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Biochemical characterization and thermodynamic study of β-mannanase from *Enterobacter asburiae*

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ABSTRACT

This study aimed for the partial purification, biochemical characterization, determination of bioprocess and thermodynamic parameters of mannanase from newly isolated strain *Enterobacter asburiae* SD26 under submerged culture. From different variables, locust bean gum and combination of peptone + yeast extract was found as best carbon and nitrogen sources respectively. The profiles of mannanases production and growth kinetics of *E. asburiae* SD26 were similar. The maximum specific growth rate (μ_{max}) and productivity (γ_{Pmax}) of enzyme was 0.02 h^{-1} and 0.006 g $l^{-1}hr^{-1}$. The highest mannanases activity was achieved at pH 6.0 and temperature 50°C. The kinetic parameters of mannanases was K_m = 25 mg.ml^{-1}, V_{max} = 2500 µmol.min^{-1}.ml^{-1} and thermodynamic parameters of enzyme, ΔH_d°, ΔG_d°, ΔS_d° were 67.74 KJmol^{-1}, 105.19 KJmol^{-1}, −115.89 J.mol^{-1}.K^{-1} respectively, recorded for mathematical description of the mannanase action at different process conditions.

1. Introduction

Mannans are polysaccharides distributed as the major integrant part of plant hemicelluloses components. It is abundant in hemicelluloses of softwoods and can also be obtained from ivory nuts, coffee beans, legume seeds, soy beans, alfalfa seeds, coconut kernel, palm kernel, konjac roots (Zyla et al., 2010) and also from the cells walls of organisms like fungi, bacteria, and yeasts. These polysaccharides have backbone of D-mannose residues joined linearly by β-1, 4-mannosidic linkages, which further characterized by addition of D-glucose and decorated with D-galactose residues as side chains (Singh et al., 2018). The types of mannans present in nature are glucomannan, galactomannan, galactoglucomannan. β-mannanase (β-1,4-D-mannan mannanohydrolase, EC.3.2.1.78) are a class of enzymes that hydrolyzes mannans by catalyzing random cleavage of β-1,4-D-manno-pyranosyl linkage in the main chain of mannans and results in linear or branched oligosaccharides of various lengths (Chauhan et al., 2012).

There has been great demand for the biotechnologically and industrially stable enzymes with higher catalytic efficiency that can work in harsh conditions (wide range of pH and temperature). β-mannanase produced by a variety of bacteria, fungi, actinomycetes, plants and animals. The extracellular production of microbial mannanases can act in wide ranging pH and temperature conditions (Srivastava and Kapoor, 2017). Industrial application of this enzyme have found in food sector, feed sector, detergent sector. It is also used in pharmaceutical product preparation, oil drilling and textile industries (Zyla et al., 2010). Application of this enzyme has also been found in treatment of coffee and tea waste (Pangsri and Pangsri, 2017).

Production of commercial enzymes from microbial sources has been successfully done by submerged culture. Microbial metabolites generation from the submerged culture process has been used majorly because of the simple and uncomplicated handling that allows the analysis of the factors such as composition of culture medium, pH, and temperature that can affect the production of enzymes (Paludo et al., 2018). Optimization of these parameters leads to the successful processes.

The bioprocess parameters was evaluated to determine factors like maximum specific growth rate (μ_{max}), maximum productivity in product (γ_{Pmax}), duplication time (t_d), total biomass productivity (γ_{Xtotal}), total product productivity (γ_{Ptotal}). The biomass generation yield on substrate utilization ($Y_{X/S}$), product generation yield from substrate consumption ($Y_{P/S}$), and product generation yield from biomass formation ($Y_{P/X}$) was also calculated (Paludo et al., 2918). The kinetics of the process was evaluated to determine parameters like Michaelis - Menten constant (K_m) and maximum velocity (V_{max}) and catalytic constant (K_{cat}). The thermodynamics study of enzyme can explain the denaturation process as temperature is major factor which influences the enzyme activity. Studies of thermodynamic parameters include

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change in enthalpy (ΔH°), entropy (ΔS°) and free energy (ΔG°) for understanding molecular behaviour at different physical parameters (Mostafa et al., 2018).

In the present work, the β-mannanase was produced from the newly found strain *Enterobacter asburiae* SD26 isolated from the wood waste site which evidently proved as the excellent extracellular mannanase producer. The strain could produce extracellular β-mannanase that degrades locust bean gum (LBG). To our foremost knowledge, this is the first report of analysis of production and determination of bioprocess, kinetic, thermodynamic parameters of β-mannanase from *E. asburiae* SD26. *E. asburiae* has been previously reported for the production of various enzymes: hemicellulose hydrolysates from genetically modified *Enterobacter asburiae* JDR-1 (Bi et al., 2009), production of cellulolytic enzyme from *E. cloacae* WPL214 (Lokapirnasari et al., 2015).

2. Materials and methods

2.1. Isolation of β-mannanase producing bacterial strain

Various bacterial strains screened from the soil collected from wood waste site (Chandigarh and Himachal Pradesh, India). Stock prepared by mixing 10 g of the soil samples with 100 ml of 0.85% NaCl solution following successive dilution from 10^{-1} to 10^{-5} was prepared. 100 µl of each diluted waste samples were spread onto LBG-agar plates containing (w/v): 0.2% LBG as carbon source, 0.4% peptone and 0.3% yeast extract as nitrogen source, 0.02% $MgSO_4.7H_2O$, 0.03% KH_2PO_4, 0.02% $CaCl_2$, 1% NaCl as metal ion sources and 2% bacteriological agar, pH 7.0, incubated at 28(± 2) °C for 24 h. The fully grown culture plates were flooded with 0.7% Congo-red dye followed by the washing of plates with distilled water for screening the mannanolytic activity. The isolates producing β-mannanase were selected on the basis of clear zone formation in LBG-agar plates around the bacterial colonies. Final screening was done by culturing the isolates in medium consisting (w/v): 0.2% LBG, 0.4% peptone, 0.3% yeast extract, 0.02% $MgSO_4.7H_2O$, 0.03% KH_2PO_4, 0.02% $CaCl_2$, 0.05% NaCl, pH 7.0.50 ml of the sterile media containing selected strains were incubated at 28(± 2) °C under shaking at 150rpm for 120 h. The culture broth was centrifuge at 8,000 for 15 min at 4°C and supernatant was collected. Standard enzymatic assay was carried to find the high activity producing strain.

2.2. Microorganism identification and taxonomic study

The bacterial strain screened from wood waste site showing highest mannanolytic activity was selected for further study. For identification, the genomic DNA was extracted (Ausubel et al., 1997; Gomes et al., 2000) from the bacterial strain and identified according to 16S rRNA sequence analysis and methods used as described in Bergey's Mannual of Systematic Bacteriology. The template 16S rDNA of the isolate was amplified using the forward primer (8F:5′AGAGTTTGATCCTGGCT CAG3′) and the reverse primer (1541R: 5′AAGGAGGTGATCCAGCC GCA3′). PCR reactions were performed under the following conditions: initial denaturation at 95^{0}C for 2 min and final denaturation then at 95 ^{0}C for 30 s, annealing at 55^{0}C for 30 s, extension at 72^{0}C for 2 min followed by final extension at 72^{0}C for 10 min. Then sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kit with AmpliTaq® DNA polymerase (Applied Biosystems) sequencer to determine nucleotide sequence. The program MUSCLE 3.7 was used for multiple alignments of sequences with databases through the NCBI server (Edgar, 2004). The resulting aligned sequences were determined using the program Gblocks 0.91b (Talavera and Castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and the program Tree Dyn 198.3 was used for tree rendering (Dereeper et al., 2008).

2.3. Optimization of medium composition for mannanase production

The newly found strain was further tested to find out best carbon and nitrogen sources in medium composition. The components tested for carbon sources were 0.5% (w/v): LBG, starch and sucrose whereas for nitrogen source components were 0.4% (w/v): peptone, tryptone and NH_4Cl. The metal ion sources added in all the flasks were same (0.02% KH_2PO_4, 0.05% $CaCl_2$, 0.01% NaCl, 0.02% $MgSO_4.7H_2O$, inoculated with 5% (w/v) 24 h old grown culture and incubated at 30°C under shaking at 150 rpm for 5 days. The cultured broth was centrifuged at 8,000 rpm for 15 min at 4°C and supernatant was collected. Standard enzymatic assay was carried out to find high activity producing carbon and nitrogen sources.

2.4. Production of β-mannanase

The isolate from the wood waste site (Chandigarh, India) was used for the production of β-mannanase in submerged culture process. Enzyme production medium was prepared in 250 ml Erlenmeyer flask containing 50 ml of medium (w/v): 0.5% LBG, 0.4% peptone, 0.3% yeast extract, 0.02% KH_2PO_4, 0.05% $CaCl_2$, 0.01% NaCl, 0.02% $MgSO_4.7H_2O$ with pH adjusted to 7.0. Before inoculating, medium was autoclaved for 20 min at 121°C and inoculated with 5% (v/v) 24h old culture. After the 5 days of growth at 30°C under shaking at 150 rpm, the cultured broth was then centrifuged at 8,000 rpm for 15 min at 4°C. The supernatant obtained was enzyme extract stored at 4°C and also pellet (biomass) was stored for further study.

2.5. Mannanase activity assay and protein determination

Mannanase activity was measured at 50°C for 20 min using the reaction mixture composed of 1 ml of 0.5% (w/v) LBG in 0.2M sodium phosphate buffer (pH 6.0) and 1 ml of enzyme sample. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method of Miller (Miller, 1959). The absorbance of colour obtained by the reaction was measured at 540 nm. One unit of mannanase activity was defined as the amount of enzyme required to produce 1 µmol of mannose sugar per minute under experimental conditions. The protein content was determined as described by Lowry (Lowry et al., 1951) using bovine serum albumin (BSA) as standard. The absorbance was taken at 630 nm.

2.6. Ammonium sulphate precipitation

All the steps performed at 4°C using 0.2 M phosphate buffer pH 7.0. The enzyme in crude cell free extract (supernatant) was partially purified by salting out with ammonium sulphate at concentrations (40-70%) saturations. The solutions were then centrifuged at 10,000 rpm for 20 min and the supernatant was discarded. After centrifugation, the collected precipitate was dissolved in small amount of 0.2M phosphate buffer (pH 7.0). The excess bound salt to protein after the ammonium sulphate precipitation was then removed by dialyzing the protein by immersing in 0.2 M phosphate buffer pH 7.0. Buffer was changed after every 1 h to achieve proper purification. The partially purified enzyme was stored at -4°C and subsequently used for characterization.

2.7. Analytical methods

2.7.1. Biomass determination

The collected biomass was filtered to remove trace of filtrate (enzyme extract) and dried in hot air oven at 60°C till the constant weight of dry biomass was achieved.

2.7.2. Bioprocess parameters and yield factors

The bioprocess parameters maximum specific growth rate (μ_{max}), maximum productivity in product (γ_{Pmax}), duplication time (t_d), total

productivity of biomass (γ_{Xtotal}), total productivity of product (γ_{Ptotal}) for mannanase were evaluated to predict the logarithmic phase of the growth and production of mannanases given in the following Eqs. (1)–(5):

$$\mu_{\max} = \left(\frac{\ln X_f - \ln X_i}{t_f - t_i}\right) \quad (1)$$

$$t_d = \frac{\ln 2}{\mu_{max}} \quad (2)$$

$$\gamma_{P\max} = \left(\frac{P_{flog} - P_{ilog}}{t_{flog}}\right) \quad (3)$$

$$\gamma_{Xtotal}\left(\frac{X_f - X_i}{t_f - t_i}\right) \quad (4)$$

$$\gamma Ptotal = \left(\frac{P_f - P_i}{t_f - t_i}\right) \quad (5)$$

where μ_{max} = maximum specific growth rate (h^{-1}), X_i and X_f = initial and final biomass concentration (g l^{-1}), t_i and t_f = initial and final time for growth and production (h), t_d = duplication time (h), γ_{Pmax} = maximum productivity (g l^{-1} h^{-1}), P_{ilog} and P_{flog} = initial and final product concentration at log phase (g $l^{-1}.h^{-1}$), t_{flog} = total log phase time (h), γ_{Xtotal} = total productivity of biomass (g $l^{-1}h^{-1}$), γ_{Ptotal} = total productivity of product (g.$l^{-1}.h^{-1}$), P_i and P_f = initial and final product concentration (g $l^{-1}h^{-1}$).

The conversion factors calculated for the enzyme were biomass generation yield on substrate utilization ($Y_{X/S}$), product generation yield from substrate consumption ($Y_{P/S}$), product generation yield from biomass formation ($Y_{P/X}$), which is given in the following Eqs. (6)–(8):

$$Y_{X/S} = \Delta X/\Delta S = \frac{X_f - X_i}{S_i - S_f} \quad (6)$$

$$Y_{P/S} = \Delta P/\Delta S = \frac{P_f - P_i}{S_i - S_f} \quad (7)$$

$$Y_{P/X} = \Delta P/\Delta X = \frac{P_f - P_i}{X_f - X_i} \quad (8)$$

Where X_i and X_f = initial and final biomass concentration (g l^{-1}), S_i and S_f = initial and final substrate concentration, P_i and P_f = initial and final product concentration (g $l^{-1}h^{-1}$).

2.8. Biochemical characterization of E. asburiae mannanase

2.8.1. Effect of temperature on mannanase activity

The reaction mixture for enzyme was incubated with 0.2M sodium phosphate buffer (pH 6.0) at different temperature degrees ranges from 30-70°C. The optimum temperature was taken as 100% activity thus the relative activity at each temperature was obtained as a percentage of 100% activity.

The E_a was calculated from the slope of the Arrhenius plot (Michel, 2018) of ln (% mannanase relative activity) versus 1000/T given by the following Eq. (9):

$$E_a = - \text{slope} \times R, \quad (9)$$

Where R is the Gas constant (R = 8.314J.K^{-1}.mol^{-1}). All results were determined in triplicate.

2.8.2. Effect of pH on mannanase activity

The effect of pH on mannanase activity was conducted in 0.2 M concentration of different buffer solutions over a range of pH 4.0 to 9.0. The different buffers used were sodium acetate buffer (pH 4.0-5.0), sodium phosphate buffer (pH 6.0-7.0), Tris - HCl (pH 7.0-8.0) and glycine-NaOH (pH 9.0). The mannanase activity was obtained as the percentage relative activity with respect to its maximum activity. All the results were determined in triplicate.

2.8.3. Kinetic and thermodynamic studies of enzyme reaction

The Michaelis-Menten constant, K_m (mg ml^{-1}) and maximum velocity, V_{max} (μmol.min^{-1}.ml^{-1}) values were calculated using Lineweaver-Burk plot (Lai et al., 2014; Kaushal et al., 2018) at different substrate (LBG) concentration of 5-50 mg.ml^{-1} prepared in 0.2M sodium phosphate buffer. The turn over number or catalytic constant (K_{cat}) value calculated as the ratio of V_{max} of mannanase to specific substrate (LBG) to the total concentration of enzyme [E_0] and expressed as sec^{-1}, which is given in following Eq. (10):

$$K_{cat} = V_{max} / [E_0] \quad (10)$$

For enzyme reaction, the catalysis occurs at a rate depending on the interactions between the enzyme and substrate molecules. According to Arrhenius theory, molecules having minimum amount of energy is sufficient for the activation of the reaction. This energy is called as the activation energy (E_a). At temperature lower than the optimum, the Arrhenius equation given in following Eq. (11):

$$k_0 = A_0.\exp(^{-}E_a/RT) \quad (11)$$

Where k_0 is the initial specific rate constant, A_0 is the molecules interaction frequency, exp (-E_a/RT) is the minimum energy for the molecules interaction to take place, R is the Gas constant R = 8.314 J.K^{-1}.mol^{-1} and T is the absolute temperature in Kelvin.

Thermodynamic parameters change in enthalpy (ΔH°), change in entropy (ΔS°) and Gibb's free energy (ΔG°) are the concept that helps in understanding enzyme reaction and interaction [7] and calculated using the following Eqs. (12) - (14):

$$_{\Delta H}{}^{\circ} = E_a - RT \quad (12)$$

$$\Delta G^{\circ} = -RT.\ln\left(\frac{k_{cat} \times h}{k_b \times T}\right) \quad (13)$$

$$\Delta S^{\circ} = \left(\frac{\Delta H^{\circ} - \Delta G^{\circ}}{T}\right) \quad (14)$$

Where h is the Planck constant (11.04 × 10^{-36} J.min), k_b is the Boltzmann constant (1.38 × 10^{-23} J.K^{-1}).

Temperature Coefficient (Q_{10}) is a factor by which reaction rate changes with increase in temperature by 10°C and was calculated by the Dixon and Webb equation (Saqib et al., 2010) which is given in following Eq. (15):

$$\ln Q_{10} = \frac{E_8}{RT^2} \times 10 \quad (15)$$

where Ea is the activation energy of the enzyme (J.mol^{-1}), R is gas constant; T is the absolute temperature (K).

2.8.4. Kinetic and thermodynamic studies of enzyme thermal denaturation

At constant temperature or the temperature higher than the optimum one, the activity of the enzyme tends to lose as a function of time. It has been proposed that the rate of enzyme activity decay is directly proportional to the enzyme activity and expressed as first-order irreversible reaction which is given in the following Eq. (16):

$$\frac{dA}{dt} = -k_d \times A \quad (16)$$

Where A is the enzyme activity, t is the incubation time at a given temperature and k_d is the first-order denaturation constant.

The k_d at different temperature range (from 50 to 80°C) was determined as the slope of straight line from the natural logarithm plot of ratio of residual enzyme activity to initial enzyme activity ln (A_{res}/A_0) versus the incubation time in min.

The activation energy for enzyme denaturation (E_d) was determined from (Eq. (1)), where k_o is replaced as k_d at each temperature. Thus the slope of plot natural logarithm denaturation rate constants (ln k_d) versus the reciprocal of absolute temperature in Kelvin gives E_d.

Also, various other important parameters like half-life ($t_{½}$), decimal reduction time (D-value), were calculated that tells the influence of temperature on enzyme denaturation process.

$t_{1/2}$ is the time after which the enzyme activity reduces to one-half of its initial enzyme activity and calculated as given in following Eq. (17):

$$t_{1/2} = \frac{\ln 2}{k_d} \quad (17)$$

D-value is the time needed for the 10-fold reduction of initial activity at a given temperature and calculated as given in following Eq. (18):

$$D = \frac{\ln 10}{k_d} \quad (18)$$

The change in enthalpy (ΔH_d°, KJ.mol^{-1}), entropy (ΔS_d°, J.mol^{-1}.K^{-1}), and Gibb's free energy (ΔG_d°, KJ.mol^{-1}) as thermodynamic parameters for mannanase denaturation were calculated using following Eqs. 19–21:

$$\Delta H_d^\circ = E_d - RT \quad (19)$$

$$\Delta G_d^\circ = -RT.\ln\left(\frac{k_d \times h}{k_b \times T}\right) \quad (20)$$

$$\Delta S_d^\circ = \left(\frac{\Delta H_d^\circ - \Delta G_d^\circ}{T}\right) \quad (21)$$

where E_d is the activation energy of enzyme denaturation, T is the absolute temperature (K), R is the gas constant, h is the Planck constant and k_b is the Boltzmann constant.

3. Results

3.1. Isolation and identification of bacterium producing high β-mannanase activity

Two bacterial isolates were selected on the basis of clear zone formation around their colonies on LBG-agar plates that showed the production of extracellular mannan degrading enzyme. The bacterial strain (W1) from the soil of wood waste sample from a Chandigarh, India was selected for further studies based on the colonies having largest diameter and highest enzyme activity. Colonies were large, raised, mostly round having slightly mucoid surface. Gram staining results showed W1 to be Gram-negative short rods. From 16s rRNA nucleotide sequencing analysis, the GenBank reported the relationship with closely related *Enterobacter asburiae* L1 strain (Accession number KU212142.1) with an aligenment score of ≥ 200 and 91% homology. Therefore, our strain was identified as *Enterobacter asburiae* SD26 under the GenBank Accession number MH718845. In this study, the β-mannanase produced from this strain referred as ManSD26 for the convenience (Fig. 1).

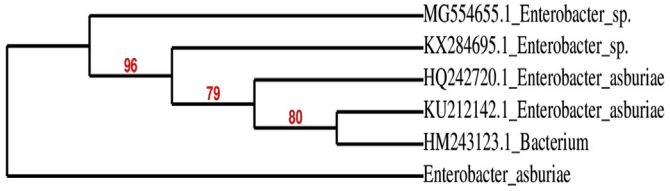

Fig. 1. Phylogenetic tree based on 16s rRNA nucleotide sequencing showing the position of *Enterobacter asburiae* SD26 and other related species of genus *Enterobacter*.

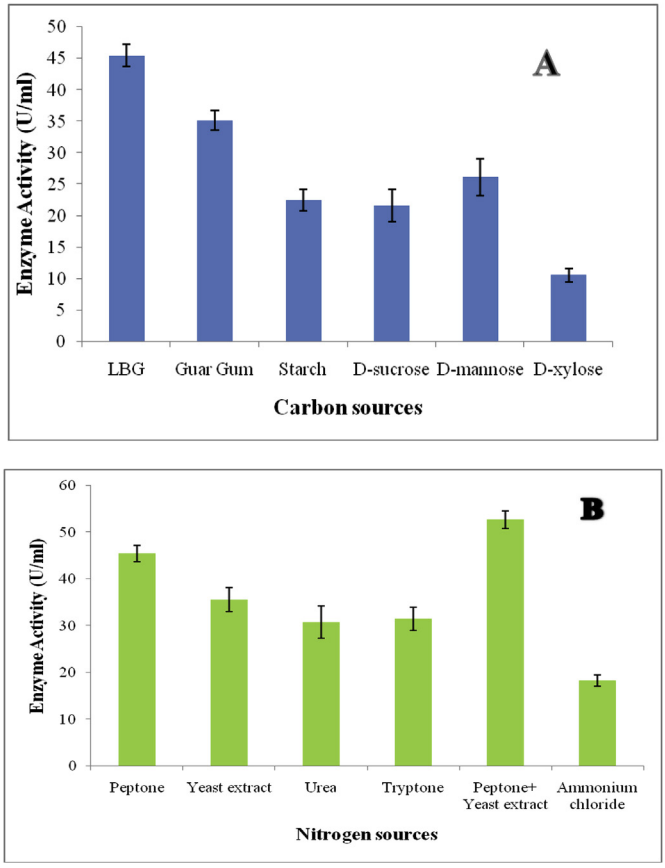

Fig. 2. Mannanase activity on different (A) carbon sources and (B) nitrogen sources.

3.2. Medium composition for enzyme production

The ManSD26 production data presented in Fig. 2 (A and B) revealed that the different carbon source (0.5% w/v of LBG, guar gum, starch, D-sucrose, D-mannose and D-xylose) and nitrogen source (0.4% w/v of peptone, yeast extract, urea, peptone + YE, ammonium chloride) support enzyme production form *E. asburiae* SD26.

LBG and guar gum galactomannan yield 45.5 and 35.2 U/ml of mannanase respectively. With starch as polysaccharides, mannanase production was less 22.4 U/ml whereas with sugars such as D-sucrose, D-mannose and D-xylose yield 21.1, 26.1 and 10.5 U/ml mannanase respectively (Fig. 2A). Therefore, it is evident that *E. asburiae* SD26 utilizes LBG (carbon source) as substrate for the production of β-mannanase.

The effect of nitrogen sources on the mannanase production was determined using 0.5% w/v LBG as sole source of carbon. All the evaluated nitrogen sources showed the increased production than that obtained with ammonium chloride (18.3 U/ml) used in the media as inorganic nitrogen source. High enzyme activity was obtained in media having combination of peptone and yeast extract yielding 52.7 U/ml of mannanase (Fig. 2B).

3.3. Production of β-mannanase

E. asburiae SD26 excreted that gave high yield of extracellular mannanases when grown on 0.5% (w/v) LBG as a substrate in submerged culture process incubated at 30°C, 150 rpm and neutral pH. The maximum mannanase activity of 2200 U was observed after 120h. This is the first report showing the production of extracellular β-mannanase from *E. asburiae* SD26 secreting mannanases at 30°C. Maximum enzyme production was observed in incubation period of 3-5 days after which

Table 1
Summary of β-mannanase from *E. asburiae* SD26.

Form of enzyme	Total Volume (ml)	[a]Total protein (mg)	[b]Total mannanase activity (U)	[c]Specific mannanase activity (U/mg)	Purification fold	% yield
Crude enzyme	100	18.4	2200	119.56	1	100
Ammonium sulphate precipitated and dialysed	50	7.5	1175	156.67	1.31	53.4

[a] The total protein content was determined by Lowry method using bovine serum albumin (BSA) as standard.
[b] Mannanase activity was calculated in 0.2M sodium phosphate buffer at 50°C in 0.5% (w/v) locust bean gum substrate by DNS method.
[c] The specific mannanase activity was determined by ratio of total mannanase activity to total protein content, expressed as U/mg.

the enzyme activity decreased considerably (data not shown). However, there have been reports on the mannanase production from *E. ludwigii* MY271 after incubating for 24 h at 30°C and 160 rpm (Yang et al., 2016) and from *Enterobacter sp.* Strain N18 for 48 h at 37 °C (You et al., 2016). Two step partial purification procedures were done. The crude protein was obtained from 70% saturation ammonium sulphate precipitation at 4°C for an overnight, followed by dialysis that resulted in increased specific activity 156.67 U/mg and concentration increased up to 1.31 fold and 53.4% yield (Table 1).

3.4. Bioprocess parameters and yield factors

The result of mannanase production and growth kinetics of *E. asburiae* SD26 was observed for 5days i.e. 120h represented in Fig. 3, to optimize all the parameters throughout. The culture was grown in previously optimized best carbon and nitrogen sources in culture medium composition (w/v): 0.5% LBG, 0.4% peptone, 0.3% yeast extract, 0.02% KH_2PO_4, 0.05% $CaCl_2$, 0.01% NaCl, and 0.02% $MgSO_4.7H_2O$ (pH 7.0) at 28°C under shaking at 150 rpm. After every 12 h, the sample was collected for the analysis of bioprocess parameters of the enzyme production. The strain *E. asburiae* SD26 showed the lag-phase in ~48h for adaptation in the provided culture conditions. After that, the log phase was observed between 48h to 120h and after this time period stationary phase was achieved that showed the stabilized growth of the culture (data not shown), therefore the calculations was done considering log phase only. Similar profile was observed for the product formation i.e. enzyme production. The biomass and product during log phase was in the range between 111.6 and 140.6 g l^{-1} and 0.48-0.93 g l^{-1}. After 120h, the growth and production remained constant whereas substrate (LBG) consumption was decreased up to 0.14 g l^{-1} in 120th h. This showed that LBG as the substrate enhanced the production for both culture and enzyme.

Table 2
Bioprocess parameters and yield factors of *E.asburiae* SD26 mannanase.

[a]Bioprocess parameters	
μ_{max} (h^{-1})	0.02
t_d (h)	34.65
γ_{Pmax} (g $l^{-1}.h^{-1}$)	0.006
γ_{Xtotal} (g $l^{-1}.h^{-1}$)	1.08
γ_{Ptotal} (g $l^{-1}.h^{-1}$)	0.007
[b]**Yield Factors**	
$Y_{X/S}$	129.78
$Y_{P/S}$	1.15
$Y_{P/X}$	0.007

[a,b]Calculated from Eqs. (1)–(5) and Eqs. (6)–(8) respectively.

Based on the data represented in Fig. 3, bioprocess parameters of mannanase production was calculated (described in section 2.7.2) and shown in Table 2. The results showed the biomass production by *E. asburiae* SD26 in culture medium. In this medium, the strain showed maximum specific growth rate (μ_{max}) of 0.02 h^{-1} and duplication time (t_d) of 34.65h. Enzyme production profile being associated with growth profile of *E. asburiae* SD26, the maximum ManSD26 productivity (γ_{Pmax}) was 0.006 g $l^{-1}.h^{-1}$, total productivity in biomass and product was 1.08 and 0.007 g $l^{-1}.h^{-1}$ respectively. The conversion factors calculated for the enzyme were biomass generation yield on substrate utilization ($Y_{X/S}$ = 129.78 g biomass g^{-1} substrate), product generation

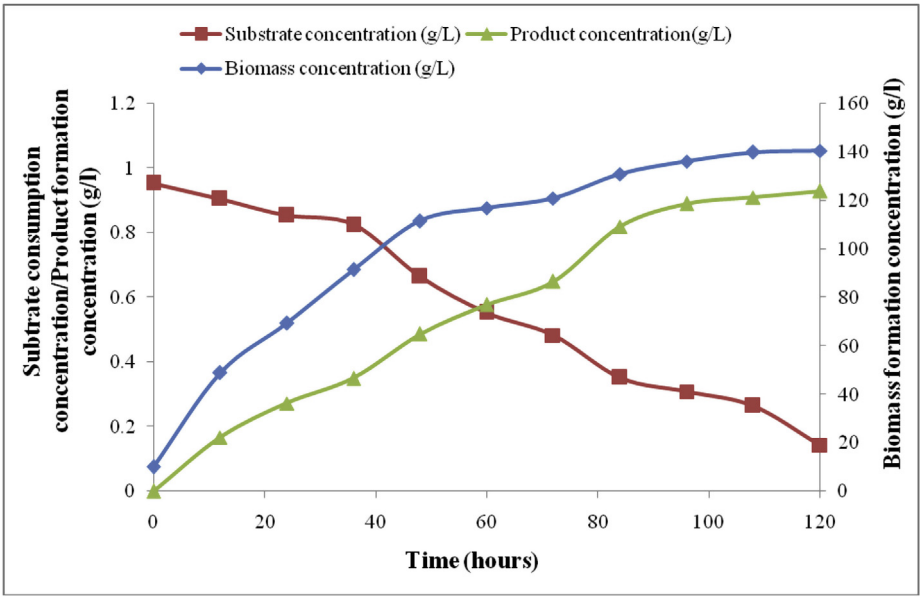

Fig. 3. Substrate consumption (g l^{-1}), biomass and production formation (g l^{-1}) profile versus time (hours).

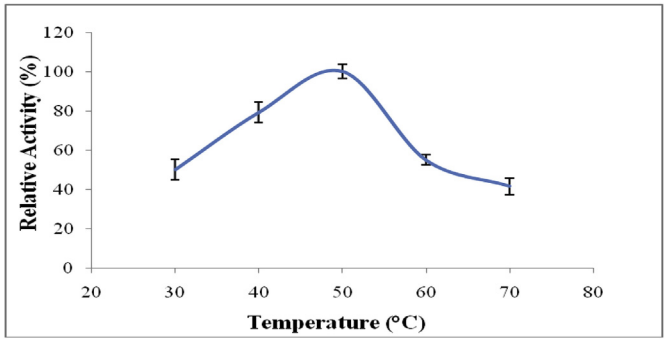

Fig. 4. Relative activity profile versus temperature (°C) for determination of optimum temperature for ManSD26.

yield from substrate consumption ($Y_{P/S}$ = 1.15 g product g^{-1} substrate), product generation yield from biomass formation ($Y_{P/X}$ = 0.007 g product g^{-1} biomass). For any industrial process applications, it depends on the choice of microorganisms to be used, their culture conditions and operational criteria. Hence, the evaluation of bioprocess parameters could give idea about reduction of expenses together with productivity parameters.

3.5. Biochemical characterization of *E. asburiae* SD26 mannanase

3.5.1. Effect of temperature and pH on mannanase activity

The excreted mannanases from *E. asburiae* SD26 was incubated at a temperature range from 30°C to 70°C to determine the effect of mannanase activity. ManSD26 showed highest relative activity of 96 ± 3.06% at 50°C (Fig. 4). It has been reported that mannanase from different bacterial sources having optima temperature ranging from 45°C to 85°C (Gibbs et al., 1999; Gomes and Steiner, 1998). However, the mannanase from *Enterobacter sp.* strain N18 (You et al., 2016) and *Klebsiella oxytoca* CW23 (Titapoka et al., 2008) had same optimum temperature 50°C as that of ManSD26. However, the optimal value of ManSD26 was closely related to mannanases from *E. ludwigii* MY271 having optimum temperature 55°C (Yang et al., 2016).

The mannanase activity produced from *E. asburiae* SD26 was determined over a wide range of pH 4.0 to pH 9.0 that showed optimum activity (90.3 ± 1.1%) at pH 6.0 (Fig. 5). Below the optimum pH, the activity deteriorated to 41 ± 1.6% at pH 4.0, implies extracellular β-mannanase *E. asburiae* SD26 (ManSD26) is slightly acidic in nature. However, the activity retained up to 77.6 ± 4.6% at pH 9.0. It has been reported previously that mannanases from *Paenibacillus thiaminolyticus* (Takeda et al., 2004) showed same optimum pH 6.0 as *E. asburiae* SD26 whereas mannanases from *E. ludwigii* MY271 (Yang et al., 2016) and *Klebsiella oxytoca* CW23 (Titapoka et al., 2008) showed maximum activity at neutral pH and from *Enterobacter sp.* strain N18 (You et al., 2016) showed optimum pH 7.5. Moreover, microbial mannanases were known to have maximum activities in wide pH ranging from 4.0 to 10.0 (Takeda et al., 2004).

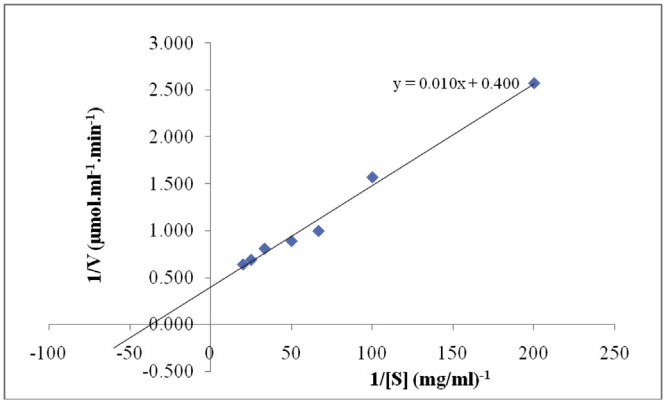

Fig. 6. Lineweaver-Burk plot determining kinetic and thermodynamic parameters.

3.5.2. Kinetic and thermodynamic parameters of enzyme activation

The kinetic parameters of ManSD26 from *E. asburiae* SD26 were determined using Lineweaver-Burk plot, 1/[V] versus 1/[S] gave a straight lines as shown in Fig. 6. The kinetic parameters (V_{max}, K_m, V_{max}/K_m and K_{cat}) represented in Table 3 (described in section 2.8.3) were determined to be 2500 µmol.min^{-1}.ml^{-1}, 25 mg.ml^{-1}, 100 µmol.min^{-1}.mg^{-1} and 7.57 sec^{-1} respectively showing affinity for LBG. The K_m and V_{max} values for ManSD26 was higher than previously reported values for mannanase from different bacterial strain using LBG galactomannan as substrate like *E. ludwigii* MY271 (K_m 26.6 ± 0.1 mg.$ml^{-1}$and V_{max} 961 ± 6.0 µmol.min^{-1}.mg^{-1}) (Yang et al., 2016), *Enterobacter sp.* strain N18 (K_m 3.427 mg.$ml^{-1}$and V_{max} 134.05 µmol.min^{-1}.mg^{-1}) (You et al., 2016), *Klebsiella oxytoca* KUB-CW-3 (K_m 1.06 mg.$ml^{-1}$and V_{max} 6.15U.ml^{-1}) (Chantorn et al., 2013), *Bacillus subtilis* BE-91 (K_m 7.14 mg.$ml^{-1}$and V_{max} 107.5 µmol.min^{-1}.ml^{-1}) (Cheng et al., 2016). K_{cat}, turn over number that corresponds to the number of substrate converted into product per active site per unit time. The k_{cat} value of ManSD26 towards LBG was 7.57 sec^{-1} which is

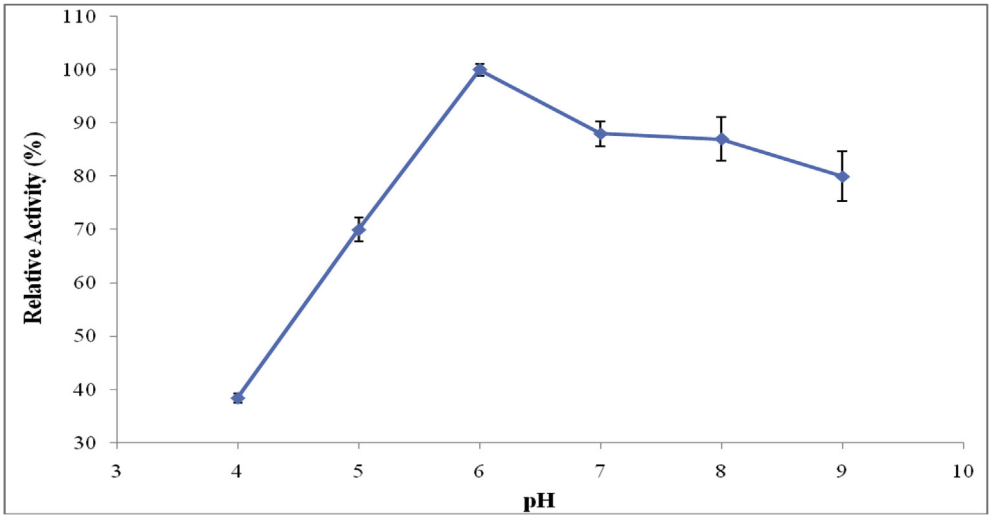

Fig. 5. Relative activity profile versus pH for determination of optimum pH for ManSD26.

Table 3
Kinetic and thermodynamic parameters of *E. asburiae* mannanase activation.

Kinetic and thermodynamic parameters	
[a]K_m (mg.ml^{-1})	25
[b]V_{max} (μmol.min^{-1}.ml^{-1})	2500
V_{max}/K_m (μmol.min^{-1}.mg^{-1})	100
K_{cat} (sec^{-1})	7.57
[c]E_a (KJmol^{-1})	28.19
ΔH° (KJ mol^{-1})	25.50
ΔG° (KJmol^{-1})	73.66
ΔS° (J.mol^{-1}.K^{-1})	149.1
[d]Q10	1.38

[a] Calculated from (Fig. 6).
[b] Calculated from (Fig. 6).
[c] Activation Energy calculated from (Fig. 7).
[d] Temperature coefficient measured at 50°C.

this first report showing the catalytic constant for β-mannanase from *E. asburiae* SD26. However, the kinetic parameter depends on the source and techniques used from which mannanase produced such as type of substrate used, substrate concentrations and optimal pH and temperature (Tuntrakool and Keawsompong, 2018).

The thermodynamic parameters (E_a, ΔH°, ΔG° and ΔS°) are crucial to discuss and understand the previous results represented in Table 3. The activation energy (E_a) for ManSD26 was 28.19 KJmol^{-1} determined from the slope of the Arrhenius plot (Michel D., 2018) of ln(% mannanase relative activity) versus 1000/T (Fig. 7) showing enzyme required lower energy to overcome the barrier to utilise its substrate to convert into product. E_a leads to another parameter of activity, temperature coefficient Q_{10} value (Table 3), found to be 1.38 at 50°C. The Q_{10} values for enzymes normally range between 1 and 2, divergence from this range indicates involvement of other factors than temperature in controlling of reaction rate (Saqib et al., 2010). This is the first report showing the thermodynamics of β-mannanase activation.

3.5.3. Kinetic and thermodynamic parameters of enzyme thermal denaturation

Enzymes are subjected to denaturation upon changes in temperature and their prolonged exposure can reduce the enzyme activity during their storage and usage in industrial applications. Some of the parameters can be evaluated to restrain the enzyme inactivation and reduce their denaturation whereas the above enzyme activation parameters are helpful to determine their catalysed reactions. Thus enzyme activity expressed as a function of both temperature and its exposed time due to the thermal inactivation. The increase in temperature leads to increase in collision energy of the molecules ultimately leading to breakage of hydrogen bond, result in loss of enzyme activity gradually over the period of time.

Estimation of first-order thermal inactivation constant (k_d) for ManSD26 was not successful below 50°C thus kinetic parameters were determined at temperature ranging from 50°C to 80°C incubating for different period of time (from 10 min to 90 min). The results illustrated from the natural logarithm plot of ratio of residual enzyme activity to initial enzyme activity ln (A_{res}/A_0) versus the incubation time in min (Fig. 8). The kinetic parameters (k_d, $t_{½}$, D-values) tells the influence of different temperature on enzyme denaturation. The k_d; $t_{½}$; D-values at temperature 50°C; 60°C; 70°C; 80°C were 0.004, 0.017, 0.031, 0.038; 173.25, 40.76, 22.35, 18.24; 575.64, 135.44, 74.27, 60.59 respectively. The kinetic parameters values obtained were decreased with increase in temperature Table 4 (described in section 2.8.4).

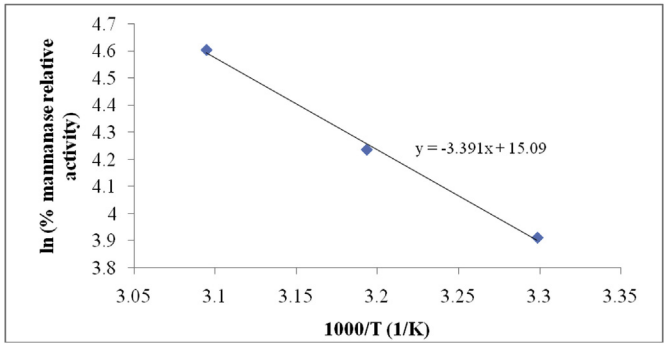

Fig. 7. Arrhenius plot to determine activation energy for enzyme reaction (E_a).

The activation energy for enzyme denaturation (E_d) was determined from the slope of plot natural logarithm denaturation rate constants (lnk_d) versus the reciprocal of absolute temperature in Kelvin (Fig. 9). E_d was calculated to 70.43 KJ.mol^{-1} which signifies that the energy needed by the enzyme to get denatured. However, high Ed values are required for industrial enzyme applications because they are considered thermally stable.The change in enthalpy of thermal inactivation (ΔH°_d) is analogous to the disruption of non-covalent bonds during denaturation of enzymes, was found to be between 67.49 and 67.74 KJ mol^{-1} for ManSD26. It was observed that ΔH°_d decreases with elevation in temperature (Table 4). The change in Gibbs free energy (ΔG°_d) measures the spontaneity of enzyme reaction i.e. it indicates how favourable the inactivation process is; higher the value of ΔG°_d, more the enzyme is thermally stable. It is worth registering that the force stabilizing the protein by several hydrogen bonds and Vander Waals interaction are lost when protein unfolds or denatures. The enzyme structure disarrangement corresponds to the randomness, increase in disorder. At all the studied temperatures (Table 4), change in entropy (ΔS°_d) was found to be negative. This could be due to the charged particle formation around the enzyme molecules because of enzyme compactness. Also some authors reported that the transition state is more ordered that results in negative entropy (Violet and Meunier, 1989; Gummadi and Panda, 2003).

4. Conclusion

β-mannanase has been beneficial for biotechnological and industrial applications. In this work, extracellular mannanases was produced from novel producer *E. asburiae* SD26. The enzyme showed its optimal activity at pH 6.0 and 50°C. High level of mannanase was produced using locust bean substrate (LBG) as a sole carbon source. LBG galactomannan comprised of mannose and galactose side chain, which significantly showed the enhanced production of mannanase from our isolated strain. The bioprocess parameters showed that maximum productivity of the product generation at log phase of the growth kinetics of the bacterial strain as the similar profile was observed for both bacterial growth and enzyme production. Kinetic parameters showed that, ManSD26 has the affinity for the LBG i.e. excellent catalysis efficiency for galactomannan degradation. The thermodynamic parameter of enzyme inactivation suggested its denaturation at higher temperature with respect to its prolonged exposure. The results indicated its satisfactory potential for the further exploitation and utilization in industrial applications.

Conflicts of interest

The authors with listed names declare no conflict of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101211.

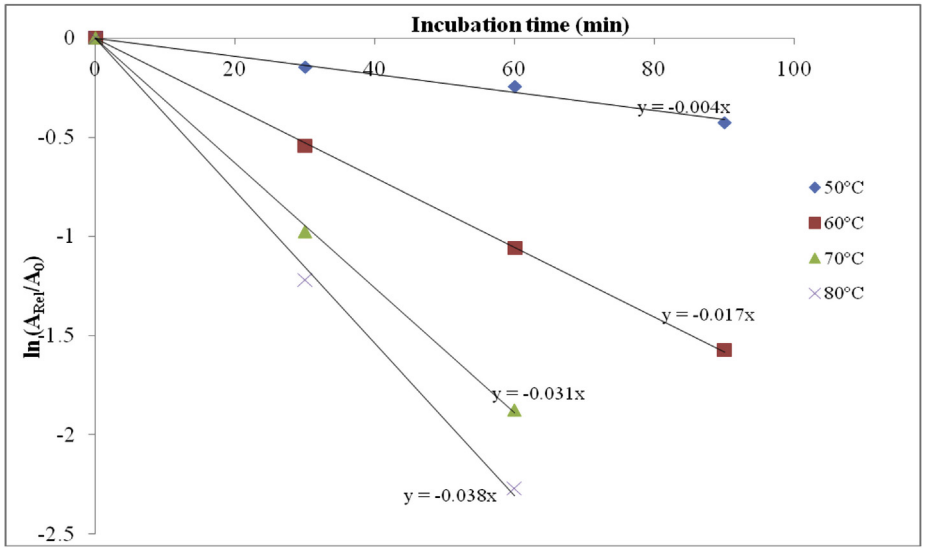

Fig. 8. Determination of first-order thermal inactivation constant (k_d) values of mannanase from *E. asburiae* SD26.

Table 4
Kinetic and thermodynamic parameters of thermal denaturation.

Temperature (°C)	k_d[a] (min^{-1})	$t_{½}$ (min.)	D-value (min)	ΔH°_d (KJ mol^{-1})	ΔG°_d (KJ mol^{-1})	ΔS°_d (J.$mol^{-1}.K^{-1}$)
50	0.004	173.25	575.64	67.74	105.19	–115.89
60	0.017	40.76	135.44	67.66	104.52	–110.64
70	0.031	22.35	74.27	67.57	106.03	–112.07
80	0.038	18.24	60.59	67.49	108.6	–116.4

[a] The value of k_d was obtained from Fig.(8), Activation energy for denaturation E_d (to calculate ΔH°_d) = - slope × R = 70.43 KJ mol^{-1}. The slope is obtained from Fig. (9).

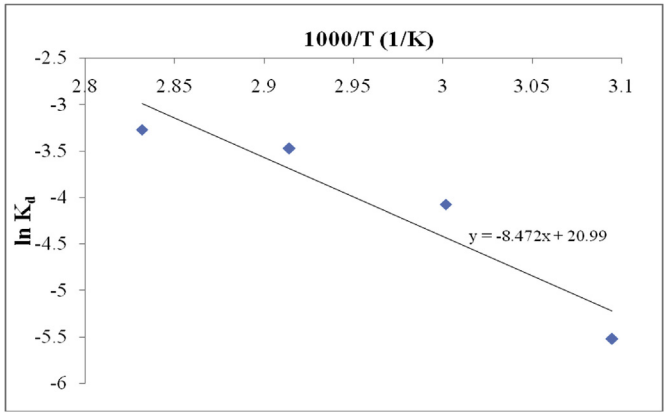

Fig. 9. Arrhenius plot to determine activation energy for enzyme denaturation (E_d).

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