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Economic production of thermo-active endo β-mannanase for the removal of food stain and production of antioxidant manno-oligosaccharides

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ABSTRACT

β-Mannanases are β-1,4-mannan-glycosidic bonds hydrolyzing enzymes that participate in various biotechnological applications. In the current study, the production of the enzyme was performed by solid state fermentation of rice straw using the locally isolated fungus *Trichoderma longibrachiatum* RS1 and the production of the enzyme was optimized to reach 89.73U/g dry substrate. The isolated fungus was identified on the base of its cultural and morphological features and by 18S rDNA sequencing.The optimum temperature for the activity of the partially purified enzyme was indicated to be 75 °C. Although production of fungal β-mannanases have been previously studied but the production of thermo-active enzymes are still challengeable. The V_{max} and K_m were 6.2U/mg protein/min and 3.33 mg/mL respectively, indicating the comparatively high affinity of the produced enzyme toward mannan substrates. The thermal stability of the produced enzyme estimated that its half lives were 633.01, 50.77 and 20.25 min^{-1} at 55, 60 and 65 °C respectively. The produced enzyme can be efficiently used in the removal of mannan based food stain. Moreover, the efficiency of the produced enzyme in the production of manno-oligosaccharides by the hydrolysis of mannan polymers was examined. The results indicated the release of 1.8 and 0.66 mg reducing sugar/mL by the hydrolysis of locust bean and guar gum for 2 h with hydrolysis percentage of 27 and 9.9% respectively. Finally, the produced manno-oligosaccharides were examined for their antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl free radical.

1. Introduction

Lignocellulose, the back bone of plant cell walls, is a matrix of ~35–50% cellulose, ~20–35% hemicelluloses and ~5–30% lignin of the plant dry weight (Zabed et al., 2016). It is considered to be the most abundant but under-utilized biomass all over the world (Kogo et al., 2017). Hemicelluloses are heteropolymers that composed mainly of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and sugar acids (Chauhan et al., 2012). They can serve as a producer of fermentable sugars used for the generation of biofuels in bio-refineries or as a producer of oligomeric and polymeric precursors used for the manufacturing of numerous high value products as food additives, prebiotics, gels, films and coatings (Bååth et al., 2018; Kaur et al., 2018; Zheng et al., 2018).

Mannan, the second major fraction of hemicelluloses after xylan present in soft and hard wood, is a heteropolysaccharide composed mainly of mannose along with some units of glucose in the main chain and some units of galactose in the side chains. Several enzymes including endo β-mannanase, β-mannosidase, β-glucosidase and α-galactosidase can be utilized in the hydrolysis of mannan (Soni and Kango, 2013). Endo β-mannanases are β-1,4-manno-glycosidic bonds hydrolyzing enzymes that randomly attack the mannan chains releasing manno-oligosaccharides (MOS) with various degree of polymerization (Chauhan et al., 2012). MOS cannot be fermented in the digestive system and recently have been found to have a great potential in the reduction of human body insulin, postprandial blood glucose and serum lipids as well as their ability in the growth inhibition of pathogens by their primary mode of action, so they can be used as additives in food and animal feed in order to health improvement (Zheng et al., 2018; Nguyen et al., 2019). Additionally, β-mannanase is useful for various industrial applications as in detergent (Singh et al., 2019; David et al., 2018), fruit juices clarification (Zhao et al., 2019), bio-fuel production (Kaur et al., 2018; Shukor et al., 2016) and oil drilling operations (You et al., 2016).

Mannanase is ubiquitous in nature but few microorganisms (mainly fungi) can be used to produce endo β-mannanase by solid state

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fermentation (SSF) (Srivastava and Kapoor, 2017). Currently, studies have been focused on devising cost effective medium for the fungal production of the enzyme by utilizing inexpensive agricultural wastes such as palm kernel cake (Soni et al., 2015, 2017; Ahirwar et al., 2016a; Li et al., 2018), copra meal (Soni et al., 2016; Ahirwar et al., 2016b; Jana et al., 2018), apple pomace (Yin et al., 2013), rice husk (Ibrahim et al., 2012) and different lignocellulosic residues (Rastogi et al., 2016; Soni et al., 2017). Rice straw is one of the most abundant lignocellulosic residues that produced and collected directly in the fields (Kamel et al., 2018). According to the Food and Agriculture Organization of the United Nations points system, the annual global production of rice straw is about 600–900million tons (Xue et al., 2017). The low bulk density, high mineral and silica contents are properties that faced its application (Jain et al., 2015). As a method to face its accumulation, burning in open fields is the most used method for its disposal all over the world. Unfortunately, using of this method leads to air pollution that consequently affects the public health (Xue et al., 2017).

The conditions of the fermentation process have a crucial impact on the microorganisms growth and their released metabolic products (Shivalee et al., 2018). Recently, statistical optimization has been widely used in enzyme production processes in order to save time and effort, decrease the number of the required experiments and consequently decrease the production process cost (Hashem et al., 2018, Ismail et al., 2019 and Ismail, 2019). Response surface methodology (RSM) was originally described by Box and Wilson (1951) and has been successfully applied in the optimization of β-mannanases microbial production (Soni et al., 2015, 2016, 2017, Ahirwar et al., 2016a&b, Jana et al., 2018 and Zhao et al., 2019).

The presence of free radicals increased the pathogenicity of several diseases as diabetes, cancer and cardiovascular disorders. Antioxidant compounds can overcome the deleterious effects of free radicals in the biological systems (Aliakbarlu et al., 2014) reflecting the importance of the production of natural antioxidant compounds.

The current study focused on the utilization of rice straw as a carbon source for the production of β-mannanase by SSF using *Trichoderma longibrachiatum* RS1 in addition to the optimization of the enzyme production. The partial purified enzyme activity was examined in respect to the effect of pH, temperature and substrate concentration and its kinetic constants were determined. Thermal denaturation of the enzyme was investigated and its half life values were determined. Wash performance test was performed to examine the efficiency of the produced enzyme in the removal of mannan based food stains. Moreover, the efficiency of the partially purified enzyme in the production of MOS by the hydrolysis of different mannan polymers was examined. Finally, the produced MOS was examined for their antioxidant activity.

2. Materials and methods

2.1. Materials

Locust bean gum (LBG) and guar gum (GG) were purchased from Sigma-Aldrich, Saint Louis, USA. Mannose and di-, tri-, tetra-oligosaccharide standards were purchased from Megazyme, Ireland. Dinitrosalicylic acid (DNS) was obtained from Panreac, Barcelona, Spain. Silica gel 60 thin-layer chromatography (TLC) plates, potato dextrose agar (PDA) were purchased from Merck, Darmstadt, Germany. All other chemicals were of analytical or HPLC grade. Rice straw was collected from Al Sharqiya, Egypt. After drying with air, rice straw was cut into pieces and ground under standard grinding condition. The ground particles moisture content was determined to be zero% and used directly without any further processing.

2.2. Microorganism and culture conditions

The fungus strain used in the current study was isolated previously during intensive course of screening program concerned with the soil isolation of microorganisms secreting non-starch hydrolyzing enzymes using various local agricultural residues as a carbon source (unpublished data).The identification of the isolate was performed according to its cultural and morphological features followed by 18S rDNA sequencing carried out in Sigma Scientific Services Co.

For the production of the enzyme, solid state fermentation method was carried out in which 5 g of rice straw in 250 mL Erlenmeyer conical flask was moistened with 10 mL of tap water. The solid substrate medium was inoculated with 2 mL of the pre-culture (prepared by scratching of the cultured slant with 10 mL distilled water containing 0.1% tween 80) then incubated for 7 day at 30 °C. After incubation, the fermented substrate was extracted with 50 mL of distilled water on an orbital shaker (150 rpm) for 1 h then centrifuged at 5000 rpm (4 °C) for 10min. The produced supernatant was used for further analysis.

2.3. Enzyme activity and protein content assays

β-Mannanase activity was assayed according to Singh et al. (2019) using LBG as a substrate in a reaction mixture consisted of 500 μL of 1% substrate in 0.05 M acetate buffer (pH 5.5) and 500 μL of the culture supernatant, then incubated at 50 °C for 30min. The released reducing sugars were measured immediately by using DNS method (Miller, 1959). One unit of the enzyme was defined as the amount of enzyme that released 1 μmol of mannose per minute under the assay conditions.The protein content was determined as described by Lowry et al. (1951) using bovine serum albumin as a standard.

2.4. Optimization of cultural conditions for β-mannanase production

2.4.1. One-variable-at-a-time

Classical approaches including removal, supplementation and replacement experiments were performed. Initially, 10 mL of Mandel's medium (composed of (g/L) $(NH_4)_2SO_4$; 1.4, KH_2PO_4; 2, urea; 0.63, $CaCl_2$; 0.3, $MgSO_4{\cdot}7H_2O$; 0.3 and peptone; 0.75) (Mandels et al., 1974) of pH 5 was used as a moistening agent instead of tap water. After that the constituent mixture of nitrogen sources in the media (peptone, $(NH_4)_2SO4$ and urea) was replaced separately by equivalent value of each nitrogen source then the most suitable concentration of the selected nitrogen source was examined in the range of 0.1–4%.

2.4.2. Statistical optimization

In this study, two step optimization approach was carried out to enhance enzyme production.

2.4.2.1. Selecting of the variables that influence enzyme production. In order to identify the variables that have the highest influence on the enzyme production, Plackett-Burman design (Plackett and Burman, 1946) including seven variables was applied. The seven independent variables were substrate concentration (% w/volume of the flask), moisture level (biomass to moistening agent ratio), pH of the moistening agent, time of autoclaving (min), age of the fungus (days), temperature (°C) and fermentation period (days), each variable has been represented in terms of high (+1) and low (−1) levels as shown in Table (1). Each generated response was calculated according to the first order linear equation:

$$Y = B_0 + \Sigma B_i X_i \quad (1)$$

Where Y is the response (β-mannanase production), B_0 is the model intercept and B_i is the linear coefficient and X_i is the level of the independent variable.

The main effect of each variable was determined by the following equation:

$$E_{(Xi)} = 2(\Sigma M_{i+} - M_{i-})/N \quad (2)$$

Where $E_{(Xi)}$ is the effect of the tested variable. M_{i+} and M_{i-} represent

Table 1
The levels of the examined variables and the observed results of β-mannanase production by applying PlackettBurman design.

Run number	Substrate concentration (% w/v)	Moisture level (biomass to moistening agent ratio)	pH of the moistening agent	Time of autoclaving (min)	Age of the fungus (days)	Temperature (°C)	Fermentation period (days)	β-Mannanase (U/g ds)
1	-(2)	-(1:2)	-(5)	+(30)	+(9)	+(30)	-(7)	45.217
2	+(3)	-(1:2)	-(5)	-(15)	-(7)	+(30)	+(10)	59.856
3	-(2)	+(1:3)	-(5)	-(15)	+(9)	-(25)	+(10)	16.331
4	+(3)	+(1:3)	-(5)	+(30)	-(7)	-(25)	-(7)	10.114
5	-(2)	-(1:2)	+(7)	+(30)	-(7)	-(25)	+(10)	44.376
6	+(3)	-(1:2)	+(7)	-(15)	+(9)	-(25)	-(7)	37.201
7	-(2)	+(1:3)	+(7)	-(15)	-(7)	+(30)	-(7)	53.630
8	+(3)	+(1:3)	+(7)	+(30)	+(9)	+(30)	+(10)	71.277

β-mannanase production from the experimental runs where the independent variable (Xi) measured was present at high and low values respectively and N is the number of runs.

2.4.2.2. Box-Behnken design. In the second step of optimization, Box-Behnken design (Box and Behnken, 1960) was applied to determine the optimum level of the selected variables. In this model (Table 3), an experimental design of 13 run and one central point was constructed with three independent variables. Each variable was examined at three different levels, low (−), high (+) and control or basal (0). A second order polynomial equation was used for the interpretation of the correlation between the variables and the response (β-mannanase production). The equation is presented in the following form:

$$Y = B0 + \sum_{i=1}^{i=3} BiXi + \sum_{i=1}^{i=3} BiiXi2 + \sum_{i=1}^{i=3} \sum_{\substack{j=1 \\ j \neq i}}^{j=3} BijXiXj \quad (3)$$

where Y is the predicted β-mannanase production; β_0 is the model intercept, $ß_i$ is linear coefficient, $ß_{ii}$ is quadratic coefficient and $ß_{ij}$is cross product coefficient where X_i and X_j are the coded levels of the independent variables.

2.5. Partial purification of the enzyme

The crude enzyme (culture free cells) was subjected to partial purification by fractional precipitation using ethanol and acetone as well as salting out using ammonium sulfate at 30–90% concentration with 10% intervals. Each fraction was assayed for enzyme activity and protein content (Ismail, 2016).

Table 2
Plackett- Burman design multiple regression analysis.

Variables	Mannanase analysis			
	Coefficient	*t*-statistics	*P*-value	Confidence level (%)
Intercept	−232.019			
Substrate weight in flask (% w/v)	13.54831	4.662546	0.00026	99.974
Moisture level (biomass to moistening agent ratio)	−1.41197	−4.29496	0.000556	99.944
pH of the moistening agent	9.370934	9.121492	9.72E-08	100
Time of autoclaving (min)	0.183785	1.315643	0.206835	79.317
Age of the fungus (days)	0.256351	0.249527	0.806128	19.387
Temperature (°C)	6.097959	14.8391	8.98E-11	100
Fermentation period (days)	3.806503	5.557768	4.33E-05	99.996
Summary of the model				
Multiple R	0.978394			
R^2	0.957255			
Adjusted R^2	0.938555			
Standard Error	5.03295			

2.6. pH and temperature effect

The effect of pH on the partially purified enzyme was evaluated in which the activity of the enzyme was determined at different pH range from 3.5 to 5.5 using 0.05 M acetate buffer and from 5.7 to 6 using 0.05 M phosphate buffer. The optimum pH was that at which the enzyme possessed the highest activity. In order to determine the stability of the enzyme, the residual activity of the enzyme was determined every 30 min at the optimum pH up to 2 h after its pre-incubation without substrate at that pH. The activity of the enzyme without pre-incubation was considered as 100% activity.

The temperature effect on the partially purified enzyme activity was determined at various temperatures (50-80°C) at the optimum pH. The activation energy (E_a) was calculated from Arrhenius plot (ln relative activity % versus reciprocal of temperature in Kelvin) according to the following equation:

$$\text{Slope} = -E_a / R \quad (4)$$

Thermal stability of the partially purified enzyme was studied by estimating its residual activity every 30min up to 2 h at the optimum conditions after pre-incubation of the enzyme at temperatures range from 55 to 65°C without substrate. The activity without pre-incubation was considered as 100% activity. Then its thermal inactivation kinetics was calculated as follow:

$$\text{Slope of Arrhenius plot (ln } K_d \text{ versus } 1/T) = -E_{a(d)}/R \quad (5)$$

$$T_{1/2} = \ln(2)/K_d \quad (6)$$

in which K_d is the thermal deactivation rate constant, T is the temperature (K), $E_{a(d)}$ is the decay activation energy ($KJmol^{-1}$) and R is the gas constant (8.3145 J/mol/K).

2.7. Effect of substrate (locust bean gum) concentration

The partially purified enzyme activity at the optimum conditions was estimated using different substrate concentrations (1–25 mg/mL). Lineweaver-Burk plot (Lineweaver and Burk, 1934) was used to calculate the kinetic constants using the following equation:

$$1/V = (1/V_{max}) + (K_m/V_{max})\ (1/S) \quad (7)$$

in which V is the specific activity of the partially purified enzyme (U/mg protein), K_m is Michaelis-Menten constant, V_{max} is the maximal activity and S is the substrate concentration (mg/mL).

2.8. Wash performance

2.8.1. Pure mannan based stain

The efficiency of the partially purified enzyme as a detergent additive in the removal of mannan based stains from cotton fabrics was

Table 3
Box-Behnken Design results.

Run number	Independent variable			Observed β-mannanase(U/g ds)	Predicted β-mannanase (U/g ds)	Residual
	X_1 pH of the moistening agent	X_2 Temperature (°C)	X_3 Fermentation period (days)			
1	6(−)	25(−)	10(0)	51.72508	51.31583	0.40925
2	8(+)	25(−)	10(0)	47.37895	55.94781	−8.56886
3	6(−)	35(+)	10(0)	23.38152	14.81253	8.568987
4	8(+)	35(+)	10(0)	10.76266	11.17171	−0.40905
5	6(−)	30(0)	7(−)	53.11488	63.8718	−10.7569
6	8(+)	30(0)	7(−)	58.45848	60.23663	−1.77815
7	6(−)	30(0)	13(+)	76.12516	74.34667	1.778495
8	8(+)	30(0)	13(+)	89.73024	78.97299	10.75725
9	7(0)	25(−)	7(−)	52.89927	42.54989	10.34937
10	7(0)	35(+)	7(−)	19.01524	16.82919	2.186042
11	7(0)	25(−)	13(+)	69.88862	72.07451	−2.18589
12	7(0)	35(+)	13(+)	6.166605	16.51581	−10.3492
13	7(0)	30(0)	10(0)	71.06046	71.0603	0.000164

examined as described by Singh et al. (2019) in which 1% (w/v) of LBG (in 0.05 M phosphate buffer pH 5.7) was mixed with mud in order to form an easily visible sticky stain. One mL of LBG/mud mixture was placed on each piece of cotton fabrics (3cm^2) and left to dry in oven (70°C) for 1 h in order to fix the stain. Each dried piece of cotton was dipped in 50 mL screw capped bottle, each containing 15 mL of tap water with 5 mL of heat inactivated (to denature any existent enzymes) commercial detergent (Arial, power gel, P&G Italia S.p.A., Palomba-Pomezia Roma, Italy), 5 mL of the partially purified enzyme (8U/mL at 50°C) or 2.5mL of heat inactivated detergent + 2.5mL of partially purified enzyme. The addition of 5mL of tap water was used as a negative control and 5mL of detergent (without inactivation) was used as a positive control. All the bottles were incubated in a shaking water bath at 50°C for 30min. At the end of incubation period, the cotton pieces were rinsed with tap water and dried in oven at 70°C for 1 h.

2.8.2. Food stain

In this experiment, ketchup (Heinz, C·F·I., Egypt) in addition to 1% (w/w) of LBG was used as a source of food stain in which it was placed on each piece of cotton fabrics (3 cm^2) and left to dry in oven (70°C) then 2.5mL of heat inactivated detergent + 2.5mL of the produced enzyme was tested in compare to the commercial detergent.

2.9. Hydrolysis activity

2.9.1. Hydrolysis of different mannans

The activity of the partially purified enzyme in the hydrolysis of LBG and GG was examined for different hydrolysis periods (1–24 h) in a reaction mixture of 2 mL substrate (1% w/v in 0.05 M phosphate buffer pH 5.7) with 1 mL of diluted enzyme to get the activity of 0.15U/mg substrate at 50°C. At the end of the hydrolysis period, the reaction mixture was boiled for 10min then centrifuged to remove the un-hydrolyzed portion of the substrate. The amount of the released reducing sugar was determined according to Miller (1959).

2.9.2. Analysis

The resulted hydrolyzates were analyzed by TLC using mobile phase of 7: 2: 1 (v/v) propanol: water: ammonia (Cabrera and Cutsem, 2005) and the produced sugars were visualized using a spraying reagent of diphenyl amine-aniline (Tanaka et al., 1999). The produced oligosaccharides as well as the final hydrolysis product were extracted from TLC then air dried and stored at 4°C for further use.

2.9.3. Chemistry of the produced oligosaccharides

The functional groups and chemical bonds of the dried final hydrolysis product and oligosaccharides (extracted from TLC) resulted from the hydrolysis of LBG were determined by Fourier transform infrared spectroscopy (FTIR-8300, Shimadzu, Japan).

2.10. Antioxidant activity

The antioxidant activity of the total dried hydrolysate resulted from the hydrolysis of LBG and GG as well as the dried extracted oligosaccharides (from TLC) in compare to the un-hydrolyzed substrates, were determined by estimating their scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as described by Brand-Williams et al. (1995). The reaction was performed by adding 0.1 mL of the sample solution (1% in 0.05 M phosphate buffer pH 5.7) to 3.9 mL of methanol solution of DPPH radical (1.1×10^{-4} mol/L). The absorbance decrease was measured by spectrophotometer at 515 nm after 30min in dark using Trolox as a standard. The results were expressed in μg Trolox Equivalents (TE)/mg of the dry sample.

2.11. Statistical analysis

The experiments were carried out in triplicates with three measurements per replicate and the results were reported as averages ± standard deviation.

3. Results and discussion

The results in this study indicated that rice straw is a promising carbon source that can be utilized for the cultivation of the fungal isolate in SSF for the production of β-mannanase. The low cost of rice straw, renewability and low impact in food production make it a suitable substrate for the large scale industrial production of the enzyme.

3.1. Microorganism

The cultural feature of the isolated fungus on PDA was shown in Fig. 1A indicating that the fungus colonies were typically green in color. Morphological features of the isolate were examined under light microscope (Fig. 1B) indicating that the fungus belongs to the genus*Trichoderma*.

The isolate identification was confirmed using 18S rDNA sequencing indicating the similarity of the isolate with *Trichoderma longibrachiatum* strain. The phylogenetic analysis was carried out on the base of the results of the partial sequence. The relation between the isolated strain and other species belong to the genus *Trichoderma* was estimated using MEGAX and neighbor-joining method (Kumar et al., 2018) and the phylogenetic tree was shown in Fig. (2). The data of partial sequence was submitted to gene bank under the name *Trichoderma longibrachiatum* strain RS1and received accession number of **MN025436**. *Trichoderma* sp. has been reported by Lim et al. (2012), Wang et al. (2014) and Ma et al. (2018) as β-mannanase producer.

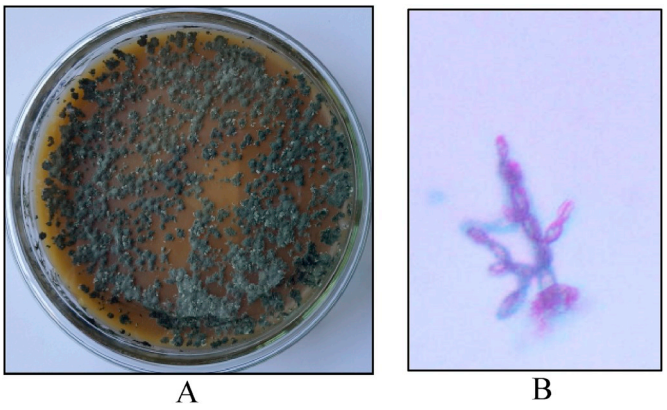

Fig. 1. Culture on PDA (A) and morphological features of the isolated fungus using light microscope (B).

3.2. Optimization of β-mannanases production

3.2.1. One-variable-at-a-time

Initially, the substitution of tap water with Mandel's medium as a moistening agent improved the productivity from 4.665U/g ds to 10.309U/g ds. Additionally, with the replacement of the constituent mixture of nitrogen source with equal value of each constituent nitrogen source separately, the highest productivity was observed by using peptone in which the productivity increased to 14.33U/g ds. By using different concentrations of peptone (0.1–4%), the productivity increased as the concentration increased to reach 35.646U/g ds by using peptone of concentration 2%. After that, the increase in concentration led to a sever decrease in the productivity (Fig. 3) that may attributed to the observed inhibition of growth.

3.2.2. Statistical optimization

3.2.2.1. Selection of the variables that influence the enzyme production. PBD was applied in which seven variables were screened for their influence on the production of the enzyme and the mean value of the

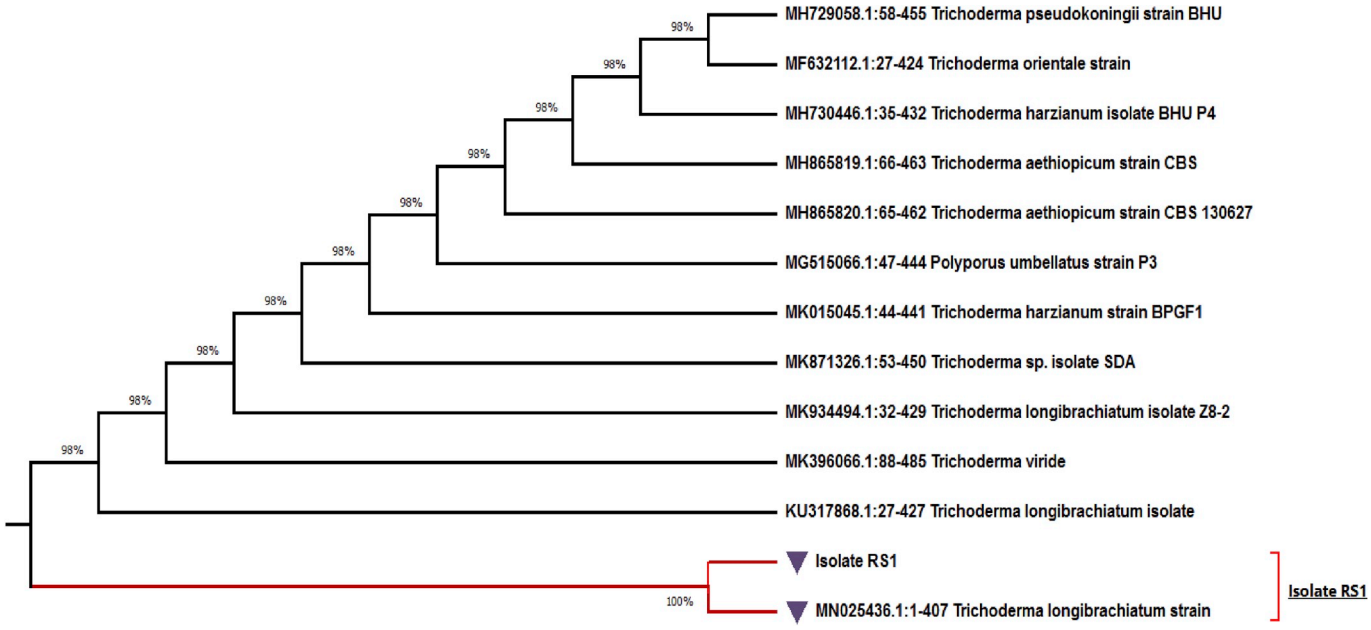

Fig. 2. Phylogenetic tree by MEGAX and neighbor-joining method.

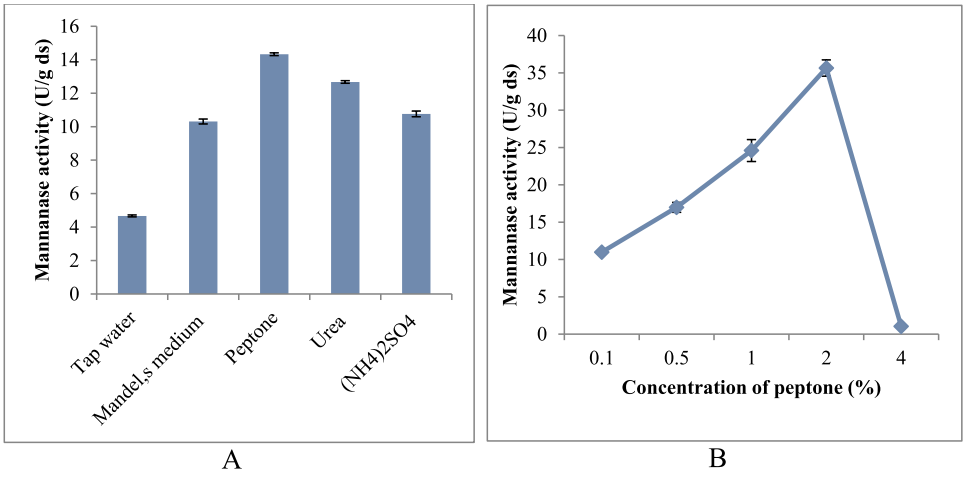

Fig. 3. The effect of different moistening agent (A) and different concentrations of peptone (B).

activity of the produced enzyme was illustrated in Table (1). The results possessed a variation in the enzyme activity ranged from 10.114 to 71.277U/g ds, reflecting the urgency of screening of the variables that influence the productivity of the enzyme. The highest activity was observed at run number 8 with 71.277U/g ds under the optimized conditions (concentration of substrate, 3%; moisture level, 1:3 biomass to moistening agent ratio; pH, 7; time of autoclaving, 30min; age of the fungus, 9days; temperature, 30 °C and fermentation period, 10days).

The examined variables main effects were calculated and represented graphically in Fig. (4). The resulted values estimated that the variables with the highest main effects were the temperature, initial pH and the fermentation period in whichall of them had positive signs. The positive sign indicated that the examined variable had more effect on the enzyme production at the high level.

The analysis of the data by multiple regression (Table 2) indicated that all of the tested variables except time of autoclaving and age of the fungus significantly affected the production of the enzyme. The effect of the examined independent variables on the production of the enzyme was estimated by the coefficient values, the variables that possessed positive effect were adjusted at positive level while the one possessed negative effect was adjusted at negative level in order to achieve in the second phase of optimization the maximum enzyme productivity.

The calculated analysis of variance (ANOVA) of the applied model indicated its overall high significance based on the very low Prob > F value that was less than 0.05 (8.98 E−10). The value of the R^2 for the applied model was 0.957 indicating the accuracy of the model as it suggested that a variation of 95.7% occur due to the independent variables. The R^2 value (>0.9) measures the degree of response exerted by the examined variables indicating the accuracy of the model (Edwards et al., 2008).The first order equation that correlated the activity of the enzyme with the selected examined variables could be presented as follows:

$$Y = -232.019 + 13.54831X_1 - 1.41197X_2 + 9.370934X_3 + 0.183785X_4 + 0.256351X_5 + 6.097959X_6 + 3.806503X_7 \quad (8)$$

where Y is the β-mannanase activity and X_1, X_2, X_3, X_4, X_5, X_6, X_7 are substrate weight in flask, moisture level, initial pH of the moistening agent, time of autoclaving, age of the fungus, temperature and fermentation period respectively.

On the base of the above results of PBD the temperature, initial pH of the moistening agent and the fermentation period, the variables with the highest confidence level, were selected for the second phase of optimization.

3.3. Box-Behnken design

The observed and the predicted mean value of β-mannanase activity of Box-Behnken design were illustrated in Table (3). The optimum β-mannanase activity (89.730U/g ds) was observed in run 8 and consequently the conducted optimum levels of the examined variables were as follow: initial pH of the moistening agent, 8; the temperature, 30 and the fermentation period, 13days.The production of β-mannanase by *Trichoderma longibrachiatum* strain RS1 in the current study was improved by changing the pH of the initial moistening agent from 5 to 8, indicating the alkaliphilic nature of the used fungus. Initial alkaline pH of the moistening agent in SSF is favorable for the fungal production of β-mannanases (Soni et al., 2015, 2016, Ahirwar et al., 2016a&b and Jana et al., 2018).

The results of the multiple regression analysis of the data were illustrated in Table (4) and the R^2 value of the applied model was 0.907 indicating that 90.7% of the variation in the observed β-mannanase activity is due to the independent variables. The second order polynomial equation, used for the prediction of the activity of produced enzyme, conducted from the multiple regression analysis was:

$$Y = -1403.91 + 43.45804X_1 + 87.94011X_2 + 10.33729X_3 - 2.69183X_1^2 - 1.40226X_2^2 + 0.109839X_3^2 - 0.41364X_1X_2 + 0.688457X_1X_3 - 0.4973X_2X_3 \quad (9)$$

Where Y is the β-mannanase activity and X_1, X_2 and X_3 are the initial pH of the moistening agent, the temperature and the fermentation period respectively.

The calculated analysis of variance (ANOVA) indicated the significance of the model terms used in the current study since the model terms had Prob > F value of 1.26 E−12 (less than 0.05). Moreover, by plotting of the observed - the predicted values (residuals) against the response (observed β-mannanase activity) (Fig. 5), indicated that the residuals were constantly and symmetrically spread throughout the range, manifesting that the model is correct on average for all the experimental results. The applied model was validated by performing an experiment under the optimized conditions. The β-mannanase production was 83.086U/g ds which is in accordance with the predicted value 78.973U/g ds, reflecting the validation of the model.

The optimum β-mannanase production of 89.73024U/g ds was achieved by applying the classical one-variable-at-a-time optimization approach followed by applying statistical designs for the SSF of rice straw. The achieved β-mannanase activity in the current study was higher than 10.45U/g ds reported by Rastogi et al. (2016) by the SSF of the same substrate using *Pyrenophora phaeocomes* S-1 and higher than 54.47U/g ds reported by Kaur et al. (2018) using *Aspergillus niger* P-19. The low cost of rice straw in addition to its renewability and its minimal impact in food production attract the research focus for its utilization in the production of various enzymes and high value compounds (Kogo et al., 2017; Zheng et al., 2017, 2019; Kaur et al., 2018; Kanti and Sudiana, 2018; Shah et al., 2019).

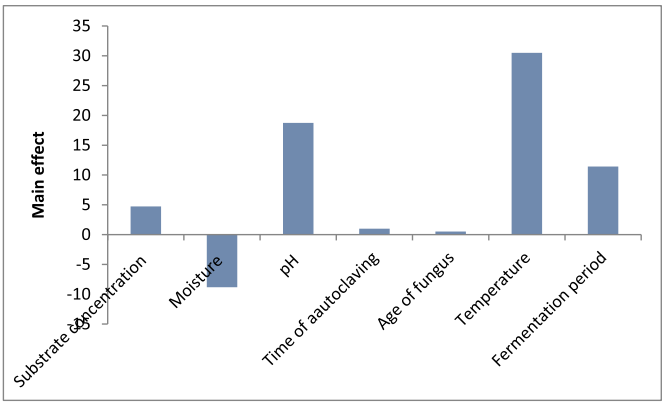

Fig. 4. The examined variables main effects on the production of β-mannanase.

Table 4
Box-Behnken Design analysis.

Term	Regression coefficient	t- test	P-value
Intercept	−1403.91	−4.70863	5.7E-05
X_1	43.45804	0.844881	0.405094
X_2	87.94011	9.555476	1.83E-10
X_3	10.33729	0.932528	0.358763
X_1^2	−2.69183	−0.78185	0.440638
X_2^2	−1.40226	−10.1824	4.37E-11
X_3^2	0.109839	0.28713	0.776054
X_1X_2	−0.41364	−0.79467	0.433262
X_1X_3	0.688457	0.793589	0.433881
X_2X_3	−0.4973	−2.8662	0.007656
Summary of the model			
Multiple R	0.952515		
R^2	0.907285		
Adjusted R^2	0.878511		
Standard Error	9.015568		

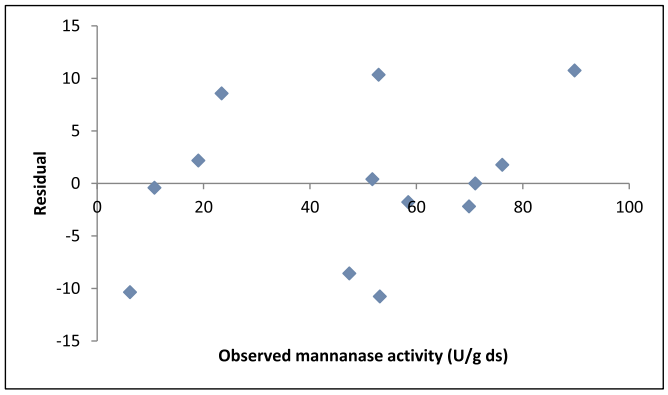

Fig. 5. Residual plot.

3.4. The produced enzyme partial purification

Ethanol and acetone fractional precipitation in addition to salting out using ammonium sulfate was performed to partially purify the produced enzyme. The fraction of 70% ethanol led to improve the specific activity to reach 1.899U/mg protein with 24.517% recovered activity yield and 2.11 fold of purification. The ethanol precipitation of β-mannanase has been reported by Soni et al. (2015) and Ahirwar et al. (2016a).

3.5. Effect of pH and temperature

The enzyme possessed optimum activity at pH 5.7 using 0.05 M phosphate buffer (Fig. 6A) at which the enzyme retained its complete activity up to 2 h. This result is consistent with other researches expressing acidic pHs as the optimum pH for the activity of fungal β-mannanases (Ahirwar et al., 2016b; Rastogi et al., 2016; Jana et al., 2018; Yang et al., 2019).

Measuring the activity of the enzyme as a function of the reaction temperature indicated that the enzyme possessed optimum activity at temperature 75 °C and by raising the temperature to 80 °C, the enzyme possessed 61.7% of its initial activity (Fig. 6B). The optimum temperature for fungal β-mannanases has been reported to be 55 °C (Rastogi et al., 2016), 60 °C (Katsimpouras et al., 2016; Jana et al., 2018) and 70 °C (Ahirwar et al., 2016b; Soni et al., 2016) while higher temperature (80 °C) has been reported by Yang et al. (2019). The high optimum temperature of the produced enzyme in the current study makes it a suitable candidate in various biotechnological applications that required high temperatures to achieve optimal results.

The thermal stability of the partially purified enzyme indicated that the enzyme retained about 95% of its activity at 55 °C after 2 h and retained more than 50% of its activity at 60 °C for 30min (Fig. 6C). Soni et al. (2016) reported that *Aspergillus terreus* FBCC 1369 β-mannanase retained 85% of its activity at 50 °C after incubation for 1 h. β-Mannanase produced by the thermophilic fungus *Thielavia arenaria* (ManXZ7) was stable at 60 °C for 20 min (Lu et al., 2013) while *Malbranchea cinnamomea* NFCCI 3724 β-mannanase retained 70% of its activity at 60 °C after incubation for 5 h (Ahirwar et al., 2016b).

Arrhenius plot is always used for studying the temperature effect on the rate of the reaction. The plotting of ln relative activity % against the reciprocal of the temperature in Kelvin, a straight line was observed (Fig. 7A) in which $-E_a/R$ is the slope value. E_a represents the activation energy and R is the gas constant. The E_a value for the partially purified enzyme was calculated to be 29.126 ± 1.276 kJ mol^{-1} for the hydrolysis of LBG between 50 and 75 °C at pH 5.7. Jana et al. (2018) reported E_a of 12.42 and 23.31 kJ mol^{-1} for *Aspergillus oryzae* MTCC 1846 β-mannanase for the hydrolysis of LBG and konjac gum respectively. Zhang et al. (2015) reported 36 kJ mol^{-1} as E_a for *Sphingobacterium* sp. GN25 β-mannanase and Regmi et al. (2017) reported E_a of 31.36 kJ mol^{-1} for *Bacillus subtilis* subsp. inaquosorum CSB31 mannanase while higher E_a of 45.18 kJ mol^{-1} was reported for *Bacillus* sp. CFR1601 mannanase (Srivastava et al., 2014).The lower the value of the E_a indicates the lower energy required for the enzyme active sites conformation for their substrate complex formation leading to a decrease in the enzyme total cost.

Not only the temperature can activate the enzyme catalytic rate but also it affects the enzyme inactivation rate. The heat inactivation rate of the partially purified enzyme was also investigated at 55, 60 and 65 °C and the half life values of the enzyme were calculated to be 633.01, 50.77 and 20.25min^{-1} respectively. Similar results was achieved for *Aspergillus terreus* FBCC 1369 β-mannanase (Soni et al., 2016) while Rastogi et al. (2016) reported that *Pyrenophora phaeocomes* S-1 β-mannanases revealed half life of 2 h at 50 °C. By plotting of ln $K_d$versus the reciprocal of the temperature in Kelvin (Fig. 7B), $E_{a(d)}$was calculated to be 318.28 kJ mol^{-1}. High $E_{a(d)}$ is an indication of the thermal adaptation of the enzyme resulted from the decrease in the unfolding rate of the enzyme (Jana et al., 2013).

3.6. Substrate concentration effect and determination of the kinetic constants

The activity of the partially purified enzyme using different concentrations of LBG was estimated and maximum activity was indicated by using substrate concentration of 20 mg/mL (Fig. 8A). The kinetic constants were determined from Lineweaver-Burk plot (Fig. 8B). K_m and V_{max} were calculated to be 3.33 mg/mL and 6.2U/mg protein/min respectively. The values of K_m and V_{max} depend mainly on the enzyme source reflecting the enzyme substrate sensitivity i.e. the higher the K_m value and the lower the V_{max} value indicate the higher sensitivity of the enzyme toward the substrate (Horn et al., 2006). The K_m and V_{max} values of the produced β-mannanase toward LBG in the current study were almost similar to the results reported by Jana et al. (2018). However, the affinity of the produced enzyme in the current study was higher than β-mannanase produced by other fungal species since the K_m value of the produced enzyme was lower than 3.71 mg/mL reported for β-mannanase produced by *Gloeophyllum trabeum* CBS900.73 (Wang et al., 2016), 4 mg/mL for *Malbrancheacinnamomea* NFCCI 3724 (Ahirwar

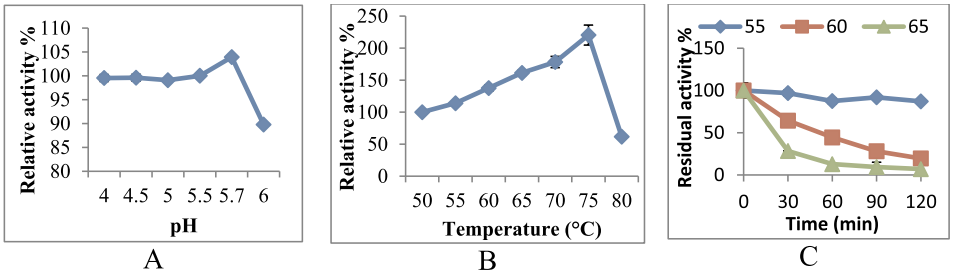

Fig. 6. The effect of pH on the activity of the enzyme (the control is pH 5.5) (A), temperature effect (B) (50°C is the control) and (C) the thermal stability of the enzyme.

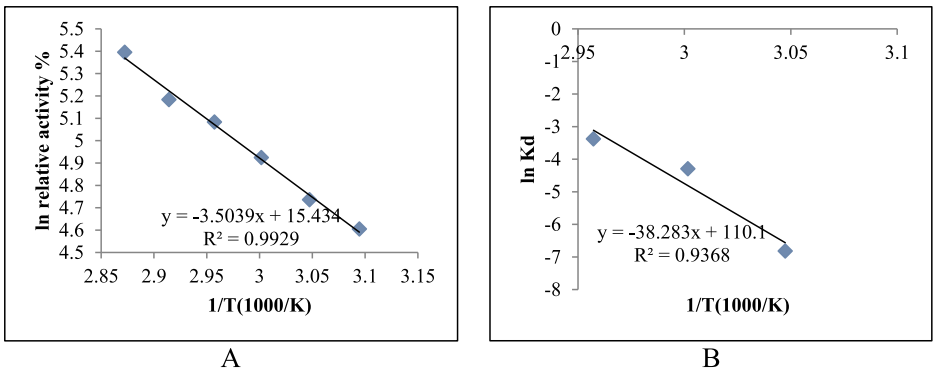

Fig. 7. Arrhenius plot for (A) thermal activation and (B) thermal denaturation of the enzyme.

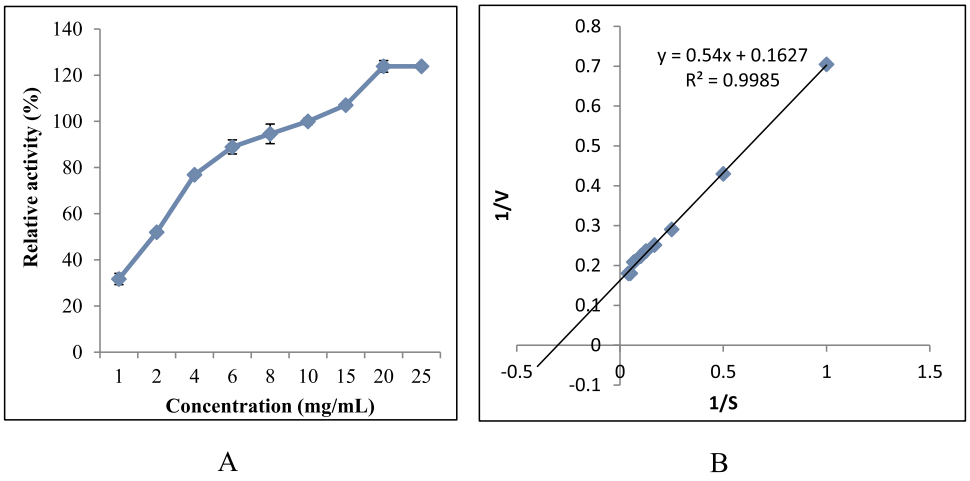

Fig. 8. The effect of different concentrations of LBG on the activity of the partially purified enzyme (A) and Lineweaver-Burk plot (B).

et al., 2016b), 5.9 mg/mL for *Aspergillus terreus* FBCC 1369 (Soni et al., 2016) and 7.6 mg/mL for *Penicillium oxalicum* GZ-2 (Liao et al., 2014).

3.7. *Washing performance of the produced partially purified enzyme with commercial detergent*

The efficiency of the partially purified enzyme in detergency was initially examined using mannan based stain since mannan is found in food such as chocolate syrup, ketchup preperations, gravy and mix fruit pulp (Singh et al., 2018). The result shown in Fig. (9) indicated that the produced enzyme when added to the heat inactivated detergent formulation can efficiently used in the removal of mannan based stains as well as ketchup stain as that of the commercial detergent. Similar results have been reported by Singh et al. (2019) and David et al. (2018) for the addition of β-mannanase in detergents.

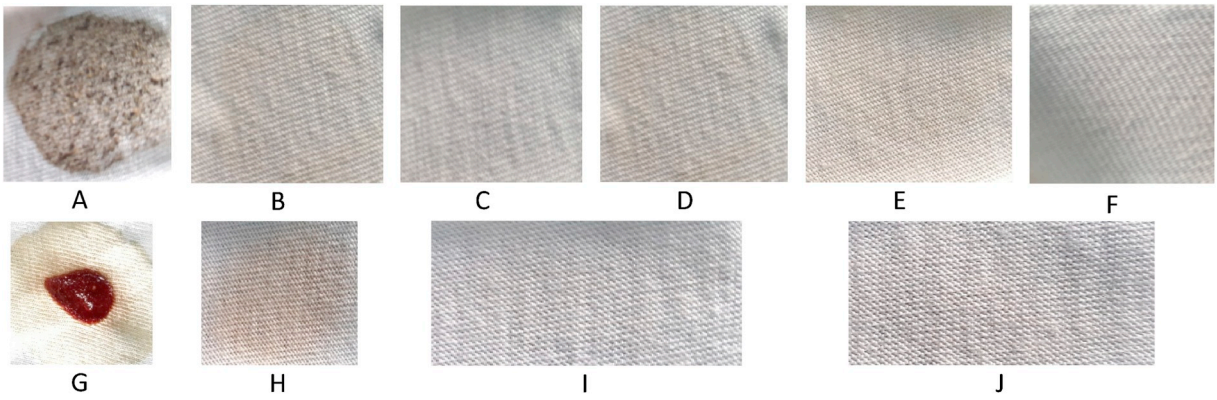

Fig. 9. Washing performance for mannan based stain in which (A) LBG/mud stain, (B) negative control (using tap water), (C) positive control (using commercial detergent), (D)heat inactivated commercial detergent, (E) the produced partially purified enzyme, (F) a mixture of the heat inactivated commercial detergent and the produced partially purified enzyme and washing performance for ketchup stain (G) in which(H)negative control (using tap water), (I) positive control (using commercial detergent), (J) a mixture of the heat inactivated commercial detergent and the produced partially purified enzyme.

3.8. Hydrolysis of different mannans

The hydrolysis of LBG and GG was examined for different hydrolysis period at 50 °C using the partially purified enzyme and the amount of the released reducing sugars was determined (Fig. 10A). The results indicated the high rate of hydrolysis at the initial period of the reaction reaching maximum after 2 h, releasing 1.8 and 0.66 mg reducing sugar/mL for the hydrolysis of LBG and GG respectively. The hydrolysis activity of the produced enzyme was almost similar to the results reported for *Aspergillus terreus* FBCC 1369 β-mannanase (Soni et al., 2015, 2016). Moreover, the amount of the released reducing sugars in the current study indicated that the produced enzyme was more active against LBG than GG, similar to *Aspergillus oryzae* MTCC 1846 β-mannanase (Jana et al., 2018). However β-mannanase produced by *Malbranchea cinnamomea* NFCCI 3724 was more active toward konjac gum (linear mannan) than LBG (Ahirwar et al., 2016b). TLC analysis (Fig. 10B) of LBG and GG resulted hydrolyzate indicated that the produced enzyme can be utilized for the hydrolysis of mannan polymers with the production of oligosaccharides.

The FTIR spectrum of the produced oligosaccharides confirmed the structures proposed for mannose subunits as shown in Fig. (11). The band of O–H vibration was observed around 3428 $cm^{-1}$and that for CH_2 was observed around 2930 cm^{-1}. The band of C═O group was observed at 1632 cm^{-1}.The band observed around 1127 $cm^{-1}$of C–O–C was broader in case of oligosaccharide. Moreover, the FTIR spectrum of the end product of LBG hydrolysis using the produced enzyme was similar to that of the oligosaccharide. This result indicated that the enzyme is an endo-enzyme since the end product is not the mono-sugar (mannose).

3.9. Antioxidant activity

The determination of the antioxidant activity (Fig. 12) of the LBG and GG after their hydrolysis using the produced enzyme indicated that the antioxidant activity of the resulted hydrolyzate of LBG was almost 4 times higher than that produced by the hydrolysis of GG without the detection of any antioxidant activity for LBG and GG before hydrolysis. This result may be attributed to the higher amount of MOS present in LBG resulted hydrolyzate than that present in GG hydrolyzate. This result is confirmed by determining the antioxidant activity of equal amount of the purified MOS (extracted from TLC) resulted from the hydrolysis of LBG and GG since antioxidant activity of 83.2 μg TE/mg dry sample was determined for both of them. Antioxidant activity of MOS result from the hydrolysis of polymannan was also reported by Amna et al. (2018). It is well accepted that the DPPH free radical scavenging by antioxidants is attributed to their hydrogen-donating ability (Chen and Ho, 1995).

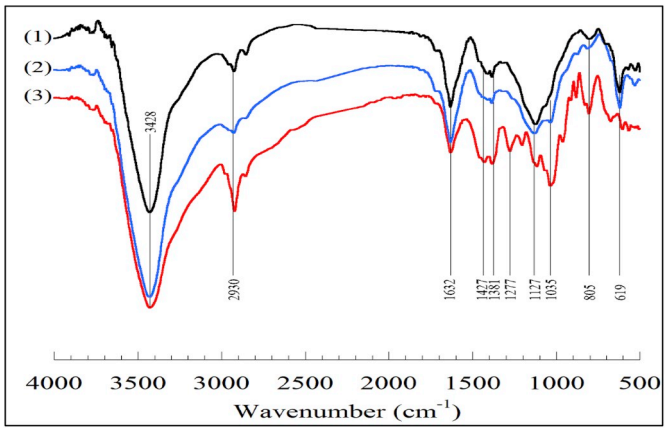

Fig. 11. FTIR analysis of (1) final hydrolysis product, (2) the produced MOS of LBG hydrolysis and (3) the standard mannose.

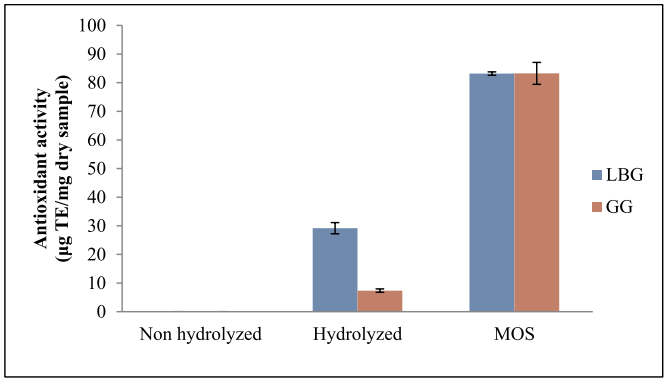

Fig. 12. Antioxidant activity.

4. Conclusion

In the current study, rice straw was indicated to be an economic substrate for the production of β-mannanase by SSF using *Trichoderma longibrachiatum*RS1in which the production of the enzyme was optimized to reach 89.730U/g ds.The optimum temperature for the activity of the partially purified enzyme was 75 °C, indicating its thermoactivity. This property makes the enzyme a suitable candidate in various biotechnological applications that required high temperatures to achieve optimal results. Moreover, the $K_m$value of the partially

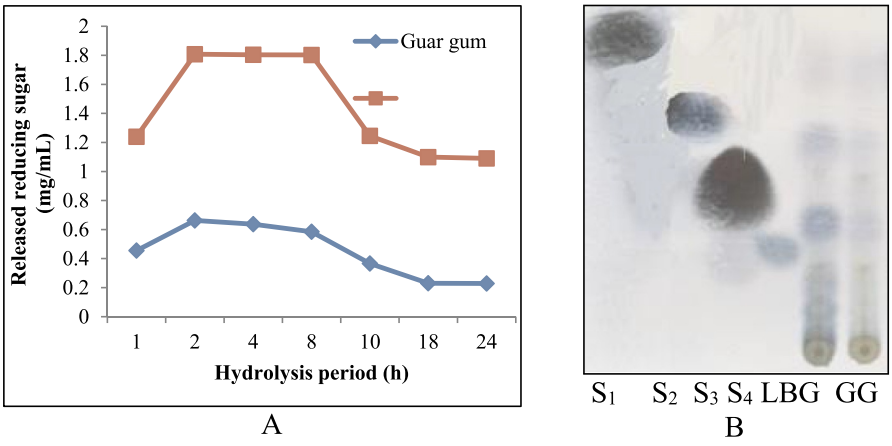

Fig. 10. The released reducing sugar by the hydrolysis of LBG and GG for different hydrolysis periods using the partially purified enzyme (A) and (B) TLC analysis of the produced hydrolyzate after hydrolysis for 2 h in which S_1, S_2,S_3 and S_4 are standards of mannose and di-, tri-, tetra-oligosaccharides respectively.

purified enzyme was 3.33 mg/mL, indicating its high affinity toward mannan substrate. The produced enzyme was efficiently used in the removal of mannan based food stains. Moreover, the produced enzyme was used successfully in the hydrolysis of mannan polymers releasing MOS confirmed by TLC and FTIR analysis.The produced MOS possessed antioxidant activity of 83.2 μg TE/mg sample.

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