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EVALUATION OF CELLULASE ENZYME SECRETED BY SOME COMMON AND STIRRING RHIZOSPHERE FUNGI OF *Juglans regia* L. BY DNS METHOD

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Abstract- The aim of this study was the isolation and screening of the rhizosphere fungi of *Juglans regia L.* for the cellulase activity. Enzymatic analysis of six fungal isolates viz., *Aspergillus niger k32*, *A. niger mtc872*, *A. niger B42*, *Penicillium camembertii*, *Trichophyton terrestre* and *Cladosporium cladosporoides* was done. The cellulase activity was assayed in carboxymethyl cellulose (CMC) solution by 1, 3-dinitrosalicylic (DNS) method. Testing the enzyme at different temperatures by DNS method determined the temperature at which the enzyme cellulases from fungal isolates worked the most efficiently. Analyzing the glucose released in the CMC solutions with spectrophotometry determined the reaction rate. The enzyme cellulase extracted from *Trichophyton terrestre* worked much proficiently at wider range of temperatures.

Key words- Cellulase, Fungi, CMC, DNS, Glucose, Protein estimation

Abbreviations- CMC- carboxymethyl cellulose, DNS- dinitrosalicylic, BSA- Bovine Serum Albumin

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Introduction

Cellulose is the world's most abundant natural biopolymer and has a great significance for the production of industrially valuable materials such as fuels and chemicals. Cellulose contains simple repeating units of glucose, but has a complex structure because of the long chains of glucose subunits joined together by β-1, 4 linkages [23]. The stabilizing factors in cellulose are weak individually, but collectively form strong bonds. The chains are in layers held jointly by Vander Waals forces and hydrogen bonds- intramolecular and intermolecular [12]. About thirty to forty individual cellulose molecules are arranged into units called protofibrils, which are further arranged into larger units called microfibrils. These in turn assemble into cellulose fibers [27]. The polymer is not entirely crystalline in nature even though the microfibrils are tightly packed to form a crystalline structure. The tightly packed and well ordered sites are spaced by loosely arranged ones called amorphous regions [20].

Cellulase, recognized as one of the key enzyme for degrading cellulose and biosynthesis of cellulase protein has been found to be most expensive process [18,36]. Fungi that have been modified through their genetic make up for the enhanced enzymatic secretion have been successfully used in a number of applications including animal feed, pharmaceutical and textile industries [5].

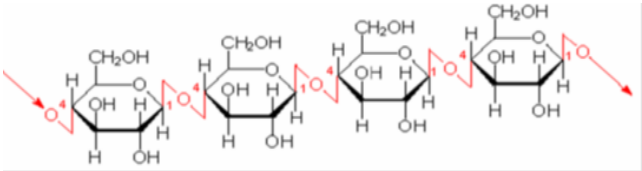

Fig. 1- Diagram showing the β-1, 4-linkages in cellulose chain [34]. The red dotted lines are the intermolecular H-bonds. Such formed cellulose chains are held by intramolecular H-bonds.

There are numerous microorganisms capable of degrading cellulose, but only a restricted group is capable to produce a sufficient amount of cell-free cellulose, which is capable of completely hydrolyzing crystalline cellulose *in vitro*. For the degradation of cellulose, fungi utilize the hydrolytic enzymes viz., exocellulase, endocellulase, cellobiohydrolase, endogluconase and β-glycosidase [7]. However, some cellulolytic fungi produce extracellular cellobiose-oxidizing enzymes besides cellulase. From the human perspective, the power of fungal enzymes is janus-faced. Fungal cellulases provide a good example of the contrasting faces of a single enzymatic capability. Continuing research on *Trichophyton terrestre* identified to have a complete set of cellulase enzymes required for the breakdown of cellulose to glucose [29,33]. These enzymes now promise the potential of converting waste cellulosics into foods for

our burgeoning population and have been the subject of intense molecular biology research [11, 25].

Material and Methods

In the present study, *Aspergillus niger k32*, *Penicillium camembertii*, *Trichophyton terrestre* and *Cladosporium cladosporoides* were isolated from the rhizosphere of *Juglans regia L.* in the Northern regions of India (Kashmir valley) by serial dilution method to get more manageable results [3]. 1g of soil was transferred to 10 ml of distilled water in test tubes. Dilutions were made up to 10^{-6} and fungal culturing was done by using Czapek-Dox agar medium with following composition (g/l); sucrose- 30, $NaNO_3$- 2, K_2HPO_4- 1, $MgSO_4$– 0.5, KCl- 0.5, $FeSO_4$– 0.01, agar agar- 15, pH of the medium was adjusted to 7.3. After autoclaving at 121°C and 15 lbs pressure, 20 ml of sterile medium were transferred to sterile petri plates and allowed for solidification (Chloramphenicol 250mg/100ml was also added to check the bacterial growth). After solidification of the medium, 0.1 ml of soil suspension was spread with the help of spreader and incubated at 28 °C for 7 days. The fungal cultures grown on the medium were transferred on to the Potato Dextrose Agar (Hi Media) medium and pH was maintained at 5.6 for further studies. Other fungal strains were collected from the additional sources like *A. niger B42* was secluded from the wheat field of Bhopal by serial dilution method and *A. niger mtc872* was collected from the Institute of Microbial Technology (IMTECH), Chandigrah, India.

Production of Cellulases from Fungal Isolates

The isolated fungal cultures were used to know their potential for cellulase production and activities. A volume of 100 ml of Czapek-Dox broth medium amended with 1% cellulose was distributed into separate 250 ml Erlenmeyer conical flasks. The pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lb. pressure, the fungal spore suspensions were inoculated into the conical flasks. The flasks were incubated at 32 °C on a rotary shaker at 120 rpm for 7 days. After incubation, the contents of the flasks were passed through Whatman filter paper No.1 to separate mycelial mat from culture filtrate. The filtrate thus obtained was used for the estimation of extracellular protein content and total activity of cellulases.

Folin Lowry's Method

Principle- Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex which is the result of reaction between alkaline copper and the protein, containing tyrosine and tryptophan [22,14,41].

Materials

1. Alkaline sodium carbonate solution (2% Na_2CO_3 in 0.1 N NaOH).
2. Copper sulphate: sodium tartarate solution (0.5% $CuSO_4$ in 1% Na, K tartarate), prepared freshly by mixing stock solutions.
3. Alkaline solution: prepared on day of use by mixing 50ml of (1) and 1 ml of (2).
4. Folin-Ciocalteau reagent: diluted the commercial reagent with an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid.
5. Bovine serum albumin was used as a standard protein.

Preparation of a BSA Standard Curve- 500 mg of Bovine Serum Albumin (BSA) was dissolved in 50 ml of distilled water (10 mg/ml). From this stock solution, 5 ml solution was dissolved in 50 ml distilled water (1 mg/ml) which was used as a working solution.

Method

5 ml of the alkaline solution was added to 1 ml of the test solution. After thoroughly mixing, it was allowed to stand at the room temperature for 10 minutes or longer and 0.5 ml of diluted Folin-Ciocalteau reagent was added followed by immediate mixing. After 30 minutes, the optical density reading was noted by spectrophotometer against the appropriate blank at OD_{750nm} and BSA standard curve Fig. 2 was made by using the experimental data Table- 1.

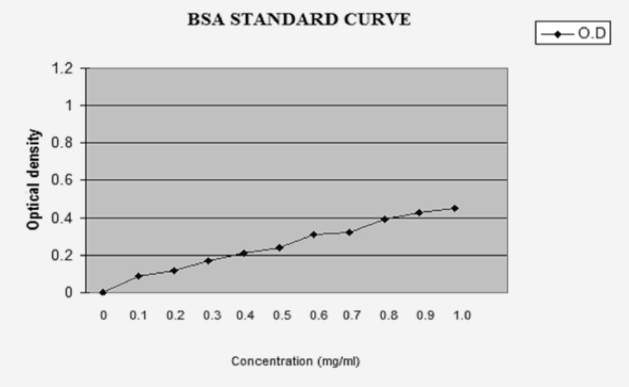

Fig. 2- BSA Standard Curve

Table 1- OD_{750nm} of different dilutions of BSA

Sr. No.	Standard solution (ml)	Distilled water (ml)	BSA (mg/ml)	Optical density
1	0.0	5.0	0.0	0.0
2	0.5	4.5	0.1	0.09
3	1.0	4.0	0.2	0.12
4	1.5	3.5	0.3	0.17
5	2.0	3.0	0.4	0.21
6	2.5	2.5	0.5	0.24
7	3.0	2.0	0.6	0.31
8	3.5	1.5	0.7	0.32
9	4.0	1.0	0.8	0.39
10	4.5	0.5	0.9	0.43
11	5.0	0.0	1.0	0.45

Protein Estimation

Protein content of the isolated fungi was estimated by using the Folin Lowry's method and optical density Table- 2 of each strain was compared with the BSA standard curve to calculate the amount of protein (mg/ml) present Fig. 3 in the supernatant used in cellulase assay.

Table 2- Protein content of six fungal strains

Sr. No.	Fungal Strains	Optical density	Protein (mg/ml)
1	A. niger k32	0.43	0.78
2	A. niger mtc 872	0.48	0.87
3	A. niger B24	0.52	0.94
4	P. camembertii	0.32	0.58
5	T. terrestre	0.09	0.16
6	C. cladosporoides	0.11	0.22

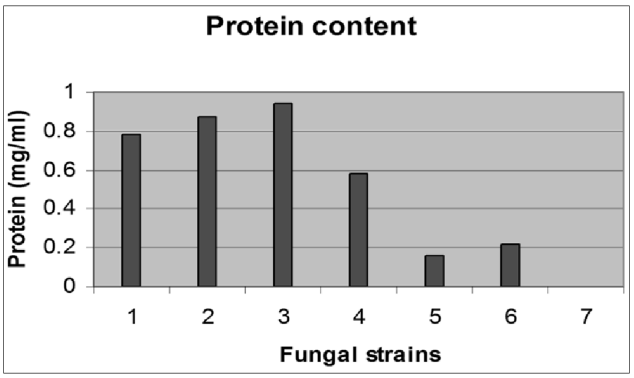

Fig. 3- Protein Estimation of Six Fungal Strains

Preparation of a Standard Curve for Glucose Estimation by DNS Method

Glucose Stock- 100 mg of glucose was dissolved in 10 ml distilled water (10mg/ml).

Citrate Buffer- 210g citric acid monohydrate C6H80.7H2O was dissolved in 750 ml distilled water. NaOH was added until pH equals to 4.3 and final make up was 1000ml. This is 1M citrate buffer having pH 4.5, when it was diluted to 0.05, pH was maintained at 4.8 (0.05 M Citrate buffer pH 4.8)

Dinitrosalicylic Acid (DNS)- 30g K-Na tartarate was dissolved in 50 ml of distilled water which was further added by 1g DNS and 20 ml 2N NaOH and final make up was 100 ml.

Methodology

Dilution of Stock in Citrate Buffer

1. 1 ml glucose stock + 0.5 ml citrate buffer (6.7mg/ml or 3.35mg/0.5ml).
2. 1 ml glucose stock + 1 ml citrate buffer (5.0mg/ml or 2.5mg/0.5ml).
3. 1 ml glucose stock + 1.5 ml citrate buffer (4.0mg/ml or 2mg/0.5ml).
4. 1 ml glucose stock + 2 ml citrate buffer (3.3mg/ml or 1.65mg/0.5ml).
5. 1 ml glucose stock + 3 ml citrate buffer (2.5mg/ml or 1.25mg/0.5ml).
6. 1 ml glucose stock + 4 ml citrate buffer (2.0mg/ml or 1.0mg/0.5ml).
7. 1 ml glucose stock + 5 ml citrate buffer (1.6mg/ml or 0.8mg/0.5ml).

The experimental data was collected and glucose standard curve Fig. 4 was used as a standard [17].

Assay of Cellulases at Different Temperatures

0.5 ml of CMC solution taken from each dilution was added by 0.5 ml culture supernatant of each fungal strain. Reaction mixture was incubated for 30 minutes at three different temperatures i.e., 4^0C, 20^0C and 50^0C. After incubation, reaction was terminated by adding 3 ml DNS and reaction mixture was boiled for 5 minutes. After boiling, 20 ml of distilled water was added and optical density was taken at 540 nm against the blank.

Reaction Mixture

0.5 ml CMC solution

0.5 ml supernatant

3.0 ml DNS

5 minute boil

20 ml distilled water

Blank was Prepared as Below

0.5 ml citrate buffer

0.5 ml distilled water

3.0 ml DNS

5 minute boil

20 ml distilled water

Blank (control) was used to set the spectrophotometer at zero absorbance. The amount of glucose liberated by six fungal strains at different temperatures has been depicted in the Fig. 5.

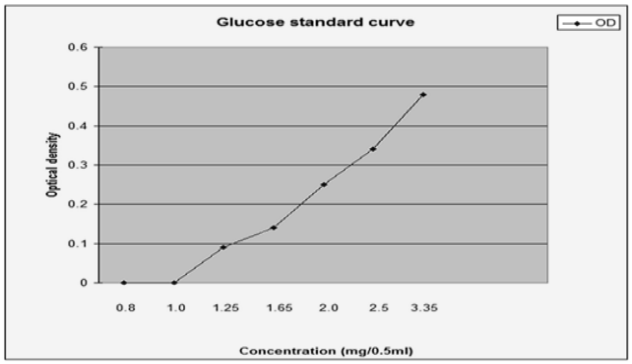

Fig. 4- Glucose Standard Curve

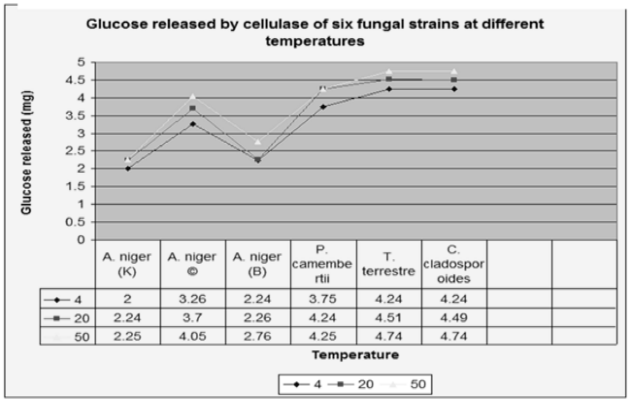

Fig. 5- Glucose Released (mg) by Cellulases of Six Fungal Strains at Different Temperatures

Results and Discussion

In this comparative study, the enzymatic activity of six fungal strains was analyzed. All the six strains were exploited and the enzyme cellulases liberated in the CMC solution were evaluated by DNS method [24]. The cellulase activity of fungal strains was math-

ematically calculated by using the data of protein content (mg/ml) present in the supernatant of isolates which was estimated by Folin Lowrey's method. The amount of protein present in the fungal strains was estimated in 1ml of the supernatant (mg/ml), but only 0.5ml of the supernatant was used to show the activity (V/ mg/30minute) of protein. The activities of enzyme cellulases were determined by using the following formula:

Enzyme activity = amount of glucose liberated/mg protein/30minutes [13].

Among the six tested fungal strains, *Penicillium camembertii, Cladosporium cladosporoides* and *Trichophyton terrestre* showed higher activities as compared to the *Aspergillus niger k32, A. niger B42* and *A. niger mtc872*. The efficiency of the enzyme liberated by *T. terrestre* was highest than all and found to be 53.00, 56.37 and 59.25mg glucose liberated/mg protein/30minutes at 4^0C, 20^0C and 50^0C respectively, whereas for *A. niger mtc 872,* it was found to be 7.56, 8.58 and 9.39mg glucose liberated/mg protein/30minutes at 4^0C, 20^0C and 50^0C respectively. It means that the activity of enzyme cellulase from *Trichophyton terrestre* is 7.01, 6.56 and 6.30 times more at 4^0C, 20^0C and 50^0C respectively than *A. niger mtc872,* which is one of the most promising fungal strain having great industrial importance in terms of enzyme efficiency depicted in Fig. 6. Maximum activity of the cellulase enzyme was seen at 50°C which coincides with the results of the Nataraja *et al.* [28]. Obviously, the activity of the immobilized cellulase varied in a wide range under the effect of ascends in temperature as compared to the activity of the free enzyme. As temperature is increased, the viscosity of the CMC solution was decreased and the mobility of the CMC molecules increased considerably. All these events will help the access of the substrate to the immobilized enzyme and will provide a proper atmosphere for reaction between the enzyme and the substrate. Thus, the activity of the immobilized enzyme will increase to a higher value, whereas it will be lower at the lower temperatures. The most important finding was the activity of the enzyme cellulase liberated by *Trichophyton terrestre,* at a wide range of temperatures. Although it remains active at lower temperature of 4^0C, yet shows the efficient thermal tolerance at 50^0C. There are several reports on such use of filamentous fungi in the production of optimal enzyme complex for degradation of host lignocelluloses [6,8,31,37]. The purification and properties of cellulases have been thoroughly described [4,9,15,16,19,21,26,30,38,40, 42].

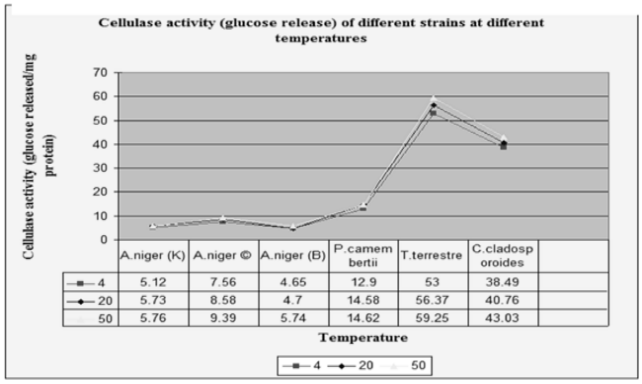

Fig. 6- Cellulases Activities (V/mg Protein) of Six Fungal Strains at different temperatures by DNS Methos

Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization [39]. The potential of using microorganisms as biotechnological sources of industrially relevant enzymes has enhanced interest in the exploration of extracellular enzymatic activity in several microorganisms [1-2]. As cellulose can be regarded as the most abundant and biologically renewable resource for bioconversion, its exploitation can be maximized on hydrolysis to glucose and other soluble sugars which can be further fermented into ethanol for use as liquid fuel [10]. Cellulases are the enzymes responsible for the cleavage of the β–1, 4–glycosidic linkages in cellulose. They are members of the glycoside hydrolase families of enzymes that hydrolyze oligosaccharides and/or polysaccharides [35]. The major goals for future cellulase research would be: (1) reduction in the cost of cellulase production and (2) improving the power of cellulases to make them more effective, so that less enzyme is needed [32].

Conclusion

Cellulose is the primary product of photosynthesis in terrestrial environments and is the most abundant renewable bioresource produced in the biosphere (~100 billion dry tons/year). Cellulases produced by microorganisms are either cell associated or in free form, which metabolize the insoluble cellulose.

Among the fungal isolates that were evaluated for the cellulase production and activity, the cellulase of *T. terrestre* was very efficient as determined by its high specific activity as compared to other isolates particularly the most promising strain *A. niger mtc872* having higher industrial applications. The activity of the enzyme cellulase of *Trichophyton terrestre,* at a wide range of temperatures, exhibits higher activity at low temperature and thermal resistance even at 50^0C. All these properties qualify this fungal species to be used as an important source of cellulase enzyme which has janus-faced applications in the industries. More basic research is needed to make designer enzymes suited for specific applications.

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References

[1] Abu E.A., Ado S.A. and James D.B. (2005) *Afr. J. Biotechnol.,* 4(8), 785-790.

[2] Akpan I., Bankole M.O., Adesemowo A.M. and Latunde-Dada G.O. (1999) *Trop. Sci.*, 39, 77-79.

[3] Aneja K.R. (2005) *New Age Publishers*, 69.

[4] Anzai H., Nisizawa K. and Matsuda K. (1984) *J. Biochem.,* 96 (5), 1381-1390.

[5] Aristidou A. and Penttilä M. (2000) *Current Opin. Biotechnol.,* 11, 478-483.

[6] Awafo V.A., Chahal D.S., Simpsom B.K. and Le G.B.B. (1996) *Appl. Biochem. and Biotechnol*., 57/58, 461-470.

[7] Bhat M.K. (2000) *Biotech. Adv.,* 18, 355-383.

[8] Chahal P.S., Chahal D.S. and Le G.B.B. (1996) *Appl. Biochem. and Biotechnol*., 57/58, 433-442.

[9] Churilova I.V., Maksimov V.I. and Klesov A.A. (1980) *Biokhimia*, 45(4), 669-678.

[10] Eriksson T., Börjesson J. and Tjerneld F. (2002) *Enzyme and Microbial Technology*, 31, 353-364.

[11] Eveleigh D.E. (1985) *Trichoderma*, 487-510.

[12] Gan Q., Allen S.J. and Taylor G. (2003) *Proc. Biochem.*, 38, 1003-1018.

[13] Ghose T.K. (1987) *Pure Apple. Chem.,* 59, 257-268.

[14] Hartree E.E. (1972) *Anal. Biochem.*, 48, 422.

[15] Kanda T., Wakabayashi K. and Nisizawa K. (1976) *J. Biochem.,* 79(5), 977-988.

[16] Kim J.Y., Hur S.H. and Hong J.H. (2005) *Biotechnol. Lett.,* 27 (5), 313-316.

[17] Kondo A., Urabe T. and Higashitani K. (1994) *J. Fermant. Bioeng.,* 77(6), 700-703.

[18] Kotchoni O. S., Shonukan O. O. and Gachomo W. E. (2003) *Afr. J. Biotechnol.,* 2(6), 140-146.

[19] Lee Y.J., Kim B.K., Lee B.H., Jo K.I., Lee N.K., Chung C.H., Lee Y.C. and Lee J.W. (2008) *Bioresour. Technol.,* 99(2), 378-386.

[20] Levy I., Shani Z. and Shoseyov O. (2002) *Biomolecular Engineering*, 19, 17-30.

[21] Li Y.H., Guo R., Yin Q.Y., Ding M., Zhang S.L., Xu G.J. and Zhao F.G. (2005) *Acta Biochem. Biophys.*, 37(10), 702-708.

[22] Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) *J. Biol. Chem.,* 193, 265.

[23] Lynd L.R., Weimer P.J., Van Zyl W.H. and Pretorius I.S. (2002) *Microbiol. Mol. Biol. Rev.*, 66, 506–577.

[24] Miller G.L. (1959) *Analytical Chemistry,* 31, 426-428.

[25] Montecourt B.S. and Eveleigh D.E. (1985) *Gene Manipulations in Fungi*, *Academic Press, Inc., New York*.

[26] Mori Y. (1992) *Biosci. Biotechnol. Biochem.,* 56(8), 1198-1203.

[27] Mosier N.S., Hall P., Ladisch C.M. and Ladisch M.R. (1999) *Advances in Biochemical Engineering Biotechnol.,* 65, 23-40.

[28] Nataraja S., Chetan D.M. and Krishnappa M. (2010) *International Journal of Microbiology Research*, 2(2), 44-47.

[29] Nevalainen K.M., Penttila M.E., Harkki A. and Teeri T.T. (1991) *Molecular Industrial Mycology*, Marcel Dekker, Inc., New York.

[30] Ogura J., Toyoda A., Kurosawa T., Chong A.L., Chohnan S. and Masaki T. (2006) *Biosci. Biotechnol. Biochem.,* 70(10), 2420-2428.

[31] Pandey A., Francis F., Sabu A. and Soccol C.R. (2004) *Concise Encyclopedia of Bioresource Technology,* 702-708.

[32] Qin C., Zhou B., Zeng L., Zang Z., Liu Y., Du Y. and Xiao L. (2004) *Food Chem.,* 84, 107-115.

[33] Reese E.T., Sui R. G.H. and Levinson H.S. (1950) *J. Bacteriol.,* 59, 485-497.

[34] Samejima M., Sugiyama J. and Igarashi K. (1998) *Carbohydrate Research*, 305, 281-288.

[35] Schülein M. (2000) *Biochemica Biophysica Acta.*, 1543, 239-252.

[36] Solomon B.O., Amigun B., Betiku E., Ojumu T.V. and Layokun S.K. (1997) *J. Niger Soc. Chem. Eng.,* 16, 61-68.

[37] Tengerdy R.P. and Szakacs G. (2003) *Biochem Eng. J.*, 13, 169-179.

[38] Thongekkaew J., Ikeda H., Masaki K. and Iefuji H. (2008) *Pichia pastoris. Protein Exp. Purif*., 60(2), 140-146.

[39] Wen Z., Liao W. and Chen S. (2005) *Bioresour. Technol.,* 96 (4), 491-499.

[40] Whitaker D.R. (1951) *Nat*. 168(4288), 1070-1071.

[41] Wilson K. and Walker J. (2000) *Practical Biochemistry: Principles and Techniques*. Cambridge University Press.

[42] Ye X.Y., Ng T.B. and Cheng K.J. (2001) *Int. J. Biochem. Cell. Biol.,* 33(1), 87-94.