃ for specific day (Mahmood *et al*., 2013).

2.6 Sample Harvesting

After specific days, contact technique was used for the extraction of endoglucanase (Krishna *et al*., 1996). We add 50ml distilled water in flasks. These flasks were then shake at 120rpm for 30 minutes in shaking setup. After shaking, the mixture was filtered. Filtered catalyst extract was then centrifuged at 6000 rpm for 10 minutes. Supernatant was stored as a crude enzyme extract at 4℃for enzyme assay (Shafique*et al*., 2004).

Effect of Conditions on Enzyme Production

Enzyme production was optimized at different conditions to observe the maximum enzyme production by *P. spadiceus*.

Effect of fermentation period*:* The fungus was grown for the specific days *i.e.* 24 hours to 168 hours to check maximum enzyme production.

Effect of pH: Five different pH i.e. 3.3, 4.5, 5.5, 6.5, 7.5, 8.5 of distilled water were used for giving moisture (50%).

Effect of temperature and moisture:In order to visualize impact of temperature and moisture different types of temperature has been adjusted (25^{o} C, 30^{o} C, 35^{o} C and 40^{o} C) and similarly, different moisture level 30 %(1.5ml), 50 %(2.5ml), 70 %(3.5ml) have been utilized.

Effect of Carbon and Nitrogen sources: Three different Carbon sources i.e. Glucose, Fructose and Sucrose and three different Nitrogen sources i.e.Peptone, Urea and Ammonium nitrate were used in combination to observe the maximum enzyme production.

Enzyme Assay

*Standard:*Glucose (molecular weight =180g/mole) was used as a standard. Different concentrations of standard were prepared and the absorbance of each concentration was measured at 540nm.

*Substrate:*Carboxy methyl cellulose (CMC) was used as the substrate of Endoglucanse. 1% of CMC in 100mL of distilled water was used for endoglucanase essay.

Reagents used:

Dintrosalicylic Acid (DNSA) was used for colorimetric testing with the hydrolytic products of endoglucanase. 500 ml solution of DNSA was prepared.

Sodium citrate buffer was used to control the pH of assay. Sodium citrate buffer having the pH of4.8-5.0 was utilized. The pH was monitored with the help of pH meter and was adjusted with the help of NaOH and HCl.

Principle of enzyme assay*:* DNSA react with the hydrolyzed product of endoglucanase and formed colored complexes. The absorbance of these complexes was then measured by spectrophotometer at 540nm. Their concentrations were determined by comparing their absorbances with the standard. Increase in the concentration of enzyme also increase in the concentration of hydrolyzed product. More the hydrolyzed product more the colored complexes and hence absorbance of sample will be increase (Iram *et al*., 2021)

Procedure of Enzyme Assay*:* To check the activity of endoglucanase,1ml of crude enzyme was added into 1ml of 1% of substrate (CMC) in test tube. Sodium citrate buffer having the pH 5.0 was added to maintain pH (Shafique *et al*., 2004). Then the test tube was incubated at 27-28 for 30 minutes in the incubator. After incubation of 30 minutes, 3ml of DNSA was added in each test

tube and were incubated in boiling water for15 minutes. In boiling water, DNSA reacted with the digested products and formed colored complexes. The absorbance of these complexes was then measured by spectrophotometer at 540nm.

Enzyme Activity*:* One unit of enzyme activity is defined as the amount of enzyme which released one micromole of glucose per minute.

Characterization of Endoglucanase

Endoglucanase was exposed to characterization of different kinetics parameters.

Effect of temperature*:* Optimum temperature was characterized for the maximum activity of endoglucanase. This catalyst assay was performed at different temperature (20℃, 25℃, 28℃, 30℃, 35℃, and 40℃).

Effect of pH*:* Enzyme assay was performed with buffer solutions of different pH. Sodium citrate buffer of different pH*i.e.*3.5, 4.5, 7.5 and phosphate buffer *i.e.*6.5, 7.5 were used.

Effect of metals ions*:* The enzyme activity is dependent upon its structure. Metal ions alter their structure once they interact with protein either inhibit or enhance their activity. Endoglucanase assay was then performed by adding 5 completely different metal ions ($CaCl_2$, KCl, NaCl, and $MnCl_2$).

Effect of concentration of substrate on Endoglucanase for the production of Km and Vmax: Endoglucanase was additionally characterized by studying the impact of concentration of enzyme substrate on enzyme activity. Five different concentrations of CMC *i.e.* 2 mM, 4mM, 6mM, 8mM, and 10mMwere used and assay was performed in every concentration.

RESULTS AND DISCUSSIONS

Endoglucanse is the most essential enzyme which is used to reduce large amount of lignocellulotic waste. Effect of different conditions and manufacture of endoglucanase from the fungus *P. spadiceus* will help to produce more and more enzyme, decrease pollution and environmental issues and produce advantageous things from resulted fermentable sugar. The different factors were adjusted during study would discuss currently.

Optimization of Conditions

Effect of fermentation period*:* The result achieved from the fermentation period has been shown in table 3.1. The activity of endoglucanaseis increased by increasing time period and rises to maximum before 72 hours (Maximum activity= 21.05 IU/mL/min) then show small reduction up to 96 hours.

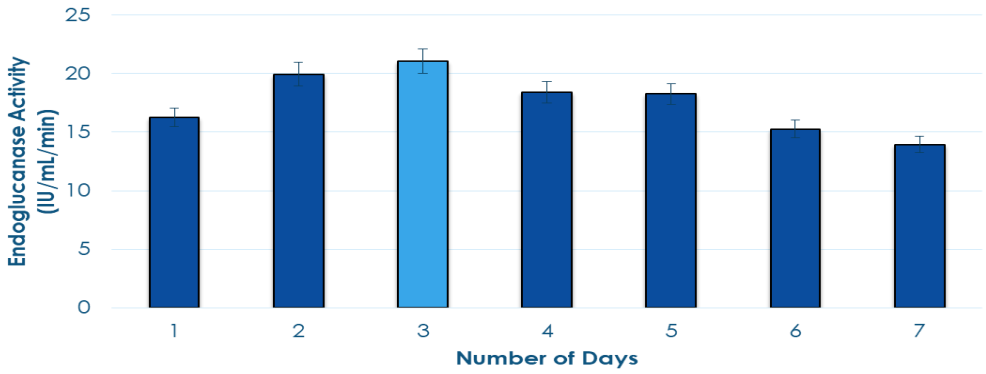

Figure 1. Activity of Endoglucanase produced from *P. spadiceus* under varying fermentation period

Effect of pH*:* Solid state fermentation was optimized to produce abundant endoglucanase from *P. spadiceus*. Change in the pH affects the ionic strength of growth media. Fungus was grown and give the maximum activity at pH 4.5 (Maximum activity = 32.28 IU/mL/min). Increase in pH of the growth media decrease the production of Endoglucanase in fig 3.2.

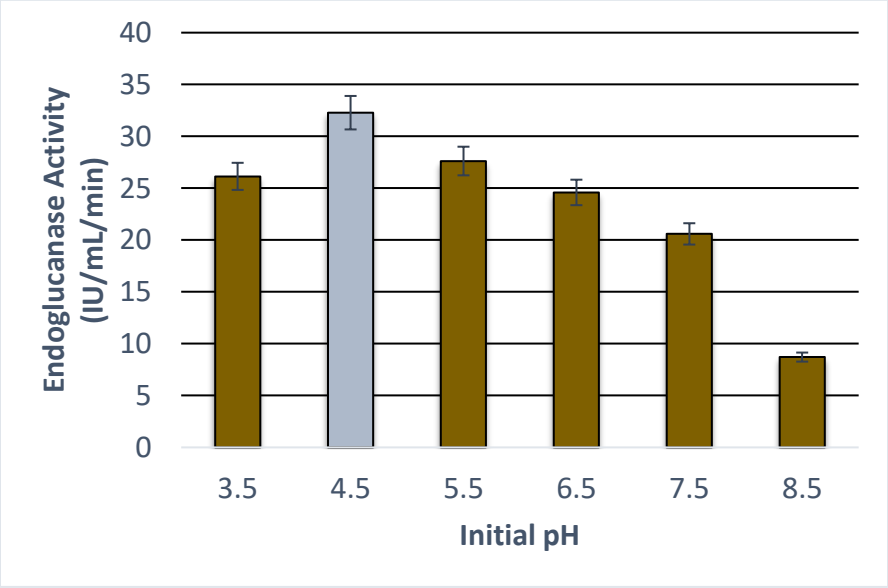

Figure 2. Activity of Endoglucanase produced by *P. spadiceus* under varying pH

Effect of temperature and moisture: Temperature and moisture play an important rolein the growth of *P.spadiceus*. During the optimization of temperature and moisture, it has been observed in my research that maximum endoglucanase was produced at 30℃ and 70% moisture level having activity 18.71 IU/mL/min**.** This indicate that the fungus has maximum growth rate at 30℃ and 70% moisture level (fig 3.3).

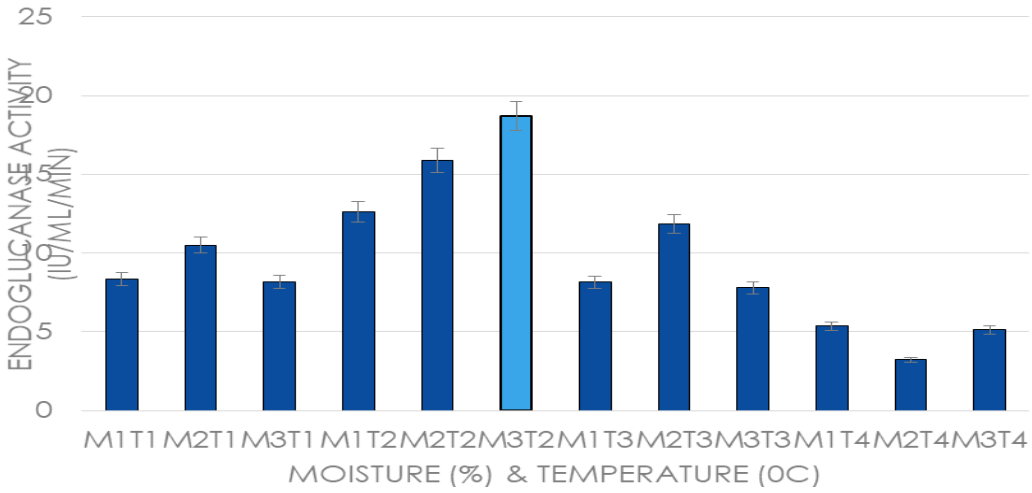

Figure 3. Activity of Endoglucanase produced by *P. spadiceus* under varying temperature and moisture

Effect of Carbon and Nitrogen sources*:* For the improvement of fungus growth, direct Carbon and Nitrogen sources are provided. Maximum enzyme activity was observed in the presence of glucose and urea.

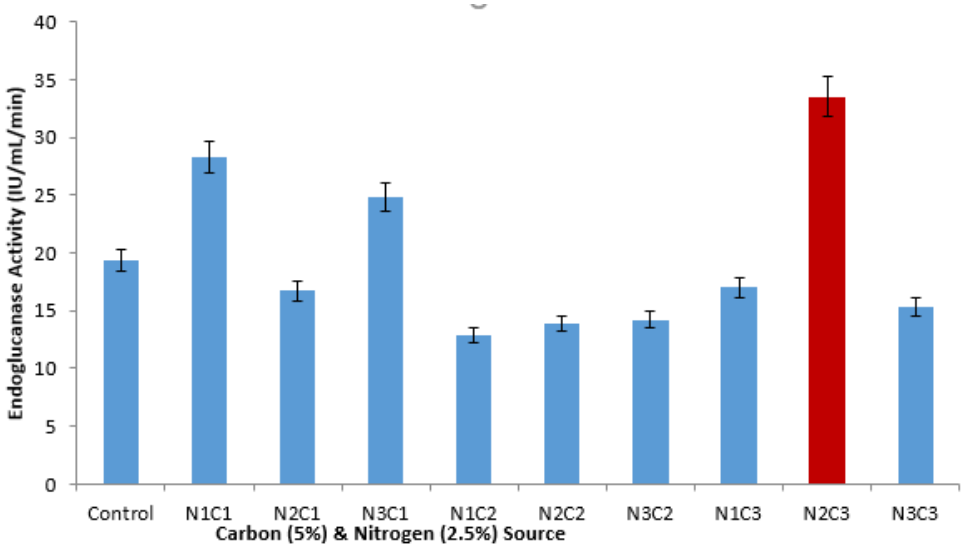

Figure 4. Effect of different Carbon and Nitrogen sources on endoglucanase activity

Characterization of Endoglucanase

Determination of optimum pH*:* Change in pH would affect the ionic strength of the growth

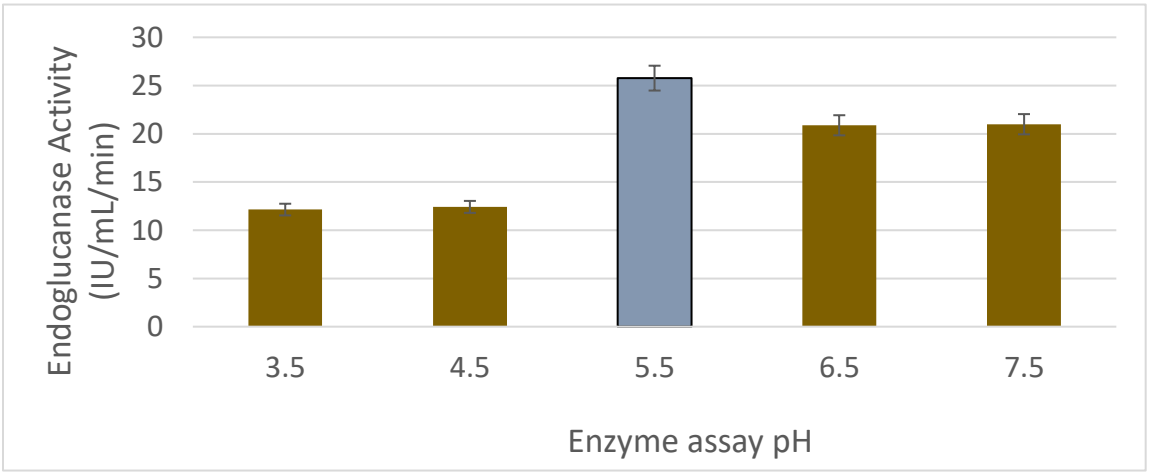

Figure 5. Activity of Endoglucanase produced by *P. spadiceus* under varying optimum pH

media. Fungus was grown at five different pH of buffer solution but maximum activity was found at pH 5.5 (Maximum activity = 25.77 IU/ml/min). Increase in the adjusted pH of buffer solution increase in the production of endoglucanase in fig 3.5.

Determination of optimum temperature*:* During the determination of optimum temperature, it has been observed that maximum endoglucanase was produced at 28℃ having the activity 27.08 IU/mL/min shown in fig 3.6.Further increase in temperature decrease the production of endoglucanase because at a very high temperature enzyme is denatured.

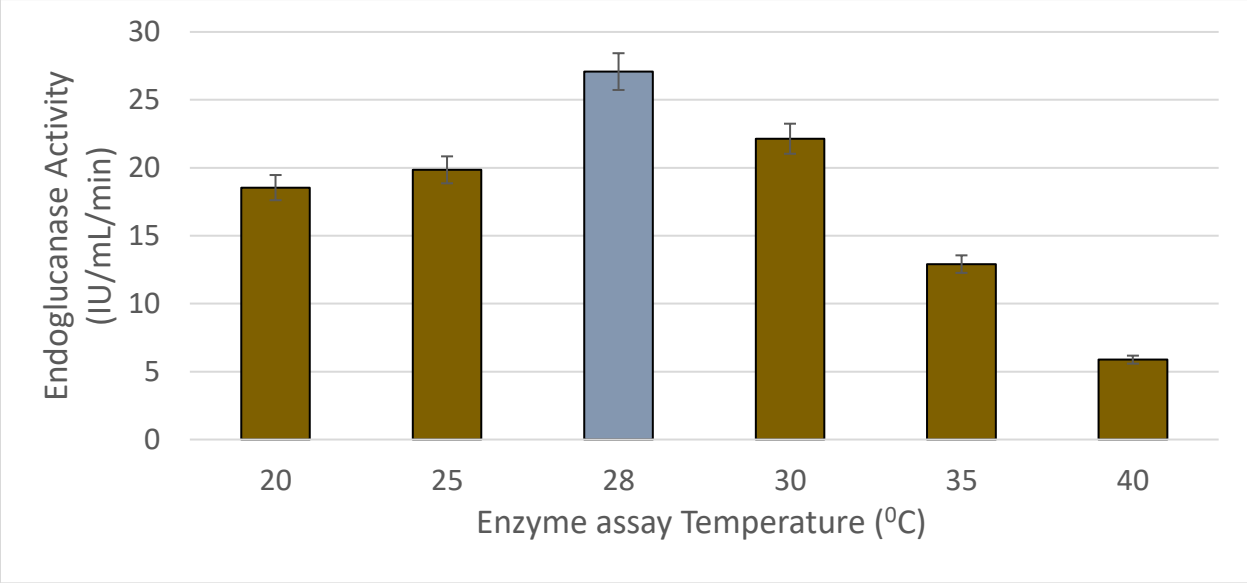

Figure 6. Activity of Endoglucanase produced by *P. spadiceus* under varying optimum temperature

Effect of metals ions*:* results showed that some metals ions have positive effect and some have negative effect on the production of endoglucanase, because those metal which act as cofactor have increase production of endoglucanse and those which act as inhibitor decrease endoglucanase production shown in fig 3.7.

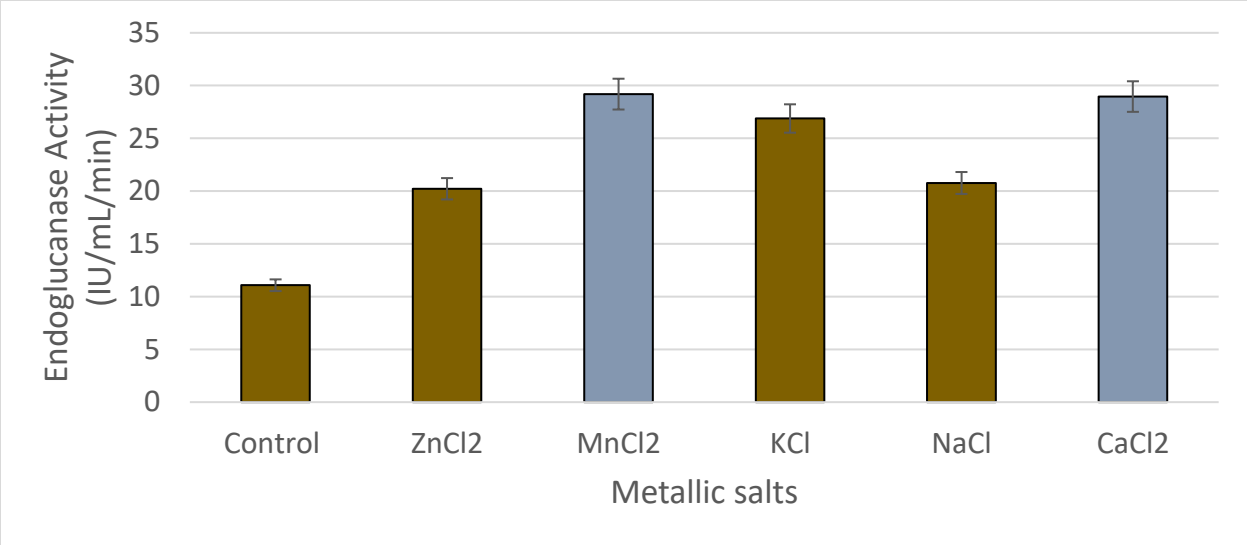

Figure 7. Activity of Endoglucanase produced by *P. spadiceus* under varying Metals Ions

Effect of substrate concentration on Endoglucanase*:* Results showed that increase in concentration of substrate caused an increase in the velocity of enzyme upto a certain limit. After that the rate became constant and further increase in substrate concentration had no effect (fig 3.8). The reason was that all the active sites were occupied with substrate. Line weaver Burk plot the graph between inverse of maximum velocity and inverse of Km respectively**.**

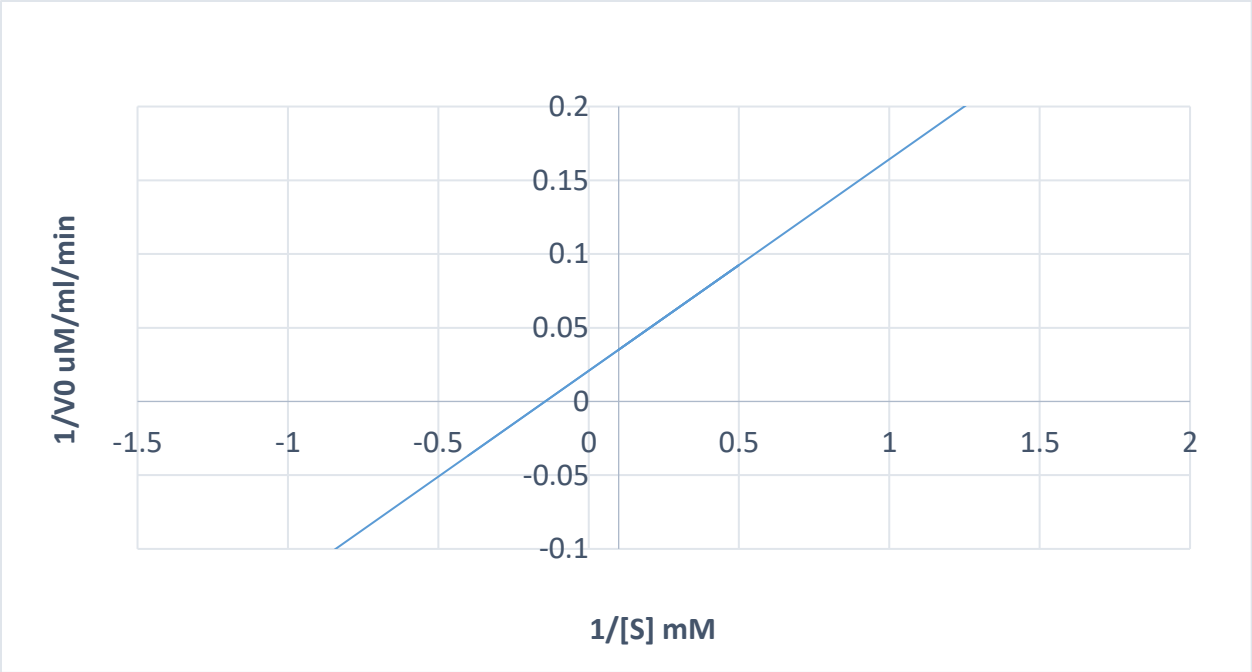

Figure 8. Line-Weaver Burk double reciprocal Plot to calculate K_m and V_{max}

CONCLUSIONS

The current study was approved out to harvest and improve endoglucanase by *P.spadiceus*. The lignocellulosic biomass in the form of wheat straw was used as a substrate for fermentation process. To find extreme yield of endoglucanase, effect of different parameterswas observed including, fermentation period, temperature, pH, moisture level and different carbon and nitrogen bases. Crude enzyme extract obtained after centrifugation was then characterized for temperature, pH, metal ions and substrate concentration for the purpose of Km and Vmax. Maximum activity of endoglucanase was observed after day 4, at 25-30˚C, at 50% moisture level at pH 4.5. Moreover, addition of carbon source (5% sucrose) and nitrogen source (2.5% urea) effect of manufacture of Endoglucanase. After description to find out its optimum temperature, pH, effect of different metal ions and Km and Vmax. Endoglucanase activity at different conditions presented that the optimum temperature was 30˚C and pH 4.5 and values of Km and Vmax showed that endoglucanase has high empathy for its substrate.

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