PRODUCTION AND CHARACTERIZATION OF EXOGLUCANASE FROM *Phaeolusspadiceus*

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ABSTRACT

In economic sector, the main backbone of Pakistan is considered agriculture that produces a hug volume of waste material which has a very few economic uses. White rot Fungi play an important role in the production of hydrolytic enzymes like cellulases. In current study *Phaeolusspadiceus*.was used for the production of exoglucanase by Solid State Fermentation of wheat straw.*P.spadiceus* produced exoglucanase having high enzymatic activity during the fermentation of wheat straw under optimum physical and nutritional conditions. There was maximum exoglucanase activity after 72 h of fermentation, at 30°C temperature, pH 4.5, 70 % moisture level and 1 mL fungal inoculum. Further production was increased with glucose as carbon source (5%) and peptone as nitrogen source (2.5%). The characterization of enzyme revealed that it has optimum temperature as 30°C and optimum pH as 4.5 using 1% Avicel aqueous solution as substrate. KCl, $ZnCl_2$, NaCl and $MnCl_2$ have positive effect on enzyme but Zn^{+2} ion has high positive effects on exoglucanase activity. The Km and Vmax were 34.48 mM and 175.43 µM/ ml/ min, respectively.

key words: Exoglucanase, *Phaelousspadiceus,* Wheat Straw, fermentation

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INTRODUCTION

Agriculture is the main backbone of Pakistan ineconomic point view. Open field burning of raw materials from agriculture cause climate change and pollution. The source of fermentable sugars, most abundant and renewable is lignocellulosic biomasson earth (Himmel *et a*l., 1999; Saleem *et al*., 2008 and Ahmed *et al*., 2009) and in nature are the most importantsource of fixed carbon (Yang *et al*., 2007 and Ahmed *et al*., 2009).Annual worldwide builds 10–50 billion tonslignocellulosic biomass from the plants (Sticklen, 2006 andPersad and Bisaria, 2014).The components of lignocelluloses consist of cellulose (∼30 to 50%), hemicellulose (∼20% to 35%), and lignin (∼15% to 25%). The renewable biomass can be used to produce building blocks for many industrial products through acidic or enzymatic hydrolysis like the production of organic acids (Xia *et al*., 1999), ethanol (Brethauer *et al*., 2010) and other important chemicals (Mahmood *et al*., 2013).Renewable lignocellulosic materials with less cost will be used to produce bioproducts and bioenergy for the local economy and national energy protection (Zhang, 2008 and Zhang and Zhang, 2013).The lignocellulosic biomass isideal for enzymatic hydrolysis due to non-repressing by-products and nontoxic discharges (Persad and Bisaria, 2014) but high cost of industrial enzymes remains a barrier commercially.

In hydrolytic enzymes, cellulase is the most important group that catalyse the breakdown of β-1, 4 linkages thatappear in cellulose to give glucose. Cellulases are the group of extracellular enzymes including endoglucanase, exoglucanase and β-glucosidase. Exoglucanase acts on oligosaccharides that have reducing and non-reducing ends and releases cellobiose units, which consist of glucose units more than two. Exoglucanase havetunnellike loop and three-dimensional(3D) structureat active sitefor interaction with substrate by hydrogen binding (Sinnott, 1997; Mahmood *et al*., 2013).Cellulases are important industrial enzymes and use in many industries like wine and brewery industry (Bamforth, 2009), textile industry (Karmakar and Ray, 2011), pulp and paper industry, food industry and bioethanol industry (Kuhad *et al*, 2011).

The researchhasfocus to improve the productivity of known enzymes, identify new and more active enzymes, and optimized characters of enzymefor lignocelluloses and less the production cost of enzyme (Merino and Cherry, 2007 and Zhang and Zhang, 2013). For enzyme production used different lignocellulosic materials are wheat straw (Norma and Guillermo, 2003; Yang et al., 2006 and Mahmood *et al*., 2013), wheat bran and corn cobs (Betini et al., 2009 and Mahmood *et al*., 2013)

Now-a-days, solid-state fermentation (SSF) is more used becauseof its advantages like lower investment spending, less cost of media for fermentation, quantity of better production, less energy needs and not essential requirement of manycontrols of fermentation parameters and low amount of waste production.

The capacity to degrade lignocellulose is high among fungi than bacteria.White-rot fungi that are responsible for efficient lignin degradation in wood decay processes. *Phaeolusspadiceus*is

present in the heart wood of the tree. It is widely study for degradation of lignocellulotic biomass (Wheat straw) by secretion of extra cellular enzyme like exoglucanase.

MATERIALS AND METHODS

Substrate collection and preparation

Wheat straw was used as substrate for the growth of *P.spadiceus*to produce exoglucanase.Wheat straw was collected from the village of SamwalSharife, Mirpur Azad Kashmir, then air dry it for 20 days.Oven dried it for 24 hours at 60 ^{0}C for the removing of remaining moisture. After that, it was ground into powder form in the laboratory of Biotechnology, MUST, Mirpur Azad Kashmir. Powder of substrate was packed in airtight plastic jars before use in fermentation process.

Fermentation Organisms

Collection and Isolation of *P.spadiceus*

The sample of *P.*spadiceus was collected from the laboratory of Biotechnology, MUST, Mirpur Azad Kashmir. It was isolated by culturing on malt extract agar media in aseptic conditions. Slant was prepared from isolated pure culture on Malt Extract Agar (MEA) media. The slant and pure culture of *P.spadiceus* were stored at 4^{o}C in refrigerator for future use.

Preparation of Fungal Inoculum Media

For inoculum broth media was contained per litter; 3g of peptone, 20g of dextrose, 20g of malt extract and adjusted pH at 4.5. The inoculum media was autoclave at 121^{o}C and 15 psi for 15 minutes. After autoclave, the fungal spores were incubated into inoculum media from the culture plate under aseptic conditions. The flask containing inoculum media was placed in shaking incubator at 120 rpm at 30 ^{o}C for 2-3 days.

Fermentation Process

Flasks containing 5g of grinded wheat straw was moist with 2.5ml (50%) of d.H_2O, having pH 5.5 that maintained with the help of 1M HCL/NaOH and autoclave these flasks at 121^{o}C and 15 psi for 15 minutes. Each flask after autoclave was inoculated aseptically with 1ml of *P. spadiceus*inoculum and incubated these flasks at 30^{o}C for species days.

Sample Harvesting

After specified day, in each of the flask added 50ml of d.H_2O (pH 5.5) for the extraction of exoglucanase. These flasks were shake at 120rpm for 30 minutes in shaking incubator. The extra cellular enzymes dissolved in water, which was filtered with help of filter paper. Filtrate extract of enzyme, then centrifuge at 12000 rpm for 10 minutes to remove all spores and impurities. Supernatant was stored as a crude enzyme at 4^{o}C before preforming enzyme assay (Shafique et al., 2004; Mahmood et al., 2013).

Effect of Physical and Nutrients Conditions

Fungal growth and production were optimized by maintaining different conditions in order to gain maximum production of exoglucanase from *P. spadiceus*.During current study following conditions were optimized.

Effect of Fermentation Period

P. spadiceus growth was optimized for different fermentation time period from 24hr to 168hr. After different period, with gap of 24 hours, the harvested crude enzyme sample was subjected to enzyme assay.

Effect of pH

*P. spadiceus*was cultured, for the optimization of pH at ranging from 3.5 to 8.5 (six varying pH of distil water used as moisture content 50% into substrate) and adjusted with the help of 1M HCl/NaOH.

Effect of Moisture and Temperature Level

P. spadiceus was incubating at four different temperatures (25 ºC, 30ºC, 35 ºC and 40 ºC) and three different moisture levels (30%, 50% and 70%) for 3 day (optimum).

Effect of Carbon and Nitrogen Sources

Carbon and nitrogen sources in different forms was used as substrate for the growth of *P. spadiceus* that increase the production of exoglucanase. For the carbon sources (Glucose, Fructose and Sucrose) and the nitrogen sources (Peptone, Urea and Ammonia Nitrate) were used during the current study.

Standard Curve

Cellobiose was used as a standard because exoglucanase act on the non-reducing end of cellulose and release disaccharide units (cellobiose). Different concentrations of standard were prepared in 0.0 µM-4 µM**.** Standard factor would be calculated by measuring absorbance of each concentration at 540nm.

Enzyme Assay

Avicel was used as substrate for exoglucanase (Sherief et al., 2010)**.** 1% solution of Avicel in distal water is used as enzyme assay. Following reagent are used for enzyme assay of exoglucanase.

DNS reagent: Dinitrosalicylic acid was used to form the coloured complexes with the product of exoglucanase. Five hundred ml solution of DNS was prepared by mixing of 5g of NaOH, 1g of phenol, 0.25g of Na_2SO_4, 91g of Roshelle salt and 5g of 3, 5-dinitrosalicylic acid**.**

Sodium citrate buffer: Five hundred ml. of buffer was prepared by mixing 3.88g of citrate acid and 1.91g of tri-sodium citrate in distal water with pH 4.8.

Procedure of Enzyme Assay

The activity of crude enzyme exoglucanase was observed by adding 1ml of crude enzyme, 1ml of 1% Avicel (substrate) and 1ml of Sodium citrate buffer having pH 4.8 in a test tube. Incubated the test tube at 30ºC for 30 minutes in the incubator. After 30 minutes added 3ml of Dinitrisalicylic acid (DNS) into each test tube to stop the reaction and tubes were placed in boiling water for 15 minutes. During boiling, DNS react with enzymatically hydrolytic product of exoglucanase to formed complexes. The concentration of these complexes was measured by spectrophotometrically at absorbance 540nm.

Enzyme Activity

One unit of enzyme activity defined as the amount of enzyme which released one micro-mole of glucose per minute.

Characterization of Exoglucanase

Purified exoglucanase was exposed to the characterization of different kinetic parameters.

Optimization of pH for Exoglucanase

To check the effect of pH on exoglucanase used two types of buffer (Sodium citrate buffer and Phosphate buffer). Enzyme assay was performed at different pH values of Sodium citrate buffer (pH-3.5, 4.5 and 5.5) and Phosphate buffer (pH-6.5 and 7.5).

Optimization of Temperature for Exoglucanase

Enzyme assay was performed at six different temperature levels e.g., 20 ºC, 25 ºC, 28 ºC, 30 ºC, 35 ºC, 40 ºC at pH 4.5.

Optimization of metal ions for Exoglucanase

Enzyme assay of exoglucanase was performed at five different metal ions solution ($CaCl_2$, KCl, $ZnCl_2$, NaCl and $MnCl_2$) and measured spectrophotometrically at 540nm. Metal ions interact with enzyme and change their structure and either enhance or inhibit their activity.

Effect of Substrate Concentration for Determination of Km and Vmax

For enzyme assay five different concentration of Avicel (2mM, 4mM, 6mM, 8mM and 10mM) were used to the determine Km and Vmax of the enzyme.

RESULTS AND DISCUSSION

The enzyme (exoglucanase) is one of important cellulose enzyme that is contributed to the degradation of large amount of wheat straw (cellulosic waste). The study of production of exoglucanase from fungus (*P. spadiceus*) and optimized it at different conditions will help to produce high quantity of enzyme that reduce environment pollution and produce appreciated

things from resulted fermentable sugar. During the current study, different factors would discuss below that are optimized.

Effect of Physical and Nutrients Conditions

Effect of Fermentation Period

After Day-3 (72 hr.) of fermentation, *P. spadiceus*gave maximum production of exoglucanase (97.11 IU/mL/min) (Fig. 1). At day-3 production of exoglucanase was increased but after day-3 production of exoglucanase decreased due to depletion of nutrients and accumulation of waste materials. Current results show the resemblance with the resultsthat reported by Shafique et al. (Shafique *et al*., 2004) for exoglucanase by fungal source (Mahmood *et al*., 2013).

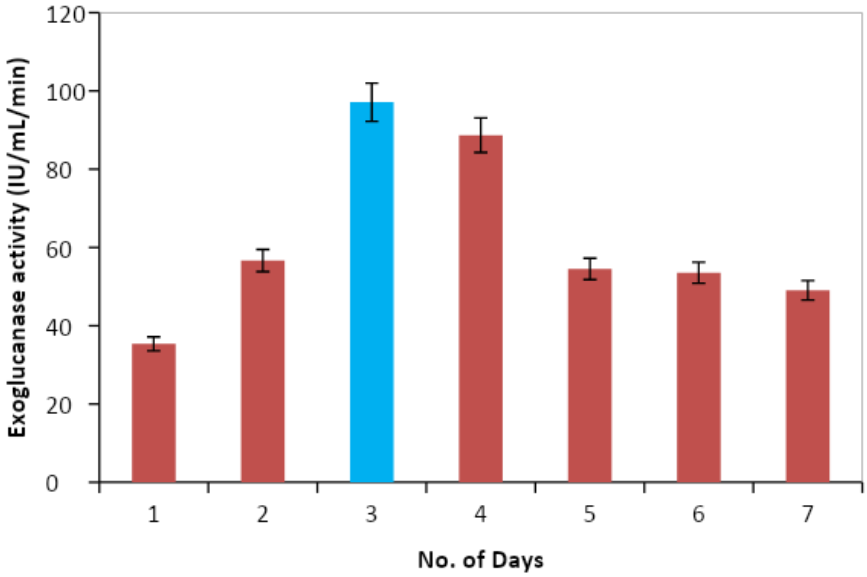

Figure 1. Effect of fermentation period for exoglucanase by *P. spadiceus*

Optimization of pH

P. spadiceus was produced maximum exoglucanase production (147.02 IU/ml/min)at pH 4.5 of fermentation (Fig. 2).After that, its activity decreased due to the acidic nature of enzyme and decreased in the stability of enzymes.

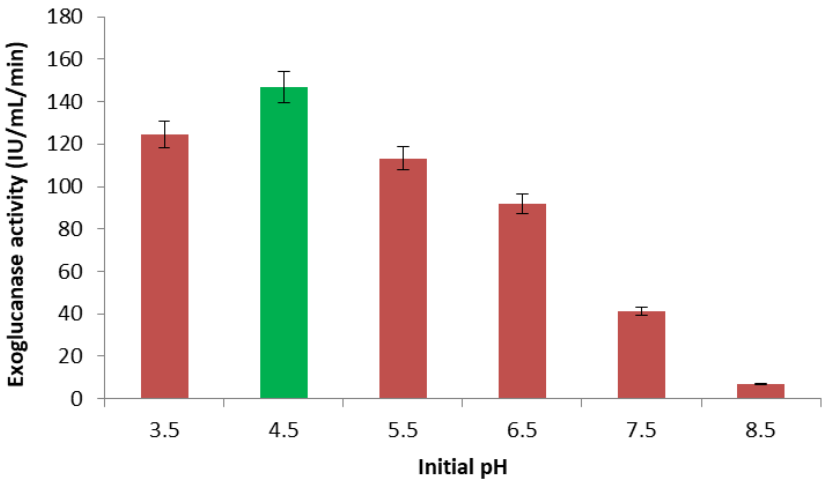

Figure 2: Effect of pH for exoglucanase by *P. spadiceus*

Effect of Moisture and Temperature Level

Maximum production of exoglucanase was observed (Fig. 3) at 70% of moisture level and 30ºC of temperature (69.47 IU/mL/min). Increase moisture level from 30 to 70% showed that increase in production of exoglucanasefrom fungus growth. Increase in temperature, initially increased the activity of enzyme because the higher movement of molecules that increase in kinetic energy of exoglucanase (Iram *et al*., 2021) . As result, the rate of reaction was enhanced, and substrate-enzyme interaction increased. Further increase in temperature caused decrease enzyme activity due to denaturation of structure of proteins, enzyme (Mahmood *et al*., 2013).

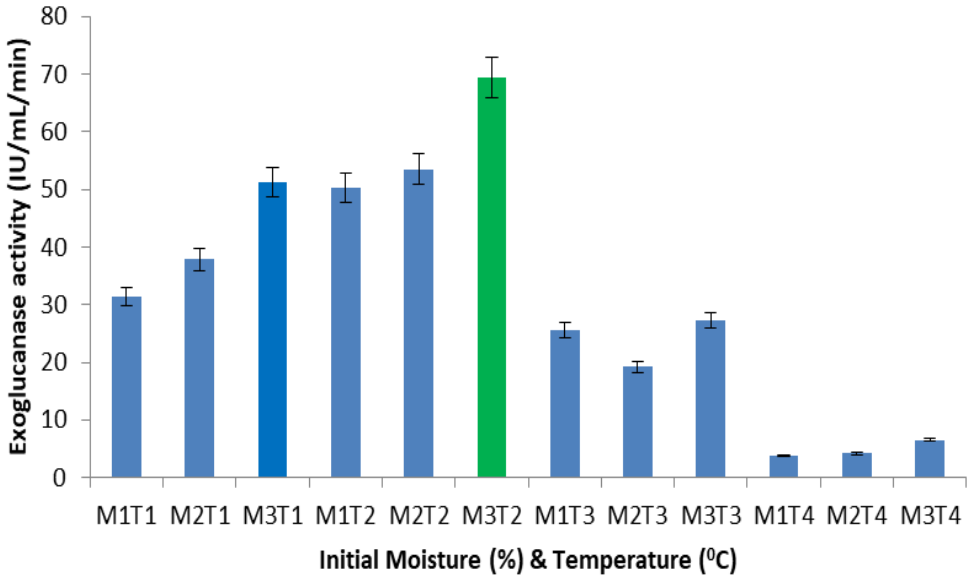

Figure 3. Effect of Moisture and Temperature Level for exoglucanase by *P. spadiceus*

Effect of Carbon and Nitrogen Sources

For carbon source used glucose, fructose and sucrose and for nitrogen source used urea, peptone and ammonium nitrate as additional components for the growth of *P. spadiceus*. Both are used for fungal growth as nutrients sources, as well as exoglucanase production. As carbon source used glucose and nitrogen source used peptone, maximum production was observed (75.8 IU/mL/min), to produce exoglucanase (Fig. 4). External carbon sources (glucose) increase the growth of fungi and production of cellulases because of easily available to fungus than substrate (Gaind and Nain, 2007 and Mahmood *et al*., 2013). Peptone is better nitrogen source as compared to urea and ammonium nitrate. Sherief et al. also described that the cellulases production enhances with peptone more than urea because amino acidscontain in peptone, readily available as nitrogen source for the growth of A. *fumigatus*and production of enzymes (Trecthewey *et al*., 2005 and Mahmood *et al*., 2013).

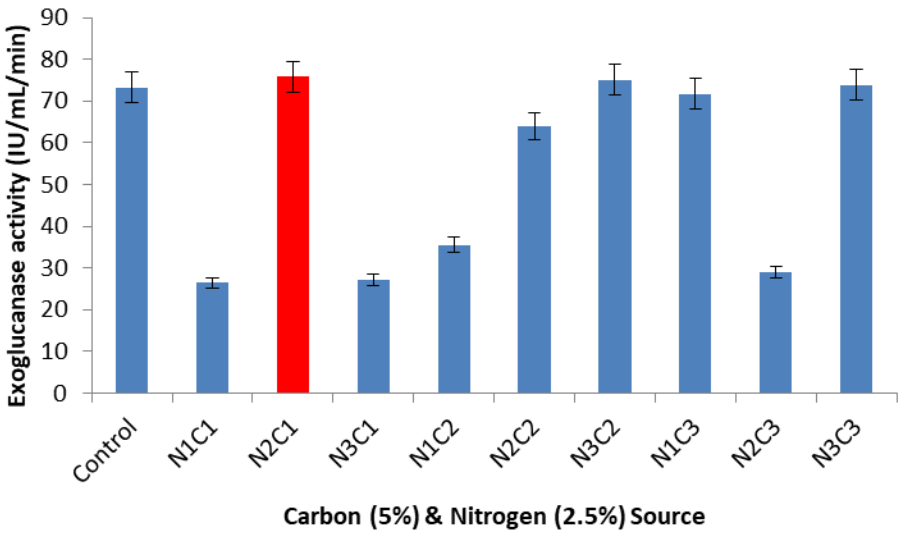

Figure 4. Effect of Carbon and Nitrogen sources on exoglucanase activity

Characterization of Exoglucanase

Optimization of pH for Exoglucanase

For optimum pH for exoglucanase, enzyme assay held under different pH values of sodium citrate buffer (3.5, 4.5 and 5.5) and phosphate buffer (6.5 and 7.5). The maximum activity (53.11 IU/mL/min) was observed at pH 4.5 of sodium citrate buffer. Future increase of pH values of different buffer (Sodium citrate and phosphate buffer),the enzyme activity was decreased probablybecause ionic strength in reaction mixture changed in that case the protein of enzyme is unstable (Fig. 5).

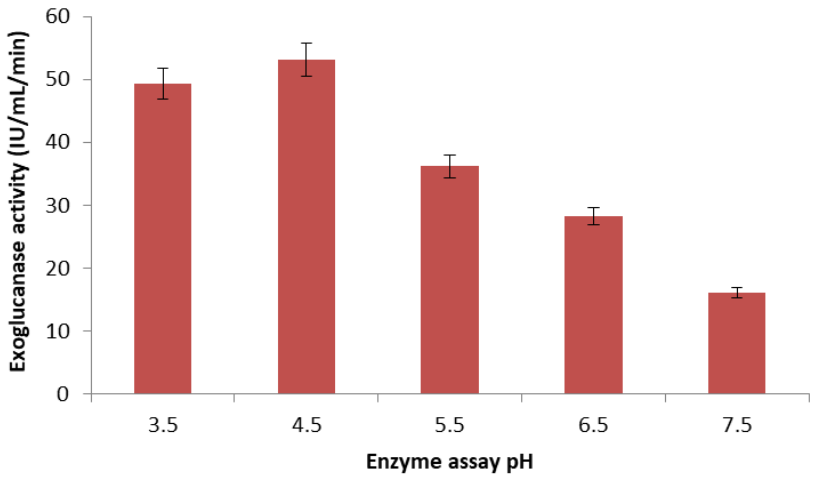

Fig 5: Optimization of pH of exoglucanase

Optimization of Temperature for Exoglucanase

For optimization of temperature, exoglucanase activity assay was held under at different temperature ranging from 20ºC, 25ºC, 28ºC, 30ºC, 35ºC and 40ºC. In each case,pH was retained at 4.5. Exoglucanase showed maximum activity (64.79 IU/mL/min) at 30°C (Fig. 6). After that, further increased temperature was decreased exoglucanase activity due to denaturation of enzyme structure.

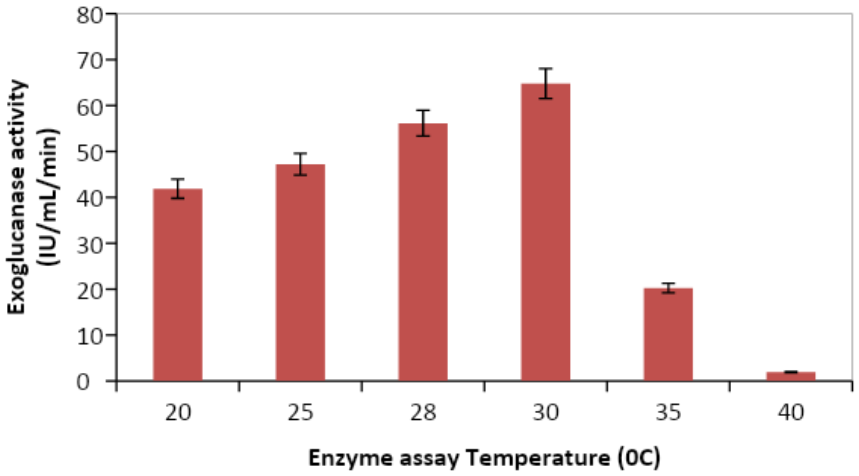

Figure 6. Optimization of temperature of exoglucanase

Optimization of Metal Ions for Exoglucanase

For the optimization of exoglucanase,the different metal ions like Ca^{+2}, K^{+}, Zn^{+2}, Na^{+}, and Mn^{+2} at different concentrations of $CaCl_2$, KCl, $ZnCl_2$, NaCl and $MnCl_2$ were used. As results described that maximum metal ions have positive effects on exoglucanase activity (Fig. 7). Maximum effect on exoglucanase activity was Zn^{+2} ion and KCl, $ZnCl_2$, NaCl and $MnCl_2$ have positive effect on enzyme. These ions increase the production of exoglucanasebecause activating many processes in the fungus like synthesis of proteins and act as a cofactor of enzymes.

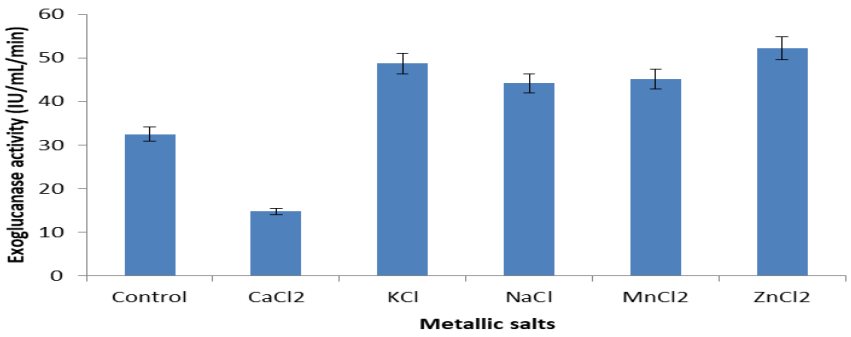

Figure 7. Effect of metal ions on exoglucanase activity

Effect of Substrate on Exoglucanase: Determination of Km and Vmax

The Michaelis–Menten constants Km and Vmaxfor exoglucanase was performed at varying concentration of Avicel (2, 4, 6, 8, and 10 mM) and that constants were measured by Lineweaver–Burk reciprocal plot. The Lineweaver–Burk reciprocal plot between $1/V_0$ (cellulase activity) on Y-axis against 1/[S] (concentration of substrate) on X-axis (Fig. 8) and generated a hyperbolic curve. The values of Km and Vmax for exoglucanase from *P spadiceus*was measured by the Linear equation from the plot to be 34.48 mM and 175.43 µM/ml/min, respectively. Dashtban et al. was stated from *Trichodermareesei,* the value of Km 3.8 mM of exoglucanase (Dashtban et al., 2009 and Mahmood et al., 2013). Ayman et al. was described the Vmax of 1.80 U/mL for exoglucanase by using as a substrate Avicel (Dabaet al., 2011 and Mahmood et al., 2013). Km value of 4.34 mM and Vmax of 7.29 µM/mL of exoglucanase from *Aspergillusfumigatus* was reported by Mahmood et al. (Mahmood et al., 2013).

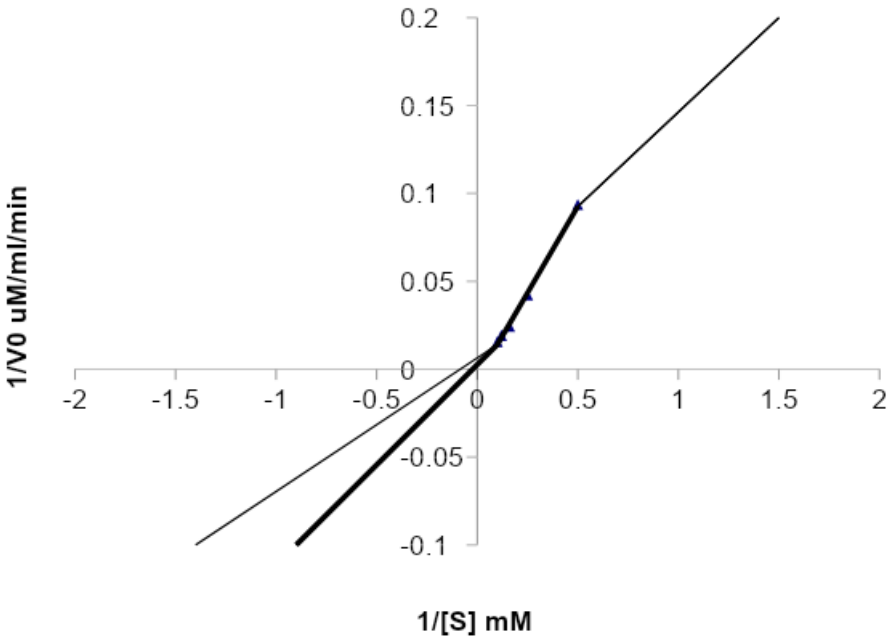

Figure 8. Line-Weaver Burk doubles reciprocal Plot to calculate K_m and $V_{max.}$

CONCLUSIONS

Exoglucanase takes part in the breakdown of cellulose along with endoglucanase and betaglucosidase. It hydrolyzed oligosaccharides produce by endoglucanase into tri- and disaccharides, called cellobiose. The Solid-state fermentation of wheat straw is producing large amount of exoglucanase by *Phaeolusspadiceus* under improved conditions. Maximum exoglucanase activity was observed at 30°C temperature, day-3 and pH 4.5. Addition of glucose as a carbon source 5% and peptoneas a nitrogen source 2.5% further enhanced the production of exoglucanase. Values of Michaelis–Menten kinetics constants (Km and Vmax) indicated that exoglucanase has high affinity for its substrate (Avicel). Different inorganic metal ions like K^+, Mg^{2+}, and Ca^{2+} and Na^+ have positive effect on enzyme activity but Zn^{2+} have more positive impact on enzyme activity. These ions act as cofactor for exoglucanase or help in the synthesis of proteins. Purification of chromatography techniques, utilization for biofuel production and application at pilot scale can be used for future perspectives.

Conflict of interest: The authors declare no conflict of interest.

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