Enhancement of β-1,3 Glucanase Production from *Penicillium oxalicum* T3.3

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Abstract *— β-1,3 glucanases are semi-constitutive hydrolytic enzymes that can degrade glucan molecules embedded in the cell wall components of cereals and some species of fungi resulted in production of D-glucose. This enzyme has a great potential and interest in biotechnology, agricultural and also industrial field. However, there is little reports on the production of β-1,3 glucanase by Penicillium oxalicum. Therefore, the cultural conditions which stimulate in vitro production of β-1,3 glucanase enzyme by P. oxalicum T3.3 and characterization of β-1,3 glucanase enzyme activity were determined.Various parameters such as different types of carbon and nitrogen sources, initial pH medium, agitation speed and surfactants were investigated. The optimization was carried out by varying and optimizing one variable at a time. The highest production of β-1,3 glucanase activity of 84.73 U/mL was obtained using seaweed Undaria pinnatifida as substrate at concentration of 1% (w/v), peptone and yeast extract as nitrogen source at 0.3% and 0.2% respectively, initial medium pH 5, agitation speed at 200 rpm and with addition of sodium dodecyl sulfate as surfactant. Under these conditions, β-1,3 glucanase activity increased by 38.6%. Enzyme characterization was also performed which indicated that this enzyme is thermostable and showed optimum activity at 50°C, pH 5 and can retained its activity around 80% up to 4 h at this condition.The optimization of β-1,3 glucanase production by P.oxalicum required adjustment of different types of carbon and nitrogen sources, initial pH medium, agitation speed and surfactants. This enzyme characterization has revealed its great potential towards detergent, beer and food fermentation industries whose manufacturing conditions are largely acidic.*

Keywords— β-1,3 glucanase, characterization, optimization, Penicillium oxalicum.

I. INTRODUCTION

β-1,3-glucanase is an important enzyme in the industrial and agricultural processing field. β-1,3 glucanase enzymes have received attention in many fields of science and biotechnology because many cultures of microorganisms widely used in industry produce β-1,3 glucanase (Kulminskaya *etal*., 2001). The resistance of this enzyme to denaturation by high temperature and pH extremes makes it particularly essential in various functions (Beshay *et al*., 2011). β-1,3-glucanases have been reported to be produced by a variety of organisms such as bacteria, fungi, and higher plants and many of them have been purified and characterized (Martin *et al*., 2007). β-1,3 glucanase can be used as biocontrol agent againstplant-pathogenic fungi and act as hydrolyticenzyme for the destruction of cell wall of fungal and structure of β-1,3-glucan (Beshay *et al*., 2003). Besides, for industrial purpose they are used commercially in combination with other enzymes in the production of beer and in the brewery, for barley-beta glucan degradation for animal feed. Furthermore, they can be used as effective additives in laundry detergents. They can also be used for saccharification of agricultural and industrial wastes to provide glucose syrups for animal use (Doughari and Hamuel 2011). *Penicillium sp* has also been reported to produce hydrolytic enzymes including chitinase and β-glucanase which are involved in the degrading of fungal cell wall (Chen *et al*., 2012; Patil *et al*., 2013). In another study, it was suggested that *P. oxalicum* secretes chitinase and β-glucanases to degrade and penetrate into the conidiophores and spores of *Nigrospora oryzae* (Sempere and Santamarina, 2008).

To the best of our knowledge there is little reports on the production of β-1,3 glucanases by *P. oxalicum.* Thus, a study on the optimization of β-1,3 glucanases production using this fungus is important to be carried out in order to enhance β-1,3 glucanases production. The most important aspects to decrease the production cost are optimization of media and process conditions (Goshal *et al.,* 2011). In this study, optimization of β-1,3 glucanases production was done by using the conventional method, which involved varying one variable at a time while the other variables were kept constant. Therefore, the objectives of this study were to determine the cultural conditions which stimulate the *in vitro* production of β-1,3 glucanase enzyme by *P. oxalicum* T3.3 and to characterize the β-1,3 glucanase enzyme activity.

II. MATERIAL AND METHOD

2.1 Microorganisms and cultivation

Penicillium oxalicum T3.3 was obtained from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. For maintenance, the fungus culture was grown on Potato dextrose agar (PDA) (Difco, USA) plates at 30ºC for 7 days. After 7 days of incubation, the matured spores were harvested by covering with approximately 10 mL of sterile distilled water. The spore suspensions of the seven day old colonies of the fungus were probed gently with a Pasteur pipette tip. Then, the collected spore suspension was transferred to a sterile tube. The spore suspension concentration was determined by using a haemacytometer. For long term stock cultures, 0.7 mL of the stock spore solution was resuspended with 0.3 mL of 80% sterile glycerol in aseptically condition. The stock spore solution was kept at -20ºC.

2.2 Enhancement of β-1,3 glucanase production

The enhancement of β-1,3 glucanases production by *P. oxalicum* T3.3 were studied by varying and optimizing one variable at a time using shake flasks under various culture conditions such as different types of substrates (fungal cell wall, seaweed, glucose and rice husk). A basal medium was used which composed of (g L^{-1} of distilled water) peptone, 3.0; $(NH_4)_2SO_4$, 2.0; yeast extract, 0.5; KH_2PO_4, 4.0; $CaCl_2.2H_2O$, 0.3; $MgSO_4.7H_2O$, 0.3 supplemented with substrates, 1.0; according to Cao *et al.*, (2009). A quantity of 50 mL of the basal medium was put into 150 mL Erlenmeyer flask and autoclaved at 121^{o}C, for 15 min. The different concentration (0.5%, 1.0%, 1.5%, 2.0% and 3.0%) of the best substrate (seaweed) was further investigated. Other parameters studied for enhancement of β-1,3 glucanases production including: incubation period, initial pH medium, agitation speed (at 120, 150, 180 and 200 rpm) and addition of different types of nitrogen sources (combinations of peptone, yeast extract and $(NH_4)_2SO_4$ at 3:2:2 ratio respectively while the control medium contained nitrogen sources in the basal medium before modifications at 3:0.5:2 ratio respectively) The effect of addition of surfactant (0.02% (v/v) Tween 20, Tween 80, Triton X-100 and 0.02% (w/v) sodium dodecyl sulphate) has been studied. Spores suspension inoculum of *P. oxalicum* T3.3 (1.0×10^6 spores mL^{-1} of culture medium) was inoculated into each flask. The cultures were grown at 30^{o}C for 5 days at 150 rpm. After 5 days, culture filtrates from the fermentation were filtered and centrifuged at 4^{o}C for 10 min at 4000 × g and the supernatants were assayed for enzyme activity and protein determination. All experiments were carried out in triplicates.

2.3 Assays of enzyme activities

In order to determine the fungal biomass, measurement of N-acetylglucosamine released as a result of acid hydrolysis of chitin is one of the indirect measures of fungal growth (Fang *et al.*, 2010; Babitha and Carvahlo, 2008). Chitin is a component of fungal biomass which is present in the fungal cell wall and it is used as a fungal growth indicator. Fungal biomass was expressed as milligrams per gram of substrate (original dry weight) according to the standard curve using glucosamine with a series of different concentrations (2-10 mg mL^{-1}) as a standard. The fungal biomass glucosamine was computed as the measured glucosamine minus the background glucosamine content of the substrates only.

For dry cell weight estimation, fermented substrate was oven dried at 60°C overnight. A 5 mL 72% (v/v) sulphuric acid was added to 0.4 g substrate. It was then agitated at 130 rpm, room temperature for 30 min. To this acid hydrolysate, 54 mL distilled water was added and the mixture was autoclaved at 121°C for 2 h. The pH was adjusted to pH 7 using 10 M, and 0.5 M NaOH. In a screw cap glass tube, 1 mL hydrolysed sample was mixed with 1 mL acetyl-acetone reagent and it was incubated in boiling water bath for 20 min. Then it was cooled under running tap water prior to the addition of 6 mL absolute ethanol. After it was mixed well, 1 mL Ehrlich reagent was added and the tubes were immediately lidded with the screw cap. Then the mixture was shaken vigorously and the excess CO_2 was released. The absorbance was read at the 530 nm using a spectrophotometer. One mL of sample was replaced by 1 mL distilled water and served as blank sample.

β-1,3 glucanase activity was assayed by adding 0.05 ml of the culture supernatant with 0.05 ml of 0.05 mol L^{-1} acetate buffer (pH 5.5), which contained 0.1 mg of laminarin (Sigma) at 50^{o}C for 30 min. Then, the reducing sugar concentration was examined by the method described by (Miller, 1960). One unit (U) of β-1,3 glucanase activity produced was defined as the total of enzyme that produced 1μmol of reducing sugar in 30 minutes under the above conditions (Cao *et al*., 2009). The soluble protein concentration was determined according to the method of Bradford protein assay using bovine serum albumin as the protein standard. Next, the absorbance of the supernatant was read at 595 nm.

2.4 Statistical data analysis

The data were statistically analyzed using software SAS (ver.9.3). The T tests of least significant differences (LSD) were used to compare the differences of means from triplicate experiments among treatment groups. Differences of ($P<0.05$) were considered significant.

III. RESULTS AND DISCUSSION

3.1 Screening of substrates for β-1,3 glucanase production

Different types of substrates were primarily screened for their abilities to produce β-1,3 glucanase enzyme which were fungal cell wall, seaweed, glucose and rice husk (Fig. 1). As shown in Fig.1, fungal cell wall was the best substrate and showed significantly ($p< 0.05$) higher β-1,3 glucanase compared to the other substrates. This was then followed by seaweed, glucose and rice husk. Maximum β-1,3 glucanase production was achieved on culture medium containing fungal cell wall as substrate which was 48.72 UmL^{-1}. From the screening of the substrates, medium containing fungal cell wall as substrate showed maximum β-1,3 glucanase production. Chernin and Chet, (2002) has suggested that most fungal cell walls contain chitin as the major structural backbone organized in regularly ordered layers and filled with β-1,3 glucan arranged in an amorphous manner. Thus, β-1,3 glucanase enzyme will hydrolyzes glucan molecules leading to the production of D-glucose, which serves as a carbon source (Tang-Yao, 2002). But in this study, fungal cell wall preparation was time consuming and laborious because it needed to undergo the fermentation process. Therefore, seaweed was chosen as the best substrates for the production of β-1,3 glucanase as it is can be used readily and is relatively cheap. On the other hand, laminarin is one of the main structural components of *Undaria pinnatifida* seaweed (Tang *et al*., 2009). It will be also hydrolyzed by β-1,3 glucanase leading to the production of glucose. Thus, these observations have shown that seaweed also has a great potential as the substrate for β-1,3 glucanase production.

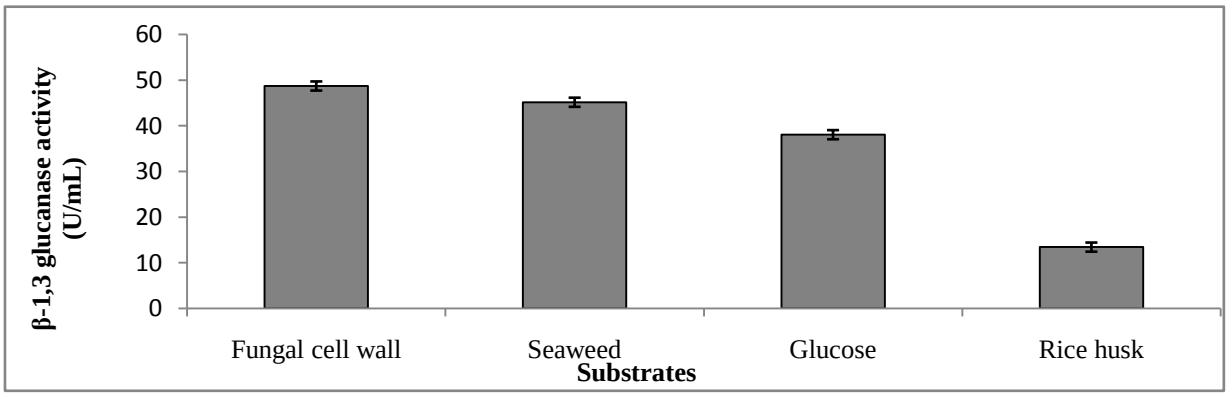

FIG. 1: Effects of different types of substrates on β-1,3 glucanase enzyme activity. Values with the same letter are not significantly different ($P<0.05$). Error bars represent standard deviation from three replicates.

3.2 Production profile of β-1,3 glucanase

The production of extracellular β-1,3 glucanase enzyme was observed during the growth of *P. oxalicum* T3.3 on basal medium containing seaweed as substrate for 7 days (Fig. 2). The highest production of β-1,3 glucanase was obtained at five days of fermentation with activity of 61.13 U mL^{-1}. After the fifth day, there was no further increase in the production of this enzyme. Protein concentration also showed the highest value on the fifth day and decreased thereafter which reflect the presence of enzyme. Fig. 2 also shows the N-acetylglucosamine content of the fungal biomass during 7-days of fermentation by *P. oxalicum* T3.3. The highest glucosamine production was detected on day 3 of fermentation with 2.78 mg g^{-1}. After day 3 of fermentation, glucosamine production continued to decrease. β-1,3 glucanase production was recorded to be maximal after five days of growth. El Katatny *et al*., (2000) also have reported that the production of β-1,3 glucanase enzyme by *Trichoderma harzianum* using laminarin as carbon source was maximum at the fourth day. In another study, *P. oxalicum* showed highest β-1,3 glucanase production on day five (Doughari, 2011). Meanwhile, the results of fungal cell growth was found to be maximal on day three and decreased thereafter as it is may due to the autolysis of the *P. oxalicum*. The results of the study are in a good agreement with Copa-Patino *et al*., (1990) who reported the production of high β-1,3 glucanase activity during autolysis of *P. oxalicum* which increases with incubation time. This activity forms part of a lytic complex

produced by this fungus. Hence, it could be inferred that the production of β-1,3 glucanase is a non-growth associated product.

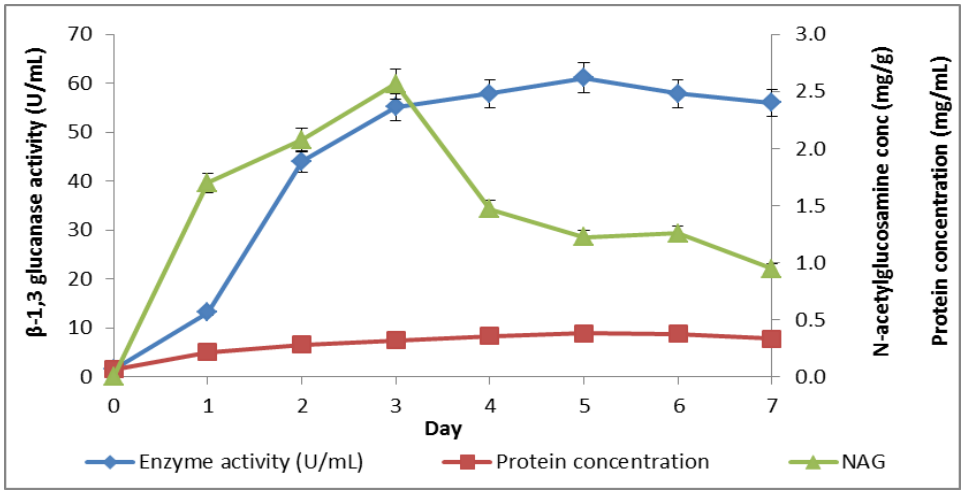

FIG. 2 Production profile of β-1,3 glucanase by *P. oxalicum* T3.3 using seaweed as substrate. Error bars represent standard deviations from three replicates.

3.3 Effects of concentration of substrates

To study the effects of concentrations of substrates, concentrations of seaweed were varied from 0.5% to 3.0%. It was observed that *P. oxalicum* T3.3 produced the highest β-1,3 glucanase in medium supplied with 1% concentration of seaweed (Fig. 3). When the concentration of the seaweed increased above 1%, the production of β-1,3 glucanase decreased. The best β-1,3 glucanase production (70.92 U mL^{-1}) was reached at 1% (w/v) seaweed concentration in the medium. It was found that when the concentration of the seaweed increased above 1%, the production of β-1,3 glucanase decreased as high concentration of substrate might inhibit the production of this enzyme. β-1,3-glucanase production was reported to be significantly affected by substrate concentration which showed that enzyme production increased up to 0.75% of laminarin concentration but decreased at higher concentrations (El Katatny *et al*., 2000). The activity of the enzyme may increase in limited carbon conditions and immediately results in autolysis, as reported in *Botrytis* spp., (Stahmann *et al*., 1992), *P. oxalicum* (Copa-Patino *et al*., 1989) and *Aspergillus nidulans* (Nuero *et al*., 1993). It also seems probable that in conditions of carbon starvation and poor growth this fungus actively secretes some level of hydrolytic enzymes.

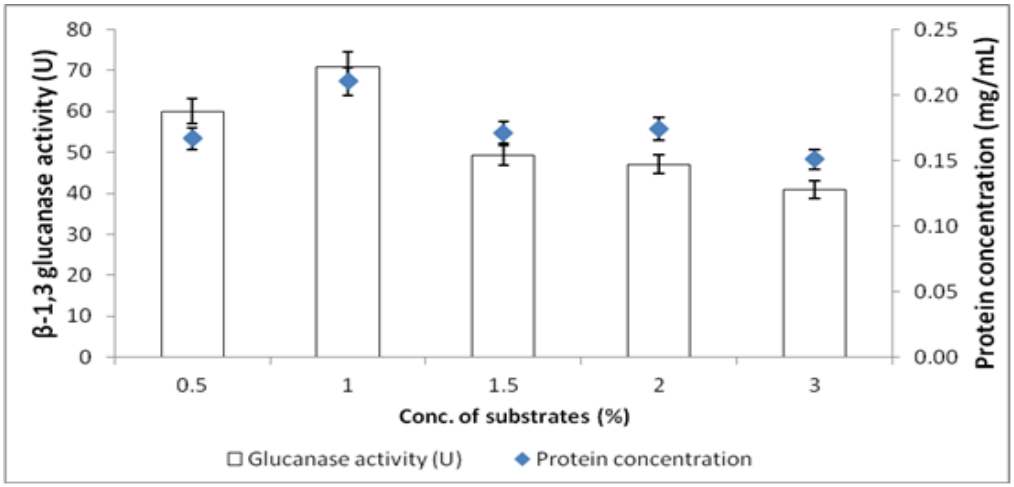

FIG. 3 Effects of concentrations of substrate on production of β-1,3 glucanase. Values with the same letter are not significantly different (P<0.05). Error bars represent standard deviations from three replicates.

3.4 Effects of nitrogen sources

The effect of different nitrogen sources on the production of β-1,3 glucanase enzyme was tested with the medium supplemented with various combinations of peptone, yeast extract and $(NH_4)_2SO_4$. This study was conducted to reduce the number of nitrogen sources used in comparison to the nitrogen sources contained in the basal medium. The combination

consisted of peptone, yeast extract and ammonium sulfate in the ratio of 3:2:2 while control medium contained nitrogen sources in the basal medium before modifications which was 3:0.5:2 respectively. Out of the several nitrogen sources tested, β-1,3 glucanase production was significantly ($p< 0.05$) higher in the presence of a combination of peptone and yeast extract as the nitrogen source (75.55 UmL^{-1}) as compared to the control. Meanwhile in the presence of peptone in the fermentation medium, it contributed to high β-1,3 glucanase production in all samples thus highlighting the importance of peptone in the production of β-1,3 glucanase . On the other hand, the use of ammonium sulfate as the sole nitrogen sources inhibited the production of β-1,3 glucanase. It has been shown that the production of β-1,3 glucanases was also significantly influenced by the nitrogen sources incorporated into the medium (Fig. 4). Combination of peptone and yeast extract was chosen as the most favourable substrate for the production of β-1,3 glucanase because it could reduce the number of nitrogen sources used in the basal medium. Furthermore, this will help to reduce the production cost. From this study, enzyme production was high in medium supplemented with peptone as nitrogen source which was in agreement with the result which reported that peptone-casein was the best nitrogen source for β-1,3 glucanase production, followed by corn steep solid and then NH_4NO_3 (El Katatny, 2000). Moreover, organic nitrogen like yeast extract and peptone significantly increased enzyme production in a novel isolate of *Alcaligenes xylosoxydans* (Vaidya *et al.*, 2001). This result is due to peptone and yeast extract contains abundant of vitamins, minerals, and amino acids, which are necessary for cell growth and enzyme production. On the other hand, the use of ammonium sulfate inhibited β-1,3 glucanase production due to ammonia reducing the culture pH excessively during NH4+ adsorption (Bazilah, 2011). The reduction in culture medium pH was not suitable for fungal growth thus inhibiting the β-1,3 glucanase production. Inhibitory effects of ammonium salts on inulinase production also had been reported by Singh and Gill (2006).

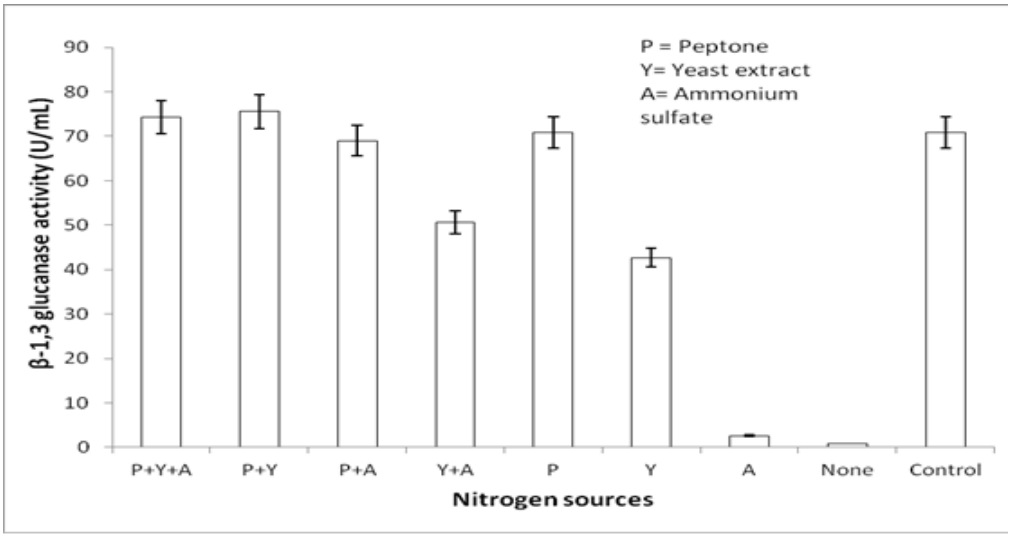

FIG. 4 Effects of nitrogen sources on production of β-1,3 glucanase. Values with the same letter are not significantly different (P<0.05). Error bars represent standard deviations from three replicates.

3.5 Effect of initial pH medium

Based on Fig.5, the medium with initial pH set range between 4.5 to 5.5 showed high β-1,3 glucanase production with the highest β-1,3 glucanase production measured when the initial pH of the medium was set to pH 5.0 (79.04 UmL^{-1}). Meanwhile, the maximum protein concentration was achieved at pH 5.5. Lower β-1,3 glucanase production was detected when the initial medium pH was set to pH 4.0 and 6.0. At pH 4.0 and 6.0, cell concentration was significantly ($p< 0.05$) reduced. Hence, it was concluded that the production of β-1,3 glucanase was favourable at initial pH range between pH 4.5 to 5.5. Culture pH is one of the factors to affect enzyme production in other fungi. From this study, β-1,3 glucanase production was favored by acidic pH range between pH 4.5 and 5.5. However, initial medium pH below 4.5 and above 6 may inhibited the production of β-1,3 glucanase. This is due to the majority of fungi not being able to grow in strongly acidic or strongly alkaline conditions accounting for the loss of enzyme activity. It was also reported that acidic pH is one of the essential growth factors in the production of β-1,3 glucanases and chitinases in thermophilic *Streptomyces* and in the mycoparasite *T. harzianum* respectively (Tweeddel *et al.*, 1993). Initial medium pH affected production of β-1,3 glucanase in *T. harzianum* with optimum β-1,3 glucanse production on laminarin being highest when the initial pH was set to pH 5.5 (El Katatny *et al.*, 2000).

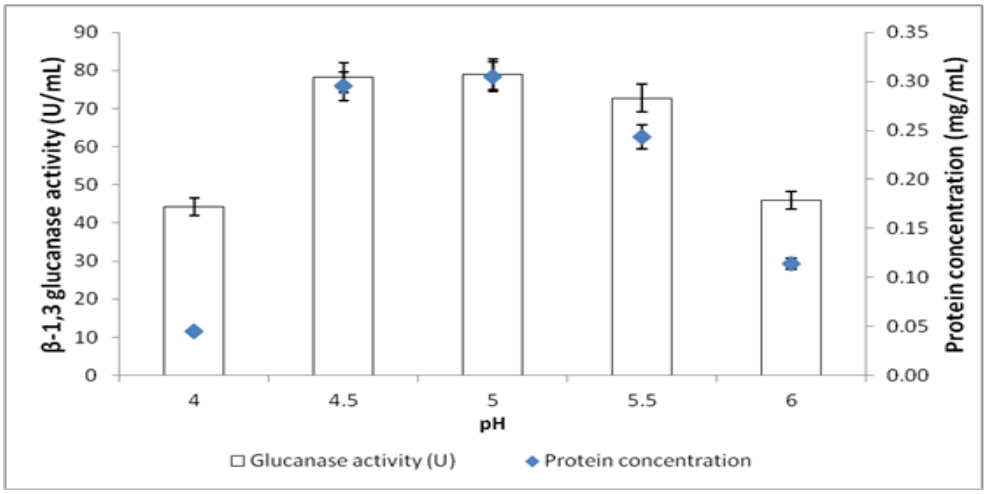

FIG. 5 Effects of initial medium pH value on production of β-1,3 glucanase. Values with the same letter are not significantly different (P<0.05). Error bars represent standard deviations from three replicates.

3.6 Effect of agitation speed

β-1,3-glucanase production was also significantly influenced by the agitation speed and the highest β-1,3-glucanase production was obtained at 200 rpm (Fig. 6). A significant ($p< 0.05$) increase in β-1,3 glucanase activity (80.03 UmL^{-1}) was observed at agitation speed of 200 rpm compared to stationary condition which was 0 rpm (0.965 UmL^{-1}). The protein concentration also showed maximum value at 200 rpm. At 0 rpm, almost no β-1,3 glucanase was produced and the protein concentration was also at its lowest. It was concluded that agitation exerted a strong influence on production of β-1,3 glucanase. The effect of the agitation speed on the extracellular protein concentration (Fig. 6) was also the same as on the enzyme activity. In this study, seaweed substrate did not dissolve in the production medium, so there was interaction between substrate and fungus in the medium. However, it was observed that at 0 rpm, this fungus only grew at the surface of the medium and had no interaction with the substrate. Thus, at 0 rpm almost no β-1,3 glucanase was produced and the protein concentration was also shown to the lowest because the uptake of the substrate was very low due to poor mixing and agitation. High agitation rate is important in fungal fermentation to provide adequate mixing and mass transfer (Abd-Aziz *et al*., 2008). On the other hand, at 120 rpm β-1,3 glucanase activity was much more lower compared to at 200 rpm. This resulted as a consequence of inadequate mixing and/or resistance in mass transfer at lower agitation speed (Arthur and Panda, 2000). It was reported by Lejeune and Barone (1994) that the optimal agitation rate for enzyme production of *T. reesei* was 200 rpm, whilst 300 rpm was recorded as showing the fastest growth. β-1,3 glucanase is the most affected and sensitive to agitation speed, followed by CMCase, and xylanase being the least affected.

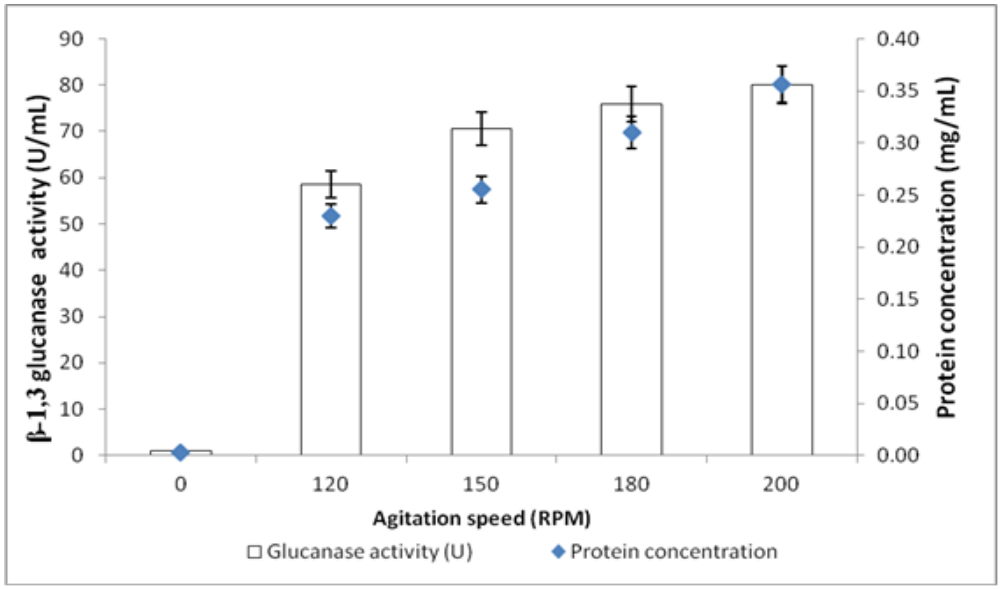

FIG. 6 Effects of agitation speeds on production of β-1,3 glucanase. Values with the same letter are not significantly different (P<0.05). Error bars represent standard deviations from three replicates.

3.7 Effect of surfactants

Two different groups of surfactants; anionic such as sodium dodecyl sulphate and non-ionic such as Triton X-100, Tween20 and Tween80 were tested for β-1,3 glucanase production. From this study it has showed that sodium dodecyl sulfate, Tween 80, Tween 20 were found to increase β-1,3 glucanase production with sodium dodecyl sulfate showing the best result (84.73 UmL^{-1}) (Fig. 7). β-1,3 glucanase production was significantly ($p< 0.05$) higher in medium supplemented with sodium dodecyl sulfate as compared to control which did not contain any surfactant. Triton X-100 was found to decrease β-1,3 glucanase production. These results showed that surfactants also gave significant effects on the production of β-1,3 glucanase. β-1,3 glucanase production was enhanced significantly when surfactants were added into the production medium. In general, surfactants may increase the cell membrane permeability through decreasing their phospholipid contents and solubilization of membrane bound proteins. Among the different surfactants, sodium dodecyl sulfate showed best results. This result is in agreement with the study by Singh and Bhermi (2008), which reported that sodium dodecyl sulfate (SDS) has an enhancing effect on enzyme production However, Triton X-100 showed decrease in β-1,3 glucanase production. This finding is in agreement with the results obtained by Pardo, (1996) on cellulase production by *Nectria catalinensis*. It was found that, triton X-100 had an enhancing effect on cellobiose activity, but is inhibited endoglucanase and exoglucanase production. Tween 80 could help in increasing cell membrane permeability leading to a more efficient uptake of nutrients, without any significant change in oxygen supply. Surfactants also had been found to facilitate the entry of compounds into cells. It was also observed that surfactants changed the cell membrane structure to assist enzyme release (Liu *et al*., 2006). Through this optimization process, it were shown that under the optimized conditions, β-1,3 glucanase activity increased 38.6%.

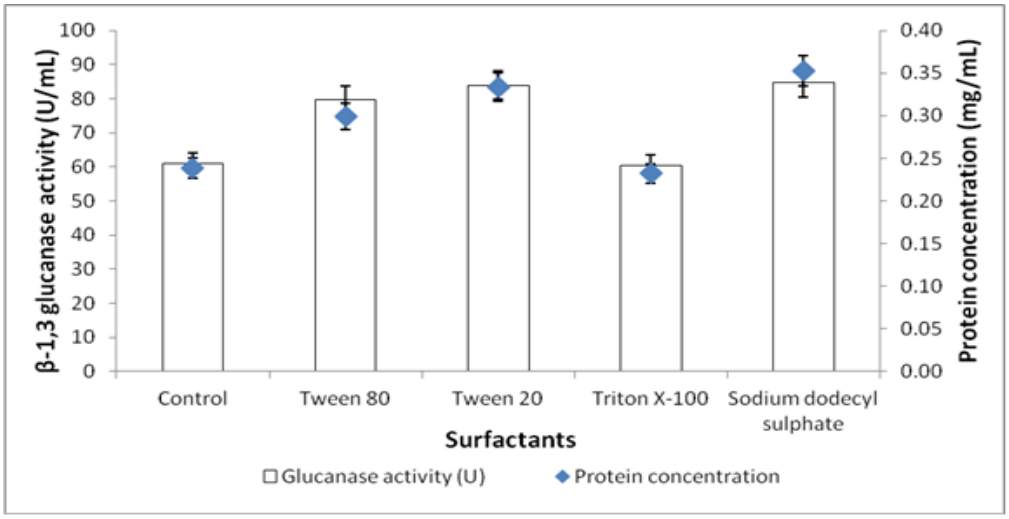

FIG. 7 Effects of surfactants on production of β-1,3 glucanase. Values with the same letter are not significantly different (P<0.05). Error bars represent standard deviations from three replicates.

IV. CONCLUSION

The optimization of β-1,3 glucanase production by *P.oxalicum* required adjustment of different types of carbon and nitrogen sources, initial pH medium, agitation speed and surfactants. This enzyme characterization has revealed its great potential towards detergent, beer and food fermentation industries whose manufacturing conditions are largely acidic.

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