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Microbial valorization of shrimp byproducts via the production of thermostable chitosanase and antioxidant chitooligosaccharides

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ABSTRACT

Chitinous materials present in marine byproducts attract the research focus for their potential agricultural and biomedical applications. In the present study, shrimp processing byproduct was used for the production of chitosanase by solid state fermentation using Bacillus cereus strain SSW1. The isolated strain was identified by Gram stain, MALDI-TOF mass spectrometry and 16S DNA sequencing. The enzyme production was statistically optimized to reach 39.774U/g dry substrate by 5.3 fold of increase. The activity of the partially purified enzyme was optimized in respect to the effect of temperature, pH and substrate concentration in which the enzyme was indicated to be thermo-active and thermo-stable. Although several chitosanases have been previously studied, thermostable enzymes are still rather rare. The K_m, V_{max} and K_{cat} values were calculated to be 7.55 mg/mL, 30.3U/mL/min and 1.9min^{-1} and the half lives of the enzyme at 55, 60 and 65 °C were 693.15, 346.57 and 77.02min^{-1} respectively, confirming the thermo-stability of the produced enzyme. The antifungal activity of the produced enzyme was indicated against the phytopathogenic fungus Fusarium oxysporum. Additionally, chitooligosaccharides were obtained by the enzymatic hydrolysis of chitosan and by the bacterial hydrolysis of shrimp byproducts that was confirmed by thin layer chromatography analysis and Fourier Transform Infrared Spectroscopy. Finally, the purified chitooligosaccharides showed in vitro antioxidant activity assessed by 1,1-diphenyl-2-picrylhydrazyl free radical scavenging assay.

1. Introduction

The industrial processing of shrimp, results to the accumulation of numerous inedible parts that may have a negative impact on the environment and consequently the human health. Recently, shell biorefinery was created for using the underutilized by-products in the production of various high-value compounds (Yadav et al., 2019; Zhang et al., 2018a). Chitin is a linear polysaccharide of β-1,4-glycosidic linkage chain of N-acetyl-D-glucosamine that compromises 20–30% of the crustaceans shell (Yan and Chen, 2015). Chitin and its deacetylated derivative (chitosan) have attracted a growing interest due to their various pharmaceutical, medical, agricultural and food industrial applications. However, their high molecular weight, poor water solubility and the increased viscosity limited their applications. The partial hydrolysis of chitin and chitosan not only improves their water solubility but it delivers various biologically active chitooligosaccharides (COS). COS are chains composed of 2–20 units of N-acetyl-D-glucosamine (GlcNAc) and/or glucosamine (GlcN) with an average molecular weight < 3900Da. The molecular weight, degree of acetylating and their amino content are the main variables that influence their biological activities (Liang et al., 2018; Liaqat and Eltem, 2018; Yadav et al., 2019). Several biological activities as antitumor, anti-inflammatory, immuno-modulatory, antioxidant, antimicrobial, anti-hypertensive, hypocholesterimic and neuroprotective effects have been reported for COS, that varied according to the variation in their physicochemical properties (Hashem et al., 2018; Liang et al., 2018; Liaqat and Eltem, 2018; Mohamed et al., 2018; Sanchez et al., 2017; Yadav et al., 2019; Zou et al., 2016).

Chemical hydrolysis is the most frequent traditional method used in the digestion of chitin and chitosan for the production of COS but the low yield of COS and the environmental hazards are considered serious drawbacks. Enzymatic hydrolysis has been recommended as an alternative method but the cost, availability and specificity of the used enzymes limited their industrial applications (Liang et al., 2018; Lodhi et al., 2014).

Chitosanases are glycoside hydrolases that specifically catalyzes the hydrolysis of chitosan with the release of COS as the main product. They have gained more attention owing to their several

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biotechnological applications. Bacterial fermentation has been primarily found to be an important source for the production of chitosanases. The common carbon sources for the bacterial production of chitosanases are chitin and chitosan that were commercially prepared by the chemical demineralization and deproteinization of crustaceans by-products using strong alkaline and acid treatments. The direct utilization of these by-products as a sole carbon and nitrogen source for the bacterial production of the enzyme attract the research focus from the perspective of reducing cost, environmental protection and minimal impact of these byproducts in food production (Doan et al., 2019, 2018a, 2018b and Liang et al., 2016, 2014).

The fermentation conditions have a crucial impact on the growth of the microorganisms and their metabolic products as well as the production cost (Shivalee et al., 2018). Experimental designs have been employed in the optimization of the fermentation conditions to overcome the time consuming limitation and decrease the number of the experiments required by the classical one-variable-at-a-time optimization approach and consequently decrease the total cost of the enzyme production process (Desai et al., 2008). Several designs including Plackett-Burman, Box-Behnken and central composite designs have been widely used in the optimization of the microbial production of various enzymes and have been successfully applied in chitosanase production optimization (Hashem et al., 2018; Liaqat et al., 2018; Nidheesh et al., 2015).

Excess oxidation is the cause of the chemical spoilage and rancidity of foods (Colbert and Decker, 1991) and excess free radicals is an important cause for the destruction of the cells. Moreover, free radicals were involved in the pathogenesis of various diseases as cancer, diabetes and cardiovascular disorders that can be overcome by antioxidant compounds (Aliakbarlu et al., 2014). Therefore, there is a growing demand for natural antioxidant compounds to overcome the deleterious effects in the biological systems.

The present study focused on the production of chitosanase using shrimp processing byproducts (SPB) as a sole carbon and nitrogen source in addition to the statistical optimization of the enzyme production by using Plackett-Burman and Box-Behnken designs. The kinetic constants of the partially purified enzyme were determined as well as the thermal activation and denaturation of the enzyme. Additionally, the antifungal activity of the partially purified enzyme was examined against the phytopathogenic fungus Fusarium oxysporum. Moreover, the potentiality of the production of COS either by the enzymatic hydrolysis of chitosan using the partially purified enzyme or by the bacterial hydrolysis of SPB were examined. Finally, the antioxidant activity of the produced COS was determined.

2. Materials and methods

2.1. Materials

Chitosan (low, medium and high molecular weight), glucosamine and N-acetyl glucosamine were purchased from Sigma-Aldrich, Saint Louis, USA. Chitopentose and chitohexose were purchased from Seilkagaku Biobusiness Corporation, Tokyo, Japan. Dinitrosalicylic acid (DNS) was obtained from Panreac, Barcelona, Spain. Silica gel 60 thin-layer chromatography (TLC) plates were purchased from Merck, Darmstadt, Germany. All other chemicals were of analytical or HPLC grade.

2.2. Preparation of shrimp byproducts substrate

Marine shrimp processing raw byproducts (SPB) composed of both cephalothoraxes and carapaces were collected from the local seafood market. The collected SPB were washed under running warm tap water to remove soluble organics and other impurities. The washed SPB were boiled in water for 1h and air dried then ground into fine powder using a standard grinder (Benhabiles et al., 2012).

2.3. Microorganism and culture conditions

The bacterial strain used in the current study was isolated from shrimp shell wastes collected from the local sea food market (data not shown). The culture was maintained on nutrient agar slants for 24 h at 37 °C before refrigeration. The identification of the culture was performed by Gram stain, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) performed at children's cancer hospital (57357), Cairo, Egypt followed by 16S DNA sequencing carried out in Sigma Scientific Services Co.

For the production of the enzyme, solid state fermentation method was used in which 5 g of SPB (with zero percentage of initial moisture content) in 250 mL Erlenmeyer conical flask was moistened with 10 mL of tape water. The solid substrate medium was inoculated with 1 mL of the pre-culture (prepared by scratching of the cultured slant with 5 mL distilled water) then incubated for 48 h at 30 °C. After incubation, the fermented substrate was extracted with 50 mL of distilled water on an orbital shaker (150 rpm) for 1h then centrifuged at 5500 rpm (4 °C) for 20min. The produced supernatant was used for further experiments.

2.4. Enzyme activity and protein content assay

The chitosanase activity was assayed using low molecular weight chitosan as a substrate in a reaction mixture consisted of 500 µL of 1% soluble chitosan (pH 5) and 500 µL of the culture supernatant incubated at 50 °C for 30min (Pagnoncelli et al., 2010). The released reducing sugars were measured immediately by using DNS method (Miller, 1959) with D-glucosamine as the standard. One unit of chitosanase was defined as the amount of enzyme that released 1 µmol of D-glucosamine per minute under the assay conditions. Soluble chitosan was prepared according to Uchida and Ohtakara (1988) in which the low molecular weight chitosan was initially dissolved in 1M acetic acid then adjusted to pH 5 using 2M sodium acetate.

Non specific chitosan hydrolyzing enzymes such as endochitinase, exochitinase and protease using colloidal chitin, 4-NitrophenylN-acetyl-β-D-glucosaminide and casein as substrates respectively, were assayed.

The protein content was determined using the Lowry-Folin method against a bovine serum albumin standard (Lowry et al., 1951).

2.5. Experimental design for chitosanase production optimization

The effect of the multiple fermentation parameters on the production of the enzyme was studied by two phase model. The first phase was the identification of the variables that have the highest influence on the productivity by applying Plackett-Burman design then in the second phase Box-Behnken design was applied.

2.5.1. Plackett-Burman design

Plackett-Burman design can be effectively used in the biochemical process for the identification of the most influencing variables in which the experimental runs were calculated as $n+1$, where n is the number of the selected variables (Plackett and Burman, 1946). In the current study, seven independent variables (fermentation period, temperature, period of microwave pretreatment of SPB, K_2HPO_4, $MgSO_4$, KCl and $FeSO_4{\cdot}7H_2O$) were evaluated in eight experimental runs. Each variable is represented in terms of high (+1) and low (-1) values (Table 1). The concentration of the added salts was selected on the base of previously available researches concerned with chitosanase production (Hashem et al., 2018; Liang et al., 2014; Muslim et al., 2016). 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 (chitosanase production), B_0 is the model intercept and B_i is the linear coefficient and X_i is the level of the independent variable.

Table 1
Plackett – Burman design with coded values and the observed results of chitosanase production.

Run number	Fermentation period (hour)	Temperature (°C)	Period of microwave pretreatment (minute)	K_2HPO_4 (%)	$MgSO_4$ (%)	KCl (%)	$FeSO_4{\cdot}7H_2O$ (%)	Chitosanase (U/g ds)
1	(24) -	(30) -	(0) -	(1.5) +	(0.1) +	(2) +	(0.01) -	25.283
2	(72) +	(30) -	(0) -	(0.15) -	(0.01) -	(2) +	(0.1) +	28.687
3	(24) -	(37) +	(0) -	(0.15) -	(0.1) +	(0.2) -	(0.1) +	20.158
4	(72) +	(37) +	(0) -	(1.5) +	(0.01) -	(0.2) -	(0.01) -	14.000
5	(24) -	(30) -	(1) +	(1.5) +	(0.01) -	(0.2) -	(0.1) +	28.532
6	(72) +	(30) -	(1) +	(0.15) -	(0.1) +	(0.2) -	(0.01) -	13.634
7	(24) -	(37) +	(1) +	(0.15) -	(0.01) -	(2) +	(0.01) -	34.162
8	(72) +	(37) +	(1) +	(1.5) +	(0.1) +	(2) +	(0.1) +	17.274

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

$$E(Xi) = 2(\Sigma Mi+ - Mi-)/N \quad (2)$$

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

2.5.2. Box-Behnken design

In order to determine the optimum level of the variables with the highest influence on chitosanase production, Box-Behnken design was applied (Box and Behnken, 1960). In this model (Table 3), an experimental design of 15 run and 3 central points was conducted with the most influencing three independent variables selected on the base of the results of Plackett-Burman design. 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 (chitosanase production). The equation is presented in the following form:

$$Y = B0 + \Sigma Bi\ Xi + \Sigma Bii\ Xi2 + \Sigma Bij\ XiXj \quad (3)$$

Where Y is the predicted chitosanase 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.

2.6. Partial purification of the enzyme

Partial purification of the crude enzyme (culture supernatant) was performed by ethanol fractional precipitation at 30–90% concentration with 10% intervals. Each fraction was assayed for chitosanase activity and protein content (Hashem et al., 2018).

Table 2
Multiple regression analysis of Plackett- Burman design.

Variables	Chitosanase analysis			
	Coefficient	t-statistics	P-value	Confidence level (%)
Intercept	43.881			
Fermentation period (hour)	-0.179	-18.423	3.38E-12	100
Temperature (°C)	-0.377	-5.623	3.81E-05	99.996
Period of microwave pretreatment (minute)	1.368	2.919	0.010044	98.996
K_2HPO_4 (%)	-2.139	-6.162	1.37E-05	99.999
$MgSO_4$ (%)	-80.644	-15.485	4.74E-11	100
KCl (%)	4.039	15.512	4.61E-11	100
$FeSO_4{\cdot}7H_2O$ (%)	21.031	4.038	0.000952	99.905
Model summary				
Multiple R	0.991			
R^2	0.983			
Adjusted R^2	0.975			
Standard Error	1.148			

Table 3
Box-Behnken Design with the observed and the predicted results.

Run number	Independent variable			Observed Chitosanase (U/g ds)	Predicted Chitosanase (U/g ds)
	X_1 Fermentation period (hour)	X_2 $MgSO_4$ (%)	X_3 KCl (%)		
1	6(−)	0.005(−)	2(0)	0.093	0.0
2	42(+)	0.005(−)	2(0)	39.175	37.602
3	6(−)	0.015(+)	2(0)	0.067	1.631
4	42(+)	0.015(+)	2(0)	33.168	33.726
5	6(−)	0.01(0)	1(−)	0.178	0.0
6	42(+)	0.01(0)	1(−)	29.558	28.589
7	6(−)	0.01(0)	3(+)	0.049	1.009
8	42(+)	0.01(0)	3(+)	38.859	40.809
9	24(0)	0.005(−)	1(−)	28.103	30.626
10	24(0)	0.015(+)	1(−)	29.825	30.216
11	24(0)	0.005(−)	3(+)	39.003	38.607
12	24(0)	0.015(+)	3(+)	39.774	37.246
13	24(0)	0.01(0)	2(0)	32.959	32.675
14	24(0)	0.01(0)	2(0)	32.397	32.675
15	24(0)	0.01(0)	2(0)	32.677	32.675

2.7. Effect of pH and temperature on the activity and stability of the produced enzyme

To evaluate the effect of pH on the partially purified enzyme, the activity of the enzyme was determined at different pH range from 4.5 to 6 using acetate buffer. The pH at which the enzyme processed the highest activity was considered as optimum. The stability of the enzyme at the optimum pH was determined by estimating the residual activity of the enzyme every 30min up to 2h after its pre-incubation at that pH. The activity of the enzyme without pre-incubation was considered as 100% activity.

The effect of the temperature on the activity of the partially purified enzyme was determined at various temperatures (50-70°C) at the optimum pH. The activation energy (E_a) for the produced enzyme was calculated from Arrhenius plot (ln relative activity versus reciprocal of temperature in Kelvin), as given in the following equation:

$$Slope = -Ea/R \quad (4)$$

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

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

$$T1/2 = \ln(2)/Kd \quad (6)$$

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

2.8. Effect of substrate (chitosan) concentration

The activity of the partially purified enzyme was determined at the optimum conditions using different chitosan concentrations (2–11 mg/mL). The kinetic constants for the partially purified enzyme were calculated on the base of Lineweaver-Burk plot (Lineweaver and Burk, 1934) using the following equation:

$$1/V = (1/Vmax) + (Km/Vmax)\ (1/S) \quad (7)$$

$$Kcat = Vmax/e \quad (8)$$

in which V is the activity of the partially purified enzyme (U/mL), V_{max} is the maximal activity, K_m is Michaelis-Menten constant, S is the chitosan concentration (mg/mL), K_{cat} is the turnover number and e is the enzyme concentration.

2.9. Chitooligosaccharide

2.9.1. Hydrolysis of chitosan

The activity of the partially purified enzyme in the hydrolysis of chitosan and COS production was initially studied in a reaction mixture of 2 mL of 1% low molecular weight chitosan prepared as described above with 1 mL of the partially purified enzyme (3U/mL) at 50°C for different hydrolysis periods (30min-6h). At the end of each hydrolysis period, the reaction mixture was boiled for 10min to stop the reaction then centrifuged to remove the un-hydrolyzed portion of chitosan. The amount of the released reducing sugar was determined according to Miller (1956) and the hydrolysis percentage was calculated according to the following equation:

$$\text{Hydrolysis percentage} = (\text{The total amount of the released reducing sugar} / \text{The total amount of added chitosan in the reaction}) * 100 \quad (9)$$

The hydrolysis of different molecular weight of chitosan was examined at the optimum hydrolysis period (maximum amount of reducing sugars was determined). An equivalent volume of the hydrolyzed products was analyzed by thin layer chromatography (TLC).

2.9.2. Chitooligosaccharides prepared in-house

The direct production of COS in the fermentation supernatant of the optimized conditions (as a result of the bacterial hydrolysis of SPB) was initially determined in terms of released reducing sugar according to Miller (1956), in which 1 mL of the culture free supernatant was added to 2.5 mL of DNS. The reaction was boiled for 10min and the produced color was measured at 540 nm with D-glucosamine as the standard. TLC was used to analyze the concentrated supernatant.

2.9.3. Analysis and purification

The produced COS were analyzed by TLC using propanol: water: ammonia (7: 2: 1 v/v) as the mobile phase (Cabrera and Cutsem, 2005) that were visualized using diphenyl amine-aniline spraying reagent (Tanaka et al., 1999).

The purification of the produced COS was performed according to Embaby et al. (2018). Briefly, the clear supernatant resulted from the bacterial hydrolysis of SPB and the enzymatic hydrolysis of chitosan were precipitated separately by cold absolute ethanol in a ratio 10:1 (ethanol:supernatant). Each mixture was left overnight at 4°C to denaturant any excitant protein then it was centrifuged and the resulted precipitate was re-dissolved in distilled water and centrifuged. Finally the clear supernatants were run on TLC and the extracted COS were subjected to air drying and stored at 4°C until further use.

2.9.4. Chemistry of the produced chitooligosaccharide

The functional groups and chemical bonds of the dried COS were determined by Fourier transform infrared spectroscopy (FTIR-8300, Shimadzu, Japan).

2.10. Antifungal activity of the partially purified enzyme

The well diffusion method according to Neto et al. (2016) was used to examine the antifungal activity of the partially purified enzyme against the phytopathogenic fungus Fusarium oxysporum. The fungus was obtained from the culture collection of the Department of Chemistry of Natural and Microbial products, National Research Center, Giza, Egypt. The experiment was performed in a Petri dish containing 20 mL of potato dextrose agar inoculated with 0.2 ml of the fungal strain spore suspension. Wells of 7 mm in diameter were made in the agar plate with a sterile glass Pasteur pipette and 0.1 mL of the partially purified enzyme of activity 4.5, 2.3 and 0.9U/mL at 30 °C were added into the wells. The plates were then incubated at 30°Cfor 48 h.

2.11. Antioxidant activity of chitooligosaccharides

The dried COS were analyzed for their antioxidant activity by determining their scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, according to the method described by Brand-Williams et al. (1995). The reaction was carried out by adding 0.1 mL of the sample solution (1%) to 3.9 mL of methanol solution of DPPH radical (1.1×10^{-4}mol/L). The decrease in absorbance after 30min in dark was measured spectrophotometrically at 515 nm using Trolox as a standard. The results are expressed in μg Trolox Equivalents (TE)/mg of the dry sample.

2.12. Statistical analysis

All experiments were performed in triplicates with three measurements per replicate and the averages of the results were reported.

3. Results and discussion

This study suggests that SPB can be used as a sole carbon and nitrogen source for the production of chitosanase, as it produced chitosanase activity of 7.5U/g dry substrate (ds) by SSF of 5 g SPB in 250 mL Erlenmeyer conical flask moistened with 10 mL of tape water, without detection of any chitinase and protease activity. The use of SPB in the microbial production of chitosanase instead of chitosan can serve as a solution for the high cost problem that faces its industrial applications.

3.1. Microorganism

The bacterial strain used in the current study is a gram positive bacillus (Fig. 1) that was identified by MALDI-TOF MS to be Bacillus cereus. Recently, MALDI-TOF MS has been proposed as a promising tool in the identification of bacterial isolates (Kim et al., 2019; Strejcek et al., 2018).

The identification of the isolate was confirmed using 16S rDNA nucleotide sequencing that indicated 100% similarity of the isolate with Bacillus cereus strain. The phylogenetic analysis was performed based on the results of the partial 16S rDNA nucleotide sequencing. The analysis showed the relation between the isolated strain and other species belong to the genus Bacillus was constructed using MEGAX and neighbor-joining method (Kumar et al., 2018) and the phylogenetic tree was shown in figure (2). The data of 16S rDNA partial sequence was submitted to NCBI under the name Bacillus cereus strain SSW1 and received accession number of **MK533796**. The former results matching the conclusions obtained by de Araujo et al. (2016), Liang et al. (2014) and Prakash and Gopal (2017), reported Bacillus cereus as a

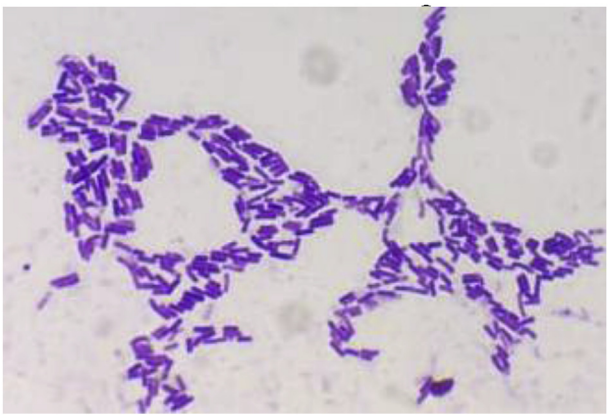

Fig. 1. Gram stain of the isolate.

chitosanase producer.

3.2. Chitosanase production optimization

3.2.1. Selection of the variables that influence chitosanase productivity using Placket-Burman design

The mean value of the observed chitosanase activity was represented in Table (1) and the results showed a wide variation ranged from 14.000U/g ds to 34.162U/g ds, reflecting the importance of the initial screening of the enzyme production using statistical strategy for the selection of the fermentation medium components and the culture conditions that influence the productivity. The maximum activity was observed at run number 7 with 34.162U/g ds using the optimized conditions (fermentation period, 24hr; temperature, 37 °C; period of microwave pretreatment, 1 min; K_2HPO_4, 0.15%; $MgSO_4$, 0.01%; KCl, 2%; and $FeSO_4{\cdot}7H_2O$, 0.01%).

The main effect of each variable was calculated and presented graphically in figure (3). The calculated values indicated that the fermentation period, temperature, K_2HPO_4 and $MgSO_4$ had negative signs while period of microwave pretreatment, KCl and $FeSO_4{\cdot}7H_2O$ had positive signs. The positive sign of the main effect indicates that the tested variable has more effect on the enzyme productivity at the high level while the negative sign indicates that the tested variable has more effect on the enzyme productivity at the low level. Low concentrations of K_2HPO_4 and $MgSO_4$ were preferred in the production of chitosanase as previously reported by Ismail et al. (2016). The use of microwave heating can homogeneously transfer heat within the sample matrix at a molecular level through the motion of molecules (Prajapat and Gogate, 2015) that may facilitate the microbial utilization of the SPB. Recently, the microwave heating attracted more interest to assist either the chemical or the enzymatic hydrolysis of chitosan and SPB as it can be utilized to shorten the reaction time and to increase the product yield (Xiao et al., 2019; Zhang et al., 2018b; Zhao et al., 2019).

The data has been analyzed by multiple regression analysis and the results represented in Table (2). The analysis indicated that all of the tested variables significantly affected the chitosanase productivity. The effect of the seven independent variables on chitosanase productivity was estimated by the coefficient values, the variable that exerted positive effect was maintained at positive level while the one exerted negative effect was maintained at negative level in order to achieve maximum chitosanase productivity in the second phase of optimization. The analysis of variance (ANOVA) has been calculated and it indicated that the model terms used in that study are statistically significant since the model terms had Prob > F value of 6.55 E-13 (less than 0.05).

The R^2 value of the selected model was 0.983. It indicated the accuracy of the model as it suggested that a variation of 98.3% occur due to the independent variables while there is only 1.7% chance that the response was not due to the experimental model variables. The greater the R^2 value than 0.9 the more the accuracy of the model since it measures the degree of response exerted by the experimental variables (Edwards et al., 2008).

The first order equation that described the correlation of the selected seven variables and the chitosanase activity could be presented as follows:

$$Y = 43.881–0.179X1 - 0.377X2 + 1.368X3 - 2.139X4 - 80.644X5 + 4.039X6 + 21.031X7$$

Where Y is the chitosanase activity and X_1, X_2, X_3, X_4, X_5, X_6, X_7 are the fermentation period, temperature, period of microwave pretreatment, K_2HPO_4, $MgSO_4$, KCl and $FeSO_4{\cdot}7H_2O$ respectively.

On the base of all the above data, the variables with the highest confidence level (the fermentation period, 24hr; $MgSO_4$, 0.01% and KCl, 2%) were selected for the second phase of optimization.

3.2.2. Box-Behnken design

The mean value of the experimental and the predicted chitosanase activity resulted from applying Box-Behnken design was represented in Table (3). The optimum chitosanase activity (39.774U/g ds) was observed in run 12 and consequently the optimized level of the selected

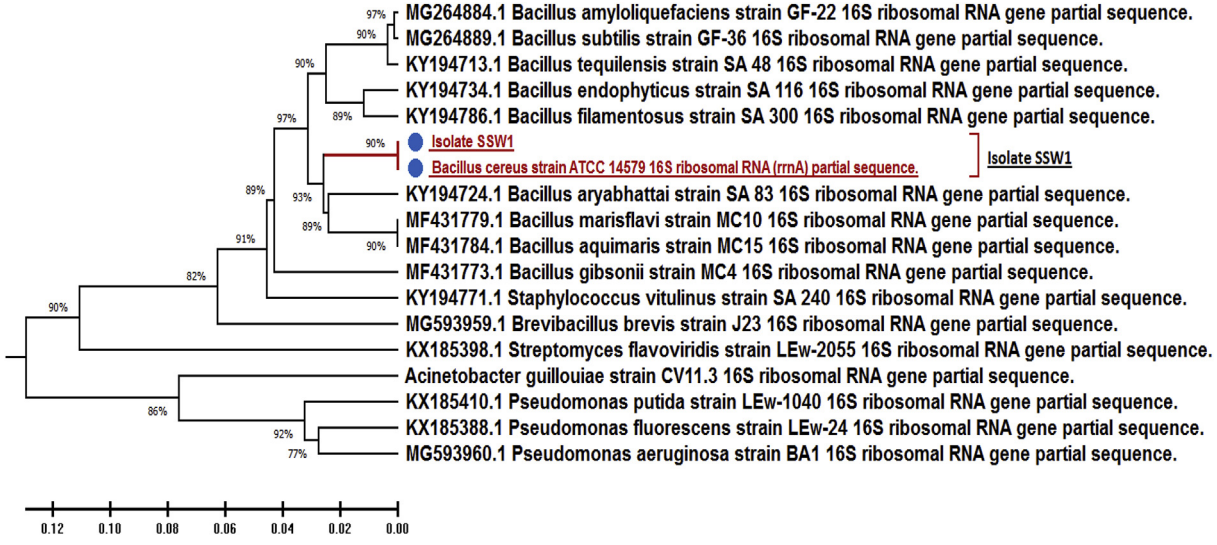

Fig. 2. The Phylogenetic tree.

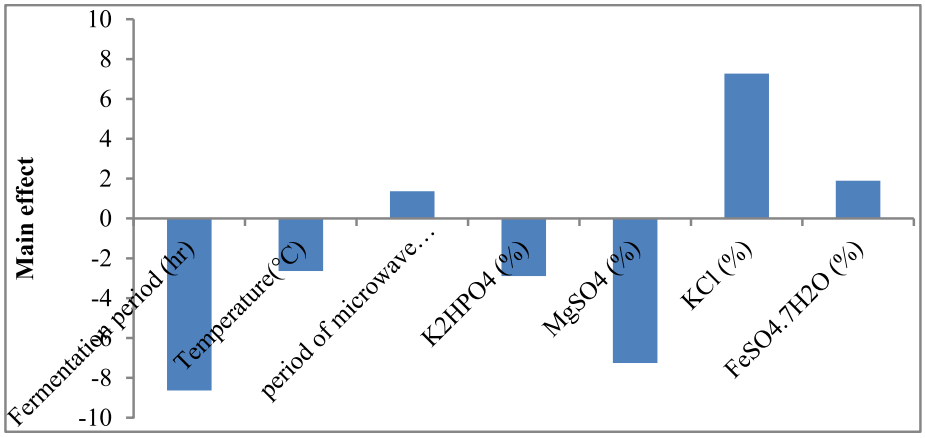

Fig. 3. Main effects of the independent variables on chitosanase production calculated from the results of the Plackett – Burman design.

Table 4
Analysis of box-behnken design.

Term	Regression coefficient	t- test	P-value
Intercept	-18.054	-3.605	0.000961
X_1	3.217	23.435	5.57E-23
X_2	-580.156	-0.972	0.337507
X_3	0.0165	0.006	0.995624
X_1^2	-0.049	-24.702	9.77E-24
X_2^2	49269.71	1.928	0.062036
X_3^2	0.267	0.418	0.678657
X_1X_2	-16.612	-2.435	0.020108
X_1X_3	0.131	3.841	0.000494
X_2X_3	-47.529	-0.387	0.701021
Model summary			
Multiple R	0.993		
R^2	0.985		
Adjusted R^2	0.982		
Standard Error	2.127		

variables was conducted to be as follow: the fermentation period, 24hr; $MgSO_4$, 0.015% and KCl, 3%.

The multiple regression analysis of the data presented in Table 4 and the R^2 value of the model was 0.9853 indicating the accuracy of the model since 98.54% of the variation in the chitosanase activity is due to the independent variables. The R value (0.993) was very close to 1, indicating a great agreement between the experimental results and the theoretical values predicted by the model equation.

The second order polynomial equation, used for the calculation of the predicted chitosanase activity, concluded from the multiple regression analysis was:

$$Y = -18.054 + 3.217X_1 - 580.156X_2 + 0.0165X_3 - 0.049X_1^2 + 49269.71X_2^2 + 0.267X_3^2 - 16.612\,X_1X_2 + 0.131\,X_1X_3 - 47.529X_2X_3$$

Where Y is the chitosanase activity and X_1, X_2 and X_3 are the fermentation period, $MgSO_4$ and KCl respectively.

The analysis of variance (ANOVA) has been calculated and it indicated that the model terms used in that study are statistically significant since the model terms had Prob > F value of 2.14 E-29 (less than 0.05). The competence of the applied model was confirmed by performing an experiment under the optimized conditions. The chitosanase production was 38.980U/g ds which is in accordance with the predicted value 37.246U/g ds, reflecting the validation of the model.

An overall increase in the enzyme productivity from 7.5 to 39.74U/g ds (5.3 fold) was achieved by applying the statistical design. The achieved optimum chitosanase activity was quite high as 41.78U/g ds achieved after statistical optimization of SSF employing the same substrate as reported by Nidheesh et al. (2015). The use of chitinous wastes in the microbial production of chitosanases has attracted the research focus as a cheap alternative to chitosan in order to decrease the total cost of the enzyme (Doan et al., 2019, 2018b; Liang et al., 2016, 2014; Nidheesh et al., 2015; Wang et al., 2014).

3.3. Partial purification of the enzyme

Fractional precipitation with ethanol was carried out in order to partially purify the produced enzyme. The fraction of 50% ethanol led to an improvement in the specific activity to reach 8.918U/mg protein with 38.8% recovery yield and 20.2 fold of purification. The use of ethanol for the precipitation of chitosanases was reported by Doan et al. (2018a, 2018b) and Hashem et al. (2018).

3.4. Effect of pH and temperature

The pH profile of the partially purified enzyme using 0.05M acetate buffer indicated that it was optimally active at pH 5 at which the enzyme retained 100% of its activity for more than 2h (Fig. 4A). This result is consistent with other research concerned with Bacillus cereus chitosanases, expressing the optimum activity in acidic conditions (de

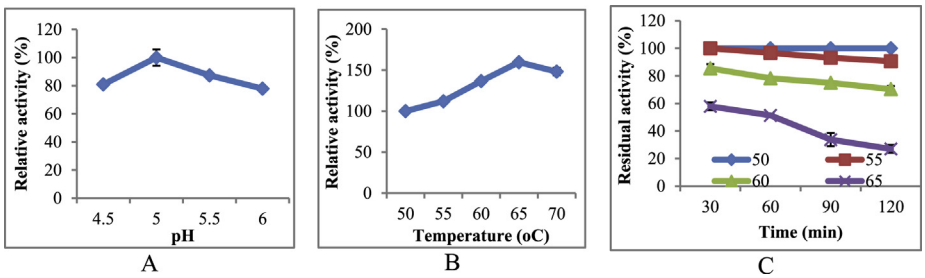

Fig. 4. The effect of pH (pH 5 is the control) (A), (B) the temperature effect (50°C is the control) on the activity of the partially purified enzyme and (C) the thermal stability.

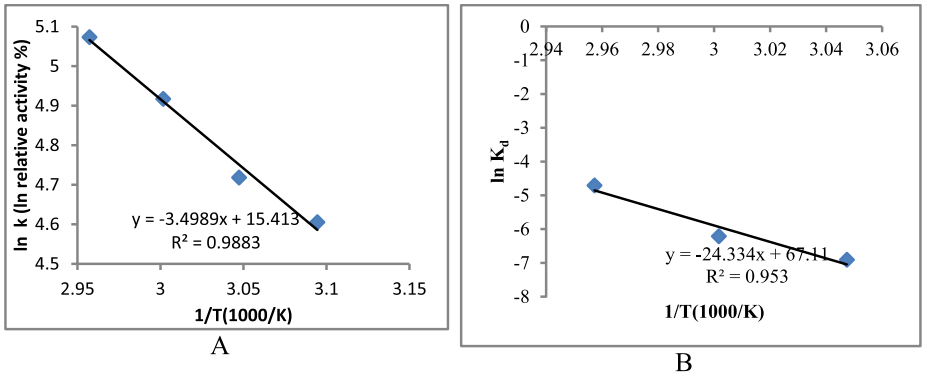

Fig. 5. Arrhenius plot of enzyme (A) thermal activation and (B) thermal denaturation.

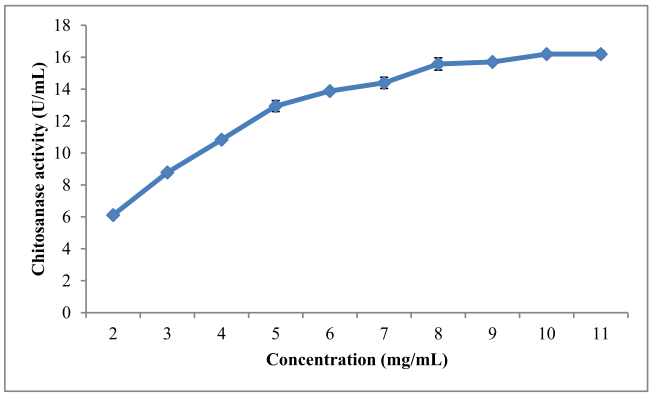

Fig. 6. The activity of the partially purified enzyme using different chitosan concentrations.

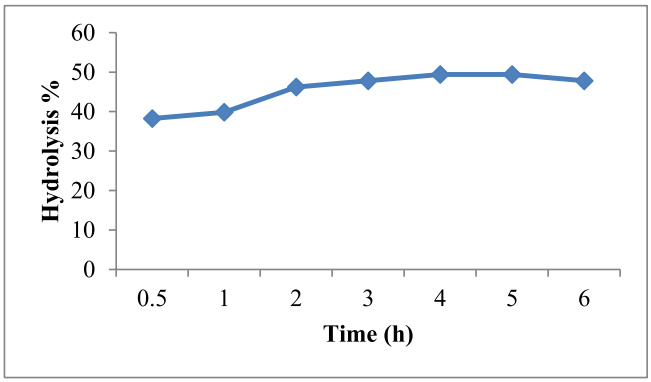

Fig. 8. The hydrolysis % of chitosan at different hydrolysis periods (the absence of error bars indicates the errors are smaller than the symbols).

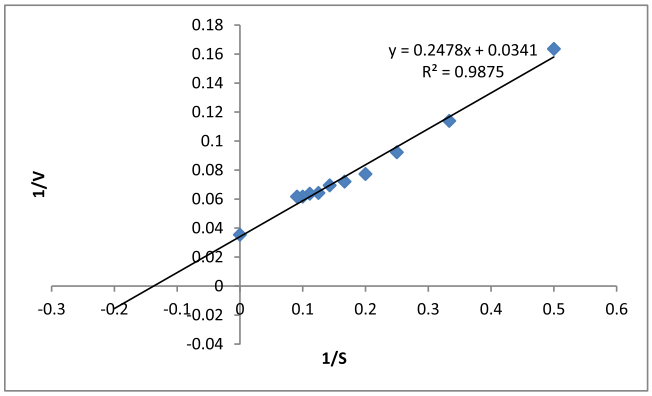

Fig. 7. Lineweaver-Burk plot.

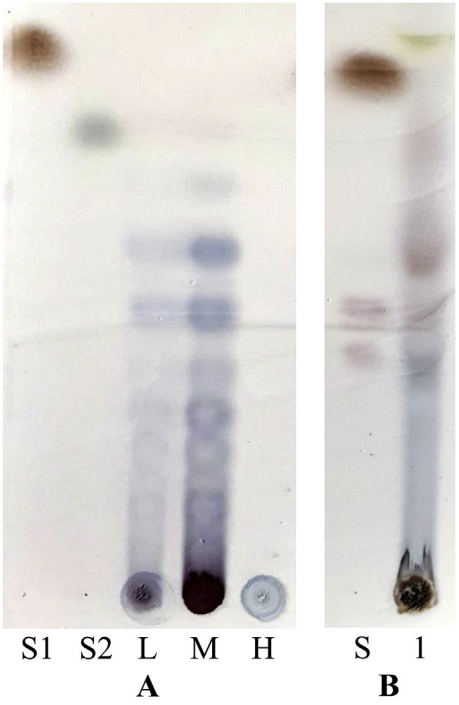

Fig. 9. TLC plate of (A) the hydrolysis of different molecular weight of chitosan using the partially purified enzyme in which S_1: N-acetyl glucosamine standard, S_2: glucosamine standard and L, M, H are the hydrolysis product of low, medium and high molecular weigh chitosan respectively (B) the bacterial hydrolysis of SPB (Lane 1) in which S are the standards from up to down (N-acetyl glucosamine, chitopentose and chitohexose respectively).

Araujo et al., 2016; Liang et al., 2014; Prakash and Gopal, 2017).

Measurements of the enzyme activity as a function of the reaction temperature indicated that the optimal temperature was 65 °C and by increasing the temperature to 70 °C, the enzyme possessed about 95% of its optimal activity (Fig. 4B). The produced enzyme demonstrated thermal stability up to 65 °C as it retained more than 70% of its activity for 2h at 60 °C and retained more than 50% of its activity for 1h at 65 °C (Fig. 4C). The optimal temperature and the thermal stability of the produced enzyme were higher than chitosanases from other Bacillus cereus (de Araujo et al., 2016; Liang et al., 2014; Prakash and Gopal, 2017) and higher than chitosanases from other bacterial strains (Doan et al., 2018a; Liang et al., 2016; Qin et al., 2018; Su et al., 2017; Zhou et al., 2019) but almost similar result was demonstrated by chitosanase produced by Paenibacillus mucilaginosus TKU032 (Doan et al., 2019). This important finding increases the economic viability of the enzyme

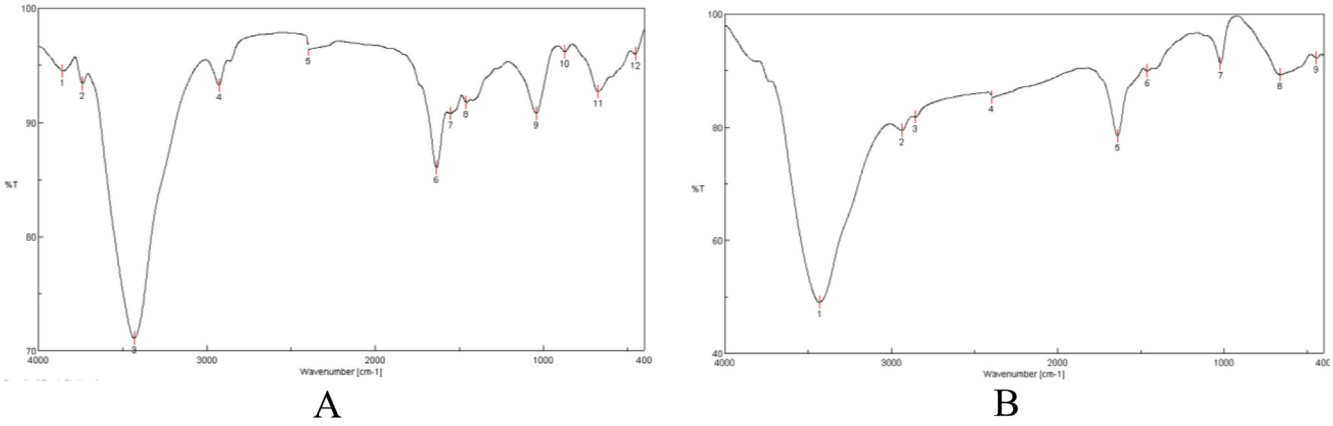

Fig. 10. FTIR analysis of COS resulted from (A) bacterial hydrolysis of SPB (B) enzymatic hydrolysis of chitosan using the partially purified enzyme.

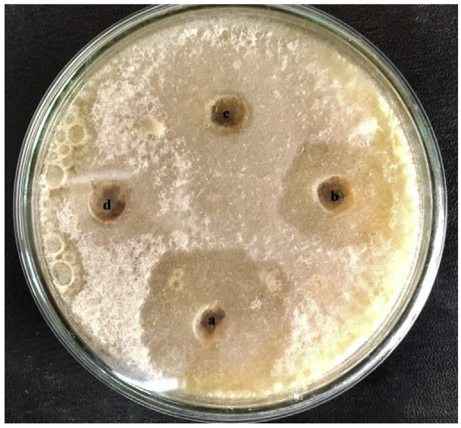

Fig. 11. Antifungal activity of the produced enzyme against the phytopathogenic fungus Fusarium oxysporum in which a, b and c are the partially purified enzyme of activity 4.5, 2.3 and 0.9U/mL and d is the crude enzyme of activity 0.8U/mL.

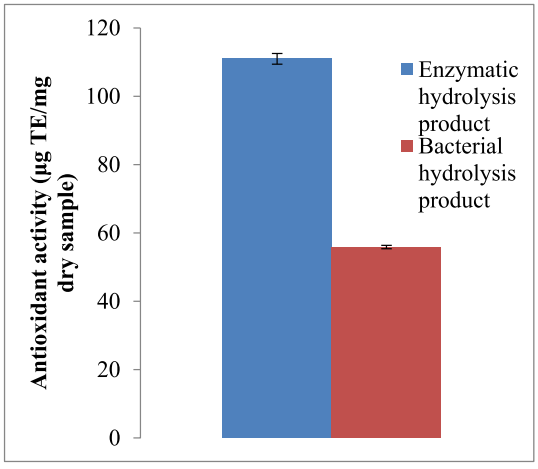

Fig. 12. Antioxidant activity of COS.

and makes it suitable in various biotechnological applications.

Arrhenius plots are always used to study the effect of temperature on the rates of the reactions. So by plotting of ln k (ln relative activity %) versus the reciprocal of the temperature in Kelvin, a straight line was observed (Fig. 5A) in which the slope represented the value of $-E_a/R$. E_a is the activation energy and R is the gas constant. The E_a of the partially purified enzyme was calculated to be 29.084 ± 0.864 kJ mol^{-1} for chitosan hydrolysis between 50 and 65 °C at pH 5. Sinha et al. (2016) reported activation energy of 28 kcal mol^{-1} for Aspergillus fumigatus IIT-004 exochitosanase however Gohi et al. (2017) reported activation energy for chitosanase from Streptomyces griseus of 12.822 kJmol^{-1} for chitosan hydrolysis between 50 and 70 °C at pH 5.

The temperature can activate the rate of the catalytic reaction of the enzyme and also it affects its inactivation. The rate of heat inactivation 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 693.15, 346.57 and 77.02min^{-1}. The results was higher than that reported by Guo et al. (2019) in which 4h was the half life for Csn21c produced from Streptomyces albolongus ATCC 27414 at 55 °C, indicating the thermo-stability of the produced enzyme in the present study. $E_{a(d)}$ was calculated from the slope of plotting ln K_d versus the reciprocal of the temperature in Kelvin (Fig. 5B) and it was calculated to be 202.29 kJ mol^{-1}. High $E_{a(d)}$ indicated the thermal adaptation of the produced enzyme as a result of the decrease in the enzyme unfolding rate (Jana et al., 2013).

3.5. Effect of substrate concentration and kinetic constants determination

The effect of different chitosan concentration on the activity of the partially purified enzyme was studied and it was indicated that the maximum activity was achieved by using chitosan concentration of 10 mg/mL and the increase in the concentration did not achieve any increase in the activity (Fig. 6). The kinetic constants for the partially purified enzyme were calculated on the base of Lineweaver-Burk plot (Fig. 7). K_m, V_{max} and K_{cat} were calculated to be 7.55 mg/mL, 30.3U/mL/min and 1.9min^{-1} respectively. The K_m and V_{max} values depend mainly on the enzyme source and reflect the sensitivity of the enzyme toward the substrate i.e. the decrease in the K_m and the increase in V_{max} indicate the increase in the enzyme sensitivity toward the substrate (Horn et al., 2006). Sinha et al. (2016) reported K_m, V_{max} and K_{cat} values of 8 mg/mL, 5.2×10^{-6}IU/mg and 3×10^3 S^{-1} respectively for Aspeyirgillus fumigatus IIT-004 chitosanase and Guo et al. (2019) reported K_m and V_{max} values of 7.5 mg mL and 263.1 µmol/min.mg for Csn21c produced from Streptomyces albolongus ATCC 27414, while Qin et al. (2018) reported K_m and V_{max} values of 2.8 mg/mL and 7142.9 µmol/min.mg for BaCsn46A produced from Bacillus amyloliquefaciens toward chitosan and Liang et al. (2016) reported K_m and V_{max} values of 0.098 mg/mL and 1.336U/min.mg for Bacillus mycoides TKU038 chitosanase toward water soluble chitosan.

3.6. Chitooligosaccharide

3.6.1. Hydrolysis of chitosan

The hydrolysis of low molecular weight chitosan was performed using the partially purified enzyme for different hydrolysis period and

the amount of the released reducing sugars was determined then the calculated hydrolysis percentage was represented in figure (8). It was observed that the hydrolysis rate was high at the initial period of the reaction and extended up to 2h, recording 38.3% after half hour and increased to reach 46.2 after 2h. By increasing the hydrolysis time more than 2h, a slight increase in hydrolysis percentage was observed. Nidheesh et al. (2015) achieve similar result by the hydrolysis of chitosan using Purpureocillium lilacinum CFRNT12 chitosanase but after 3 and 6h. The slow rate of hydrolysis observed after 2h may be attributed to either the binding of the enzyme with the substrate or the inhibition of the enzyme by the produced COS (Lacombe-Harvey et al., 2013; Nidheesh et al., 2015).

The hydrolysis of different molecular weight of chitosan for 2h was examined and the hydrolysis percentage was 46.2, 52.7 and 94.5 for low, medium and high molecular weight respectively. The resulted hydrolyzates were analyzed by TLC as shown in figure 9A. The result indicated that medium molecular weight chitosan is the most suitable substrate for the production of COS using the produced partially purified enzyme. Although high hydrolysis percentage was observed by the use of high molecular weight chitosan but no COS was observed by TLC analysis. This result may be justified to the limited resolving power of TLC as it is by far confined to COS of small DP (1–8) but the un-visualization of COS with high DP does not necessarily indicate their absence (Embaby et al., 2018).

3.6.2. Chitooligosaccharide prepared in-house

The amount of the reducing sugar released in the culture supernatant was estimated to be 761.3 ± 0.009 μg/mL without the determination of any reducing sugar before the bacterial cultivation of the SPB. The TLC analysis of the culture supernatant was shown in figure 9B, indicating the presence of COS. Chitin comprises 20%–30% of the shells from crustaceans (Yan and Chen, 2015) so the growing of the microorganisms on SPB as a sole carbon source would result on the production of chitin hydrolysis products. Embaby et al. (2018) reported the synthesis of COS by the hydrolysis of chitosan with the aid of whole cell fungus but COS synthesis with the aid of whole cells of microorganisms growing on SPB is at most negligible.

3.6.3. Fourier transform infrared analysis

The FTIR analysis of the dried COS extracted from TLC resulted from both of the enzymatic hydrolysis of medium molecular weight chitosan and the direct bacterial hydrolysis of SPB was shown in figure (10). Almost quite similar pattern of the spectra was indicated in both samples showing characteristic absorption band at wave length 3431.71 cm^{-1}, derived from O-H stretching vibration merged with that of N–H stretching band. It was broader in case of the enzymatic hydrolysis product of chitosan indicating the presence of more NH_2 groups than that present in the product of the bacterial hydrolysis of SPB. This result was further supported by the absorbencies at 1636.3 cm^{-1} (amide I, single H-bond) and 1551.45 cm^{-1} (amide II) of the product of the bacterial hydrolysis of SPB in compare to the absorbance at 1641.13 cm^{-1} (amide I, double H-bond) of the enzymatic hydrolysis product of chitosan (Brugnerotto et al., 2001).

3.7. Antifungal activity of the partially purified enzyme

The partially purified enzyme showed antifungal activity against the phytopathogenic fungus Fusarium oxysporum as shown in figure (11). The susceptibility of this fungus to chitinolytic enzymes was initially examined using chitinase prepared as described by Ismail et al. (2019) but without presence of inhibition zone. This result can be clarified based on the finding of Kouzai et al. (2012), in which it was indicated that the conversion of the fungal cell wall chitin to chitosan is one of the ways that the phytopathogenic fungi bypass their degradation by the host lytic enzymes.

3.8. Antioxidant activity of chitooligosaccharide

The COS extracted from TLC resulted from both of the enzymatic hydrolysis of medium molecular weight chitosan and the direct bacterial hydrolysis of SPB, showed average antioxidant activity of 110.95 and 55.89 μg TE/mg dry sample respectively (Fig. 12). El-Sayed et al. (2017) reported antioxidant activity range from 1.84 to 86.97 μg TE/mg for COS resulted from the enzymatic hydrolysis of chitosan in which the COS with the lowest molecular weight (< 1 KDa) possessed the highest activity. The strong antioxidant activity of low molecular weight COS with DP > 2, results from the hydrolysis of chitosan, was also emphasized by Doan et al. (2019). The amino groups in the COS are essential for the free radical scavenging activity in which the increase in the number of these groups led to a good antioxidant performance (Avelelas et al., 2019). This seems to be in line with the results of the present study, when comparing the antioxidant activity of the COS with larger number of free amino groups, result from the hydrolysis of chitosan, with that result from the hydrolysis of SPB.

4. Conclusion

In this study, low cost production of chitosanase and COS using SPB as sole carbon and nitrogen source was achieved by SSF using Bacillus cereus strain SSW1 and the productivity of the enzyme was optimized to reach 39.774U/g ds. The partially purified enzyme was indicated to be thermo-active and thermo-stable. These characteristics make the enzyme a superior candidate in various biotechnological applications. The enzyme showed antifungal activity against the phytopathogenic fungus Fusarium oxysporum. Moreover, the produced enzyme was successfully used for the hydrolysis of chitosan and production of COS. The produced COS either by the enzymatic hydrolysis of chitosan or by the direct bacterial hydrolysis of SPB can be utilized as a functional food ingredient as they both processed antioxidant activity of 110.95 and 55.89 μg TE/mg respectively.

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