Role of Metal Ions, Surfactants and Solvents on Enzymatic Activity of Partial Purified Glucoamylase from *Aspergillus niger* ER05

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Abstract: The glucoamylase requirement of different industries should vary in their physiochemical and functional properties, so the investigation of new sources for the novel enzymes is the only solution. The current study describe the production of glucoamylase from *Aspergillus niger* ER05 in a submerged fermentation. The enzyme then partially purified and then effect of thirteen different metal ions (Cs^+, K^+, Na^+, Ba^{2+}, Ca^{2+} Co^{2+}, Cu^{2+} , Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} , Fe^{3+}), surfactant as well as organic solvent on catalytic performance of glucoamylase was studied. A newly isolated *Aspergillus niger* ER05 is hyper producing strain of glucoamylase. Specific activity for the crude enzyme was found to be 6.87 KU/mg. The crude enzyme was partially purified via fractional ammonium sulphate precipitation. Ammonium sulphate saturation from 40-80% was found suitable to precipitate the enzyme. After dialyzing precipitates, the specific activities were found to be 66.33KU/mg with percent yield of 26.5. The inhibitory influence of all metal studies was interestingly found on glucoamylase activity. The strong inhibition was demonstrated in presence of Hg^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}. Only Na^+ ions were able to maintain the 101% relative activity at 1mM concentration. The SDS completely inhibits the enzyme activity and in presence of tween-80 and triton X-100 glucoamylase exhibited less than 45% relative activity. Furthermore, formaldehyde, isopropanol, ethanol, methanol, and DMSO stabilized the enzyme activity while chloroform inhibits enzyme activity by 48%.

Keywords: Activators, inhibitors, metal ions, surfactants, organic solvents, glucose, *Aspergillus niger*.

INTRODUCTION

Glucoamylase (α-1, 4-glucan-glucohydrolases, EC 3.2.1.3) have its own place in the catalytically active proteins family because of the broad possibilities of industrial applications [1]. Glucoamylase is an extracellular enzyme that yields ß-D- glucose as the end-product after hydrolyzes α- 1, 4-glycosidic bonds of starch only from the non-reducing end [2, 3]. The yield of glucose serves as a basic carbon source and driving force for fermentations, conventionally industrial production of glucoamylase was carried out by submerged fermentation [4, 5].

The industrial production of glucose after partial digested of starch with amylase majorly depends on glucoamylase. All the saccharified products from dextrin majorly required glucoamylase. Food and confectionary industries utilized high-fructose corn syrups (HFCS) produced after digestion of corn starch by glucoamylase [6]. The production of bioethanol required glucoamylase in first step to produce glucose for the fermentation. Glucose feed stocks can be further utilized in the commercial production of amino acids [7], in synthesis of drugs [8, 9] and in production of agro-chemicals [10, 11].

Aspergillus niger is the major glucoamylase producer from the microbial sources in enzyme production industries. The enzyme can easily recover from cell free filtrates after fermentation [12]. Glucoamylase reported from the various fungal sources widely differ from each other in their physico-chemical properties such as optimal conditions, influence of metal ions, stimulator or inhibitor specificity of different organic solvents and surfactants [13, 14]. Metals were required by some enzymes for their maximal catalytic activity [15, 16]. Metals also help in right orientation of active site of enzymes and also responsible for protein structure stabilization [17, 18]. One of the reported *A. niger* glucoamylase was found to be activated by metals. Presence of Mn^{2+} and Fe^{2+} increased the enzyme activity [19]. *Aspergillus flavus* glucoamylase was found to be activated by Mn^{2+}, Co^{2+} and Ba^{2+} and was inhibited by Hg^{2+}, Fe^{3+}, Zn^{2+} and Cu^{2+} [20].

As the importance of glucoamylase, it is highly desirable to enhance the glucoamylase activity.

Therefore, for the better understanding of enzymatic reactions study of different inhibitors, destabilizers, and activators, is very significant. The objective of the present investigation is to find out the environmental contaminant influences on the activity of glucoamylase obtained from *A. niger* ER05. The metal ions, surfactants and organic solvents effect is investigate on the partially purified enzyme preparations in order to

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ISSN: 1814-8085 / E-ISSN: 1927-5129/18 © 2018 Lifescience Global

characterize above elements as activators, inhibitors and stabilizers.

MATERIAL AND METHODS

Fungal Strain and Submerged Fermentation Conditions

Aspergillus. niger ER05 has been isolated from soil and identified by sequence analysis and phylogenetic studies. Sequence with accession no: MF579602 has been submitted to NCBI GenBank. Pure cultures were maintained in PDA medium at 4°C and were repeatedly subculture after 30 days period.

The submerged fermentation has been done in optimized medium consist of 1 % soluble potato starch, 0.2% yeast extract, 0.1% peptone and 0.05% of $MgSO_4$ (pH 5.0). The culture was subjected to submerged fermentation for 4 days at 25±2°C. After fermentation, the mycelia were removed by filtration and then collect cell free filtrate.

Precipitation and Desalting of Proteins

The cell free fluid was subjected to fractional ammonium sulphate precipitation. First fractionation of proteins was precipitate out at 40% ammonium sulphate saturation. Precipitates were separated out by centrifugation at 4000rpm. The supernatant was again saturated with ammonium sulphate till 80%. The collected precipitates were dissolved 25mM Na-acetate buffer (pH4.0). The sample was dialyzed overnight against the same buffer. Total protein was estimated by the method of Lowry [21].

Glucoamylase Assay

Glucoamylase activity was determined by using potato soluble starch (1.0%) as substrate in 25mM sodium acetate buffer (pH4.0). Dialyzed enzyme sample (0.075ml) mixed with 1.0 ml substrate and placed at 70°C for 5 min. After that, boil the reaction tubes for 5 min and then cooled to room temperature. Glucose oxidase (GOD-PAP) method was used to estimate the liberated glucose. The standard glucose was used as standard as described by Trinder [22, 23]. The absorbance of test and control were read at 546 nm using spectrophotometer.

One unit of glucoamylase is defined as ‘‘amount of enzyme that released 1.0 µM of glucose per minute under the standard assay conditions’’ and in the current study it is expressed as KU/ml/min. (1 kilo units/ml/min is equal to 1000 units/ml/min)

Effect of Different Metal on Glucoamylase Activity

A number of metal ions with valences from monovalent to trivalent ions were used to study their effect on the activity of glucoamylase. Chloride salts of all the metal ions were used to in this study. The concentrations, 1.0, 5.0 and 10.0 mM of three monovalent metal salts (Cs^+, K^+, Na^+), nine divalent metal ions (Ba^{2+}, Ca^{2+} Co^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}) and one trivalent metal ion (Fe^{3+}) were prepared in sodium acetate buffer (25 mM and pH 4.0). Each of the reaction mixtures contains of enzyme and metal ion solutions in 1:1 ration and kept at 37 °C temperatures for 60 min. After completion of incubation, enzymatic activity of the samples was conducted at 70 °C according to standard procedure describe above. The control of reaction was established without metal ion. Enzyme activity in the absence of metal ion consider as 100 percent was used to calculate percent relative activity of glucoamylase.

Effect of Surfactant on Glucoamylase Activity

Effect of several surface active agents on glucoamylase activity was determined by incubating enzyme with anionic (tween 20, tween 80, triton X-100) and nonionic surfactants such as sodium dodecyl sulfate. Glucoamylase was incubated with 10, 50 and 100 mM concentrations at 37 °C for 1 hour. Control (without surfactant) was setup and its enzyme activity was taken as 100%.

Effect of Solvents on Glucoamylase Activity

Various organic solvents exert prominent effect on glucoamylase activity. Effect of chloroform, DMSO, ethanol, formaldehyde, isopropanol and methanol was evaluated for one hour at 37 °C with three different concentrations of solvents i.e.1.0, 5.0 and 10.0 mM. Control enzyme activity (100%) was setup devoid of solvent.

RESULTS AND DISCUSSION

Glucoamylase from *Aspergillus niger* ER05 was produced under optimized culture conditions in submerged fermentation. After fermentation, cell free filtrate collected and proteins was precipitated out in two step ammonium sulphate precipitation. First fraction of 0 - 40% saturation was removed and CFF again precipitated till 80% saturation. The precipitate then dialyzed and the enzyme activity was determined in collected precipitates. Glucoamylase was partially

Table 1: Partial Purification Procedure of Glucoamylase from *Aspergillus niger* ER05

Purification step	Total Enzyme (KU)	Total Protein (mg)	Specific activity (KU/mg)	Fold purification	Yield (%)
Crude CFF	2828.7	411.7	6.87	1	100
40% NH_4SO_4	31.1	4.4	7.02	1.02	1.1
80% NH_4SO_4	802.7	13.98	57.41	8.35	28.3
Dialyzed PPT (80%)	751.1	11.3	66.33	9.65	26.5

purified up to 26.5 fold with specific activity of 66.33KU/mg (Table **1**).

Effect of Several Metals on Glucoamylase Activity

The interactions of several metal ions to glucoamylase from *Aspergillus niger* ER05 was studied by incubating enzyme with metal ion solutions. Three different concentrations of each metal ion were investigated. The relative activity was measured with reference to the enzyme activity present in the absence of any metal ion. In enzyme substrate reactions, metal ions have ability to influence the rate of reaction as they bind on active site of enzyme and can be grouped into inhibitors, activators and stabilizers at appropriate concentration [18].

Results outcomes presented that three monovalent ions i.e. Cs^+, K^+ and Na^+ exhibited inhibitory effect on enzyme activity at 10mM concentration as the relative activity was 61%, 67% and 57% respectively (Table **2**). Subsequently, the relative activity of Na^+ increases to 101% when the ten times decrease concentration was tested. Hua *et al.*, [24] has been found that glucoamylase activity from *Bispora* sp. MEY-1 has been increased by monovalent cations i.e. Li^+, Na^+, K^+ and 5 mM Na^+ increased relative activity of glucoamylase to 140%.

In the presence of all nine divalent metal ions, inhibition in the glucoamylase activity was represented in Figure **1**. Ba^{2+}, Ca^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+} acted as inhibitors as they decreased glucoamylase activity via 44%, 46%, 38%, 48% and 52% respectively at 10 mM concentration.

While, a higher inhibitory effect has been noticed with 10mM Cu^{2+} (40% relative activity), Zn^{2+} (33% relative activity) and Ni^{2+} (32% relative activity). Similar results have been reported by Hua *et al*., 2014 with Cu^{2+} at same concentration. However, Ni^{2+} and Zn^{2+} increased the enzyme activity [24]. One of the amyloglucosidase reported by Gudi *et al.* in 2013, showed the reduction in enzyme activity by following

Table 2: Effect of Different Metal Ions on Glucoamylase from *Aspergillus niger* ER05

Metal ions	Relative Activity (%)		
	1mM	5mM	10mM
Control	100		
CsCl	76	69	61
KCl	72	69	67
NaCl	101	63	57
$BaCl_2$	50	56	56
$CaCl_2$	51	53	54
$CoCl_2$	56	58	62
$CuCl_2$	48	44	40
$HgCl_2$	32	30	16
$MgCl_2$	42	46	52
$MnCl_2$	47	48	48
$NiCl_2$	48	40	32
$ZnCl_2$	28	33	33
$FeCl_3$	32	38	41

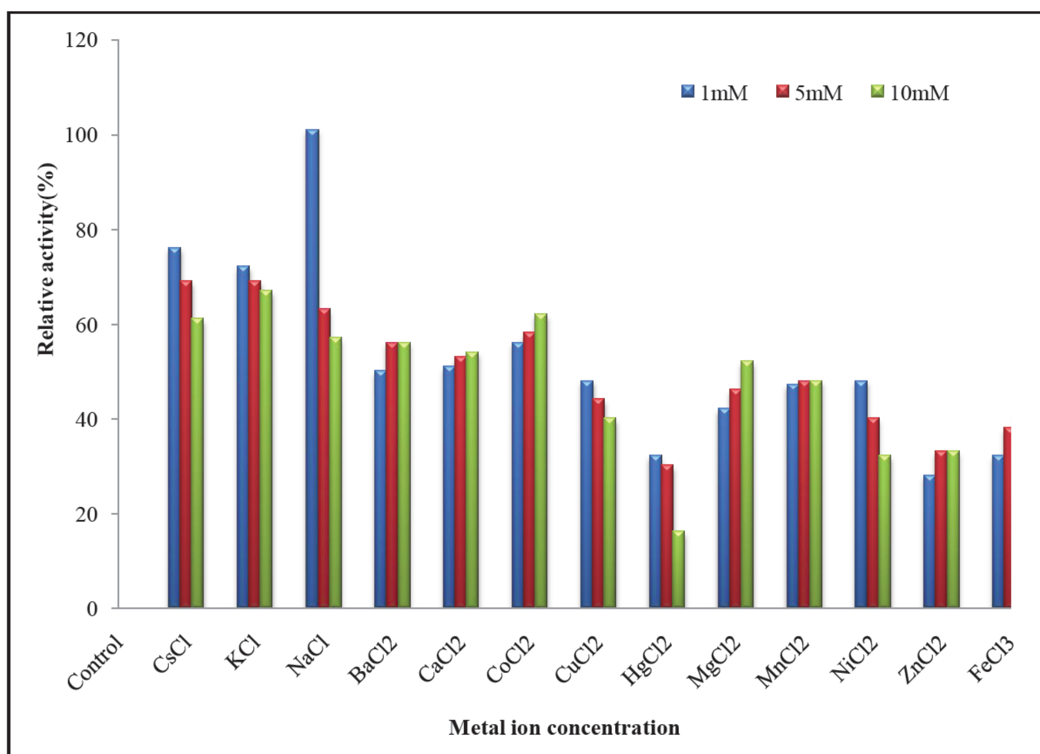

Figure 1: The effect of several metal ion concentration on activity of glucoamylase from *Aspergillus. niger* ER05.

heavy metals at 10 mM concentration i.e. Fe^{2+}, Hg^{2+}, Cu^{+2}, Zn^{2+} and Ni^{2+} reduced by 60%, 40%, 30%, 20% and 11% respectively [13].

The highest inhibitory effect was showed by Hg^{2+} on glucoamylase activity, 32%, 30% and 16% relative activity at 1 mM, 5mM and 10mM concentration. As, 10 mM Hg^{2+} ion concentration abolish 84% of enzyme activity, so emerged as the strong inhibitor [13, 20, 24].

One of the trivalent ions Fe^{3+} was studied to detect its impact on activity of glucoamylase. Results exhibited that Fe^{3+} has been inhibited the activity of enzyme by 41% at 10mM concentration [20]. However, the Hua *et al.* reported the stimulatory impact of Fe^{3+} with the same concentration [24].

Effect of Surfactant on Glucoamylase Activity

The surface active agents were recognized as a major ingredient of detergents and well-known factor for the alterations in the activity of enzymes. In this current investigation, glucoamylase of *Aspergillus niger* ER05 was subjected to the effect of nonionic and anionic detergents (Figure **2**).

According to the results, both tween-80 and triton X-100 exhibited pronounced inhibitory activity on glucoamylase at 1 mM concentration i.e., 45% and 35% relative activity respectively. It is also noted that with the increase in surfactant concentration that relative activity further declined. Gudi *et al*., 2013 repots the detergent-mediated production of Glucoamylase, which showed surfactant tolerance. The enzyme retains 90% of its activity in the presence of high concentration of detergent [13].

Sodium dodecyl sulfate as an anionic detergent exhibited complete inhibition of glucoamylase activity at all concentrations tested. Hu *et al*. [24] showed strong inhibition of glucoamylase activity i.e. 86-87% by SDS. It has been reported that the most of the anionic detergents have the ability to absorb on the surface of proteins, denature their native structure, thus eventually hinders the function of proteins [25, 26].

Effect of Solvents on Glucoamylase Activity

Incubating glucoamylase in 1mM, 5mM and 10mM concentrations of organic solvents for an hour and afterward checking the influences on catalytic performance of enzyme was investigated. The glucoamylase of *Aspergillus niger* ER05 is extraordinarily stable in the presence of all organic solvents under study. It was observed that except, chloroform and DMSO (1mM) presence of other solvents stabilized the activity (Figure **3**). These results were in accordance to the glucoamylase of

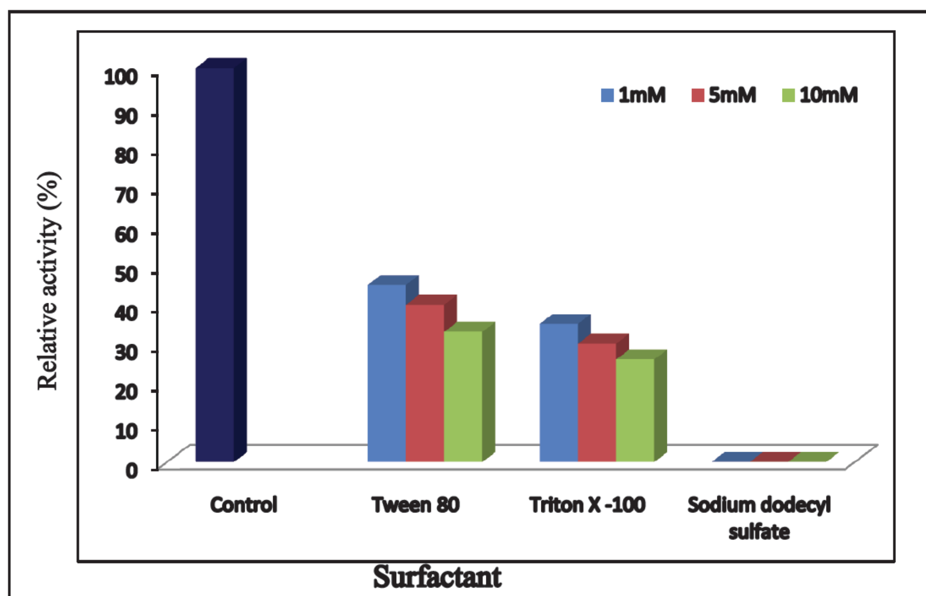

Figure 2: Effect of surface active agents on activity of glucoamylase from *Aspergillus niger* ER05.

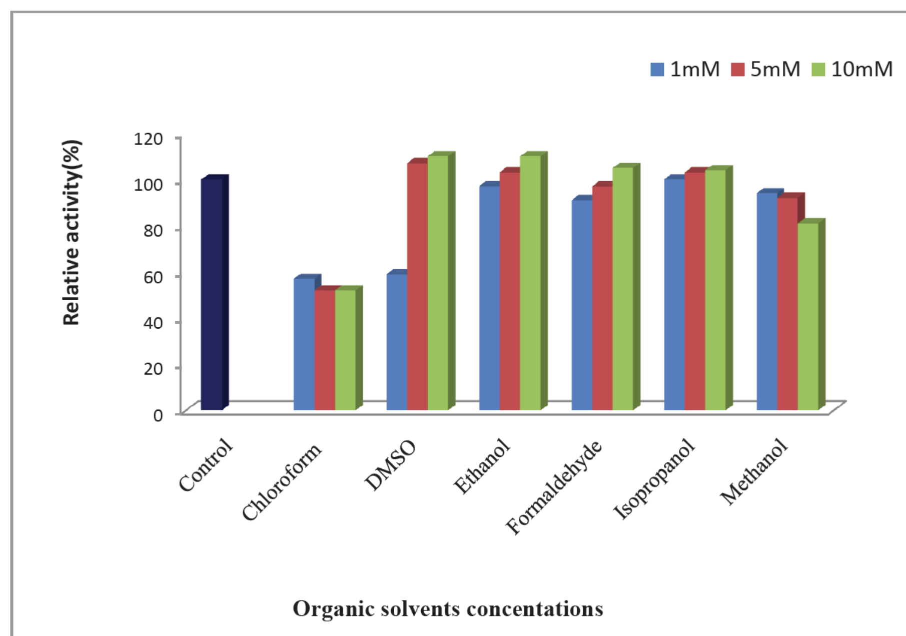

Figure 3: Effect of solvents on activity of glucoamylase from *Aspergillus niger* ER05.

Halolactibacillus sp. SK71 which showed good stability in organic solvents [27]. The interface provided by nonpolar solvents helped the enzyme to be in exposed confirmation thus stimulating the enzyme activity [28].

CONCLUSION

Our current investigation on glucoamylase of *Aspergillus niger* ER05 showed the influences of different metal chlorides, surface active agents and solvents of organic nature on the enzyme activity. It was concluded that glucoamylase was inhibited by most of the metals, in which the Hg^{2+} showed the maximum inhibition, SDS, tween-80, triton X-100, chloroform and DMSO also showed inhibitory effect.

REFERENCES

[1] Pandey A. Glucoamylase research and overview. Starch/stiirke 1995; 47: 43-45. https://doi.org/10.1002/star.19950471108

[2] Kumar GS, Chandra MRGS, Sujana YN, Reddy BR, and Choi Y-L. Enhanced production and partial purification of glucoamylase from mutated *Bacillus sp*. FME. Journal of the Korean Society for Applied Biological Chemistry 2009; 52: 412-8. https://doi.org/10.3839/jksabc.2009.073

[3] Bhatti HN, Asma Z, Rakhshanda N, Munir AS, Muhammad HR. Effect of copper ions on thermal stability of glucoamylase from *Fusarium sp*. Int J Agri Biol 2005; 4: 585-587.

[4] Kumari MS, Lakshmi MVNC, Sridevi V. Production and optimization of Glucoamylase from wheat bran by *Aspergillus oryzae* NCIM 1212 under Solid State Fermentation. Int J Appl Innov Eng Manag 2013; 2(10): 318-323.

[5] Radha K, Srivastava AK, Ramaswamy NK, Suprasanna P, Souza SF. Banana peel as substrate for alpha amylase production using *Aspergillus niger* NCIM 616 and process optimization. Ind J Biotechnol 2012; 11: 314-319.

[6] Vivian MB, Thiago MP, Fernanda DA, Joao AJ, and Maria de Lourdes T. A novel glucoamylase activated by manganese and calcium produced in submerged fermentation by *Aspergillus phoenicis*. J Basic Microbiol 2014; 54: 333-339. https://doi.org/10.1002/jobm.201200515

[7] Michelin M, Ruller R, Ward RJ, Moraes LAB, Jorge JA, Terenzi HF, *et al*. Purification and biochemical characterization of a thermostable extracellular glucoamylase produced by the thermotolerant fungus *Paecilomyces variotii*. Journal of Industrial Microbiology & Biotechnology 2008; 35: 17-25. https://doi.org/10.1007/s10295-007-0261-1

[8] Norouzian D, Akbarzadeh A, Scharer JM, and Young MM. Fungal glucoamylases. Biotechnology Advances 2006; 24: 80-5. https://doi.org/10.1016/j.biotechadv.2005.06.003

[9] Abdalwahab SA, Ibrahim SA, Dawood ES. Culture condition for the production of glucoamylase enzyme by different isolates of *Aspergillus* spices. Intern Food Research J 2012; 19: 1261-1266.

[10] Zambare V. Solid State Fermentation of *Aspergillus oryzae* for Glucoamylase Production on Agro residues. International Journal of Life Sciences 2010; 4: 16-25. https://doi.org/10.3126/ijls.v4i0.2892

[11] Dafei Y, Xiaonan Y, Xingyu W, Zhao L, Maofei W, Yuming G, *et al.* Supplementation of amylase combined with glucoamylase or protease changes intestinal microbiota diversity and benefits for broilers fed a diet of newly harvested corn. Journal of Animal Science and Biotechnology 2018; 9: 24-36. https://doi.org/10.1186/s40104-018-0238-0

[12] Pandey A, Soccol CR, Mitchell D. New developments in solid state fermentation: I-bioprocesses and products. Process Biochemistry 2000; 35: 1153-69. https://doi.org/10.1016/S0032-9592(00)00152-7

[13] Gudi SK, Chandrnsekhan G, Rather G, Chamdra MGS, Mangamuri UK, Podha SE, *et al*. Glucoamylase from a newly isolated *Aspergillus niger* FME: detergent-mediated production, purification, and characterization. J Korean Soc Appl Biol Chem 2013; 56: 427-433. https://doi.org/10.1007/s13765-012-3001-9

[14] Kazi MRK, Ahmad H, and Tasmia T. Production and characterization of crude glucoamylase from newly isolated *Aspergillus flavus* NSH9 in liquid culture. Am J Biochem Mol Biol 2017; 7: 118-126. https://doi.org/10.3923/ajbmb.2017.118.126

[15] Okwuenu PC, Agbo KU, Ezugwu AL, Eze SO, Chilaka FC. Effect of Divalent Metal Ions on Glucoamylase Activity of Glucoamylase Isolated from *Aspergillus niger.* Ferment Technol 2017; 6: 141-146.

[16] Liang XJ, Xun F. Purification and biochemical characterization of a novel mesophilic glucoamylase from *Aspergillus tritici* WZ99. International Journal of Biological Macromolecules 2018; 107: 1122-1130. https://doi.org/10.1016/j.ijbiomac.2017.09.095

[17] Sarojini R, Deepika K, Rangabhashiyam S. Production, characterization, kinetic studies of glucoamylase through solid-state fermentation by *Aspergillus niger* using agricultural residues as substrate. Int J Curr Rsrch 2012; 4: 198-201.

[18] Kellis J, Todd R, Arnold F. Protein stabilization by engineered metal chelation. Biotechnology 1991; 99: 4-995. https://doi.org/10.1038/nbt1091-994

[19] Selvakumar P, Asherhumari L, Helen A, Pandey A. Purification and characterization of glucoamylase produced by *A. niger* in solid state fermentation. Letters Appl Microbiol 1996; 23(6): 403-406. https://doi.org/10.1111/j.1472-765X.1996.tb01346.x

[20] Koç O, Metin K. Purification and characterization of a thermostable glucoamylase produced by *Aspergillus flavus* HBF34. Afr J Biotechnol 2010; 9(23): 3414-3424.

[21] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. Journal of Biological Chemistry 1951; 193: 265-275.

[22] Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol [Internet] 1969; 22: 158-61. https://doi.org/10.1136/jcp.22.2.158

[23] Trinder P. Determination of blood glucose using 4-amino phenazone as oxygen acceptor. J Clin Pathol 1969; 22: 246. https://doi.org/10.1136/jcp.22.2.246-b

[24] Hua H, Luo H, Bai Y, Wang K, Niu C, Huang H, *et al*. A Thermostable Glucoamylase from *Bispora* sp. MEY-1 with Stability over a Broad pH Range and Significant Starch Hydrolysis Capacity. PLoS ONE 2014; 9(11): e113581. https://doi.org/10.1371/journal.pone.0113581

[25] Spinelli LBB, de Lourdes M, Polizeli TM, Terenzi HF, and Jorge JA. Biochemical characterization of glucoamylase from the hyperproducer exo-1 mutant strain of *Neurospora erassa*, FEMS Microbiology Letters 1996; 138: 173-7. https://doi.org/10.1111/j.1574-6968.1996.tb08152.x

[26] Bartnik FG. Interaction of anionic surfactants with proteins, enzymes, and membranes. Surfactant Science Series 1992; 43: 1-42.

[27] Yu HY, Li X. Characterization of an organic solvent-tolerant thermostable glucoamylase from a halophilic isolate, *Halolactibacillus* sp. SK71 and its application in raw starch hydrolysis for bioethanol production. Biotechnol Prog 2014; 30: 1262-1268. https://doi.org/10.1002/btpr.1978

[28] Zaks A, Klibanov AM. Enzymatic catalysis in nonaqueous solvents. J Biol Chem 1988; 263: 3194-201.

Received on 10-03-2018 Accepted on 26-03-2018 Published on 13-04-2018

https://doi.org/10.6000/1927-5129.2018.14.21

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