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PURIFICATION AND CHARACTERIZATION OF PECTIN LYASE SECRETED BY *Aspergillus flavus* MTCC 10938

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An indigenously isolated fungal strain *Aspergillus flavus* MTCC 10938 was subjected to pectin lyase (PNL) production under submerged fermentation conditions. The enzyme was purified to homogeneity from the culture filtrate of the fungus involving concentration by ultrafiltration, anion exchange chromatography on DEAE cellulose and gel filtration chromatography on Sephadex G-100. The purified PNL gave a single protein band in SDS-PAGE analysis with a relative molecular mass corresponding to 50 kDa. Using citrus pectin as the substrate the K_m and k_{cat} values of the enzyme lyase were obtained as 1.7 mg/mL and 66 s^{-1}, respectively. The optimum pH of the purified PNL from *A. flavus* MTCC 10938 was 8.0 and up to 90% of its activity retained in the pH range from 3.0 to 11.0 after 24 h incubation. The optimum temperature of the purified enzyme was revealed at 55°C and it was completely stable up to 40°C when exposed for 30 min. The purified *A. flavus* MTCC 10938 PNL showed efficient retting of *Crotalaria juncea* fibres.

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Pectic substances are ubiquitous in the plant kingdom representing the major component of middle lamella of a plant cell wall conferring firmness and structure to the plant cells [1]. They are mainly degraded by a group of enzymes referred as pectinases which have been classified according to their modes of action and substrates used *viz.* pectinesterases (EC 3.1.1.11); polygalacturonases (EC 3.2.1.15); pectate lyases (EC 4.2.2.2) and pectin lyases (EC 4.2.2.10). Based on the potential industrial applications of pectinases in clarification of fruit juices, retting of fibers, treatment of pectic waste water, coffee and tea leaf fermentation, oil extraction, virus purifications, these enzymes have been extensively studied [2–5].

Pectin lyases (PNL) are of particular interest as they degrade pectin polymers directly by β-elimination mechanism resulting in the formation of 4,5-unsaturated oligogalacturonides while other pectinases act sequentially to degrade pectin molecule totally. Further, PNL have biotechnological potential in fruit juice industries due to the fact that they degrade pectin without disturbing the ester group which is responsible for specific aroma of the juice and also do not form methanol which is highly toxic [6]. The production, purification, biochemical characterization, molecular biology and applications of PNL have recently been reviewed [7]. These enzymes are relatively less explored enzyme as compared to other pectinases. PNL with different properties were used for different applications, e.g enzymes with pH optima in the acidic range were suitable for clarification of fruit juice [8], while PNL with pH optima in alkaline range were effective in retting of natural fibres [9]. Thus, there is a scientific need to search new PNL source with properties suitable for different applications.

The aim of the study was to purify and characterize an alkaline PNL from a new fungal isolate *Aspergillus flavus* MTCC 10938 showing efficient retting of sunn hemp (*Crotolaria juncea*) fibres.

MATERIALS AND METHODS

Chemicals. Citrus pectin, DEAE cellulose and Sephadex G-100 were purchased from Sigma (USA). Rests of the chemicals were procured either from Merck (Germany), Navi Mumbai (India) or S.D. Fine Chem. Ltd. (India) and were used without further purification.

PNL assay. Activity of PNL was detected by the method of Albersheim [10]. Assay was performed by monitoring the increase in optical density at 235 nm due to formation of unsaturated uronide product using spectrophotometer Hitachi (Japan) model U-2000. Enzyme solution (0.2 mL) was added to the reaction mixture containing 0.8 mL citrus pectin (1% w/v) and 2.0 mL of the 100 mM phosphate buffer (pH 8.0) maintained at 37°C. Optical density was measured at zero time and after 20 min of incubation and the steady state velocity was calculated as absorbance change per min. PNL activity was defined as μmole of unsaturated product released per min, based on the molar extinction coefficient value of 5500 $M^{-1}\,cm^{-1}$.

Protein estimation. Protein was determined by the Lowry's method taking bovine serum albumin as the standard.

Organism and growth conditions. The fungal strain was isolated from the contaminated tissue culture media in the Plant Tissue Culture laboratory of the Department of Biotechnology, DDU Gorakhpur University (India), using a standard serial dilution technique [11]. The isolated and purified fungal strain was screened for the secretion of pectinases by plate assay method [12]. The PNL producing fungal strain was identified and deposited as *Aspergillus flavus* MTCC 10938 by Microbial Type Culture Collection Centre and Gene Bank, Chandigarh (India). The culture was maintained by cultivation on Czapek-Dox agar slants at 25°C fortnightly. Based on prominent halo zone formation the culture was further screened for the PNL secretion in liquid culture medium containing 0.3% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% L-Asp and 0.5% pectin. The initial pH of the medium was adjusted to 4.5. Once the culture conditions for maximum secretion of the pectin lyase were optimized, the fungal strain *Aspergillus flavus* MTCC10938 was grown for purification of extracellular PNL in submerged fermentation medium in 25 × 100 mL culture flasks. One mL of spore suspension (spore density 5×10^6 spores/mL) from agar slant was inoculated aseptically into 25 mL of sterilized liquid culture medium, contained in 100 mL culture flasks. The flasks were incubated at 25°C in BOD incubator and cultures were allowed to grow under stationary conditions. On the seventh day (day for maximum production) culture was filtered through Whatman no 41 filter paper, this cell free filtrate (CFF) was used as crude for further purification.

Purification of the enzyme. *Ultrafiltration by Amicon.* The clear culture filtrate was centrifuged at 14000 × g for 10 min and 400 mL supernatant was concentrated to 2.0 mL with the help of Amicon Concentration Cell (USA) using PM 10 ultrafiltration membrane having 10 kDa molecular mass cut off value.

DEAE cellulose column chromatography. The concentrated crude PNL was dialyzed against 2.0 L of 10 mM citrate phosphate buffer (pH 7.0) with 3 changes at the intervals of 8 h. The sample was then centrifuged for 10 min at 14000 × g and the supernatant was loaded on DEAE cellulose column (6.5 × 2.0 cm) equilibrated with 10 mM citrate phosphate buffer (pH 4.0). The adsorbed protein was then washed twice by bed volume of the same buffer. The protein was eluted stepwise using 10 mL of NaCl (0.2–1.0 M) in the same buffer at the flow rate of 18 mL/h. Fractions of 3.0 mL were collected and analyzed for activity for the PNL and protein.

Sephadex G-100. The pooled fractions from DEAE cellulose showing maximum activity were concentrated with sucrose and dialyzed against 2.0 l of 100 mM citrate phosphate (pH 7.0) buffer. The dialysed PNL was centrifuged at 14000 × g and the supernatant was loaded on a Sephadex G-100 column (1.0 × 30.0 cm), pre-equilibrated with the same buffer. The flow rate was maintained at 9.0 mL/h and fractions of 1.5 mL were collected and analyzed for protein and PNL activity. All the active fractions were pooled, concentrated and tested for homogeneity by electrophoresis.

SDS-PAGE. The enzyme purity and the molecular mass were estimated by the 10% SDS-PAGE method, using phosphorylase B (97.4 kDa), BSA (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa) as standard protein markers [13]. Silver staining was done for locating the protein bands [14].

Characterization of the *A. flavus* MTCC 10938 PNL. The K_m and k_{cat} values of the purified *A. flavus* MTCC 10938 PNL were determined by measuring steady state velocities of the enzyme catalyzed reaction at different concentrations of citrus pectin and drawing double reciprocal plots [15]. Calculations were made using linear regression analysis of the data points of double reciprocal plot. The pH optimum was determined by measuring steady state velocity in the buffered reaction solution using different 100 mM buffers at in the pH range 1.0–12.0. The buffers used were: HCl-KCl (1.0–2.0), citrate-phosphate (3.0–7.0), sodium-phosphate (8.0), glycine-NaOH (9.0–10.0) and sodium phosphate–NaOH (11.0–12.0).

Steady state velocity of the PNL reaction against pH of the reaction medium was plotted. The pH stability of the enzyme was studied by exposing the enzyme to buffers of different pH for 24 h at 4°C. The residual activities were assayed and plotted in the form of percent residual activity versus pH. The optimum temperature for the *A. flavus* MTCC 10938 enzyme activity was determined by assaying the activity of the enzyme at different temperatures in the range 5–100°C and plotting a graph of the enzyme activity versus temperature of the reaction. Thermal stability of the enzyme was tested by incubating an enzyme aliquot at a particular temperature (10–100°C) for 30 min, assaying its residual activity and plotting the percent residual activity against temperature.

Effect of metal ions and protein inhibitors on the *A. flavus* MTCC 10938 PNL activity. The effect of metal ions like Ca^{++}, Mg^{++}, Mn^{++}, Co^{++}, Zn^{++}, Hg^{++}, K^+, Na^+, Ag^+ and protein inhibitors like sodium azide, potassium permanganate, potassium ferrocyanide and EDTA were studied by measuring the steady state velocity in the reaction solutions containing 1mM of the metal ions or protein inhibitors and comparing it with the value in the absence of these ions or inhibitors.

Retting of fiber by purified *A. flavus* MTCC 10938 PNL. The retting of natural fiber *Crotalaria juncea* was carried out by Zhang et al. [16] with minor modifications. Approximately 10 cm long sunn hemp sticks were put in 3 different tubes labeled as control (C) without enzyme, experimental (E1) containing en-

Purification of PNL produced by *A. flavus* MTCC 10938

Fraction	Total activity, IU	Total protein, mg	Specific activity, IU/mg	Purification, -fold	Yield, %
Crude extract	16.0	200.00	0.08	1.0	100
Amicon concentration	6.5	16.40	0.39	4.8	40.6
DEAE cellulose	2.8	4.60	0.60	7.5	17.5
Sephadex G-100	0.4	0.05	8.00	100.0	2.5

zyme without EDTA and experimental (E2) containing enzyme with 1.0 mM EDTA. All the test tubes were incubated in a water bath at 37°C for 24 h. Then the sticks were shaken vigoursly each with 10 mL hot water for 1 min and photographed.

RESULTS AND DISCUSSION

Purification of the *A. flavus* MTCC 10938 PNL. The extracellular PNL produced by *A. flavus* MTCC 10938 was purified by the steps shown in Table. The elution profiles from the anion exchange on DEAE cellulose column and gel filtration Sephadex G-100 column are shown in Fig. 1 and Fig. 2, respectively. A hundred-fold purification with specific activity 8 U/mg protein and 2.5% yield have been achieved. The electrophoretic homogeneity was confirmed on SDS-PAGE revealing a single band corresponding to a relative molecular mass of approximately 50 kDa (Fig. 3). The literature survey has indicated that the relative molecular mass of PNLs varied from 22 to 90 kDa [7]. It was revealed that PNL produced by *Aspergillus japonicus* and *Bacillus* PN-33 had relative molecular mass of 50 and 52 kDa, respectively [17, 18].

Characterization of *A. flavus* MTCC 10938 PNL. The variation in activity of the purified *A. flavus* MTCC 10938 PNL with pH of the reaction solution as well as stability when the enzyme was exposed to various pH buffers for 24 h at 4°C is shown in Fig. 4. The pH optimum was revealed at 8.0 and the enzyme retained up to 90% of its activity in pH range 3.0–11.0. The pH optima of the previously reported PNL have been found to be acidic for *Penicillium canescens* (5.5) [19] neutral for *Penicillium expansum* (7.0) [20] and basic for *Fusarium oxysporum* (9.5) [21].

The activity of the purified *A. flavus* MTCC 10938 PNL with variation in the temperature of the reaction solution is demonstrated in Fig. 5a and the temperature optimum of the enzyme was 55°C. Similar value for optimum temperature has been reported for PNL produced by *Aspergillus japonicus* [22] while an optimum temperature of 50°C has been reported for most of the fungal sources, *Penicillium italicum* [23] and *Fusarium oxysporum* [21] etc. The results of the thermal stability of the purified *A. flavus* MTCC 10938 PNL are presented in Fig. 5b. The enzyme was fully stable at temperatures up to 40°C after which it started loosing activity. The k_m and k_{cat} values determined were 1.7 mg/mL and 66 s^{-1}, respectively. The PNL from *A. fla-*

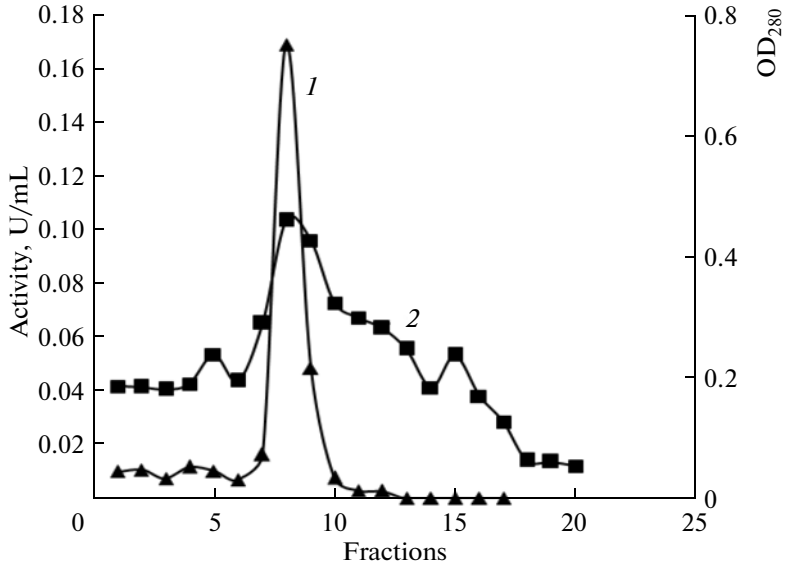

Fig. 1. Anion exchange chromatography on DEAE cellulose column of PNL by *A. flavus* MTCC 10938: *1* – protein; *2* – PNL activity.

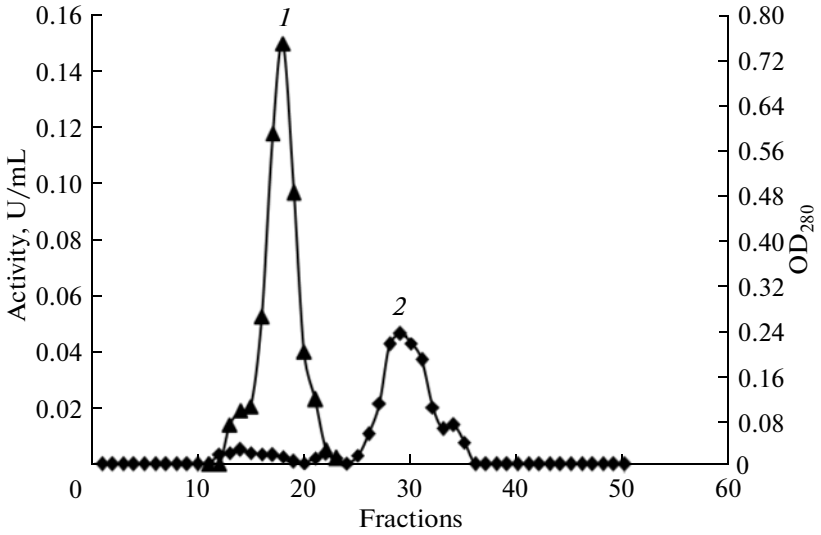

Fig. 2. Gel filtration chromatography of PNL produced by *A. flavus* MTCC 10938: *1* – protein; *2* – PNL activity.

vus MTCC 7589 had lower k_{cat} value of 52.2 s^{-1} and a low molecular weight of 38 kDa [24] as compared to enzyme secreted from *A. flavus* MTCC 10938. Km values of 3.87 and 4.6 mg/mL have been reported for PNL from, *Rizopus oryzae* [25], isolate F-7-4 [26], respectively, while k_{cat} values of 86 s^{-1} and 133 s^{-1} have been calculated for PNL of *Aspergillus japonicus* and *Penicillum canescens* [17, 19] respectively.

Effects of metal ions and protein inhibitors. It has been observed that 1 mM Zn^{++}, Ag^{+}, Hg^{++} ions totally inhibited enzyme activity while Ca^{++} and Mn^{++} stimulated it to some extent (data not shown). Though PNLs are generally not stimulated by presence of Ca^{2+} ions unlike pectate lyases, it can have an effect on enzyme activity [27, 19]. Calcium-dependent PNL from one species of *Aspergillus* and *Pythium splendens* have been found [28, 29]. Among protein inhibitors, potassium ferrocyanide and potassium permanganate showed total inhibition of enzyme activity from *A. flavus* MTCC 10938 while EDTA and sodium azide did not cause significant inhibition. Total inhibition of *Aspergillus terricola* PNL activity in the presence of potassium ferrocyanide and potassium permanganate has been previously described [30].

Retting of *Crotolaria juncea* fiber using purified *A. flavus* MTCC 10938 PNL. As the purified PNL had a pH optimum in alkaline range, its application in retting of locally available natural fiber *Crotolaria juncea*

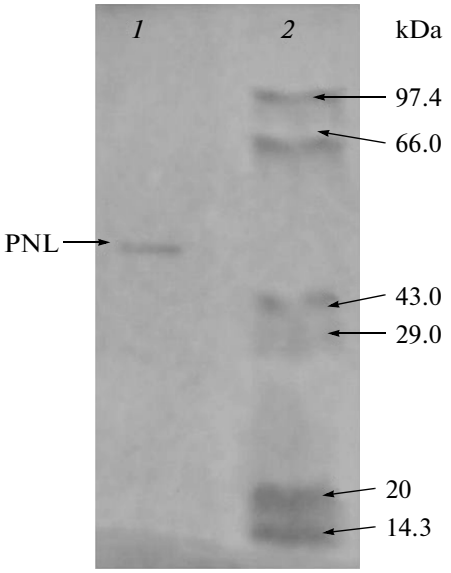

Fig. 3. SDS-PAGE of purified PNL produced by *A. flavus* MTCC 10938. Lane *1* – purified PNL, lane *2* – protein markers.

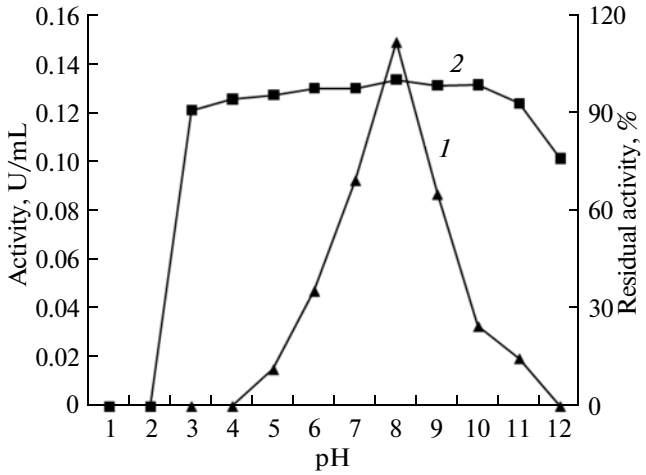

Fig. 4. pH optimum (*1*) and pH stability (*2*) of the purified PNL produced by *A. flavus* MTCC 10938.

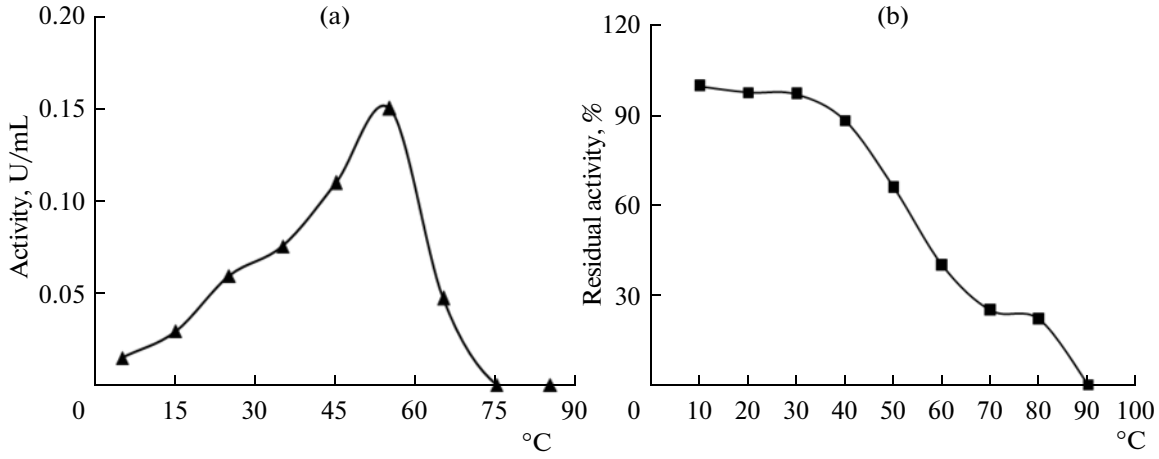

Fig. 5. Temperature optimum (a) and stability (b) of purified PNL produced by *A. flavus* MTCC 10938.

was attempted. Similar to methods reported earlier [16], sticks of fiber were subjected to purified PNL taken in variable concentrations with suitable control and in presence and absence of EDTA.

The purified 0.04 IU of pectin lyase of *Aspergillus flavus* MTCC 10938 gave efficient retting of *Crotolaria juncea* in absence of EDTA. It is an interesting observation that even though EDTA was not inhibiting the PNL activity showing 126% activity (data not shown), it caused an inhibition of retting at 1.0 mM concentration. Retting of natural fibers by PNL produced by *A. flavus* MTCC7589, *A. terricola* MTCC7588 and *P. citrinum* MTCC8897 has been reported [24, 30, 31]. Recent research has indicated that acidic pectins and Ca^{2+} are located preferentially in the epidermal regions of *Linum usitassimum* (flax) [32], contributing to the structural integrity of the stem and bast fibers. Therefore, chelators, such as EDTA have ability to remove Ca^{2+} and enhance retting of flax [33–35]. The variability in the pectin content along with Ca^{2+} in different fibers might influence the retting efficiency in the presence or absence of EDTA.

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REFERENCES

1. O'Neill, M., Albersheim, P, and Dravill, A., *Methods in Plant Biochemistry,* Dey, D.M., Ed., London: Academic Press, 1990, pp. 415–441.
2. Alkorta, I., Garbisu, G., Llama, M.J., and Serra, J.L., *Process Biochem*., 1998, vol. 33, pp. 21–28.
3. Kashyap, D.R., Vohra, P.K., and Tewari, R., *Biores. Technol.*, 2001. vol. 77, pp. 215–227.
4. Gummadi, S.N. and Panda, T., *Process Biochem*., 2003, vol. 38, pp. 987–996.
5. Jayani, R.S., Saxena, S., and Gupta, R., *Process Biochem*., 2005, vol. 40, pp. 2931–2944.
6. Taragano, V.M. and Pelosof, A.M.R., *Enz. Microbiol. Technol.,* 1999, vol. 25, pp. 411–419.
7. Yadav, S., Yadav, P.K., Yadav, D., and Yadav, K.D.S., *Process Biochem*., 2009, vol. 44, pp. 1–10.
8. Montovani, C.F., Geimba, M.P., and Brandelli, A., *Food Biotechnol.,* vol. 19, pp. 173–181.
9. Hoondal, G.S., Tiwari, R.P., Tewari, R., Dahiya, N., and Beg, Q.K., *Appl. Microbiol. Biotechnol*., 2002, vol. 59, pp. 409–418.
10. Albersheim, P., *Methods Enzymol*., 1966, vol. 8, pp. 628–631.
11. Cappuccino, J.G. and Sherman, N., *Microbiology: A Laboratory Manual.* California : Benjamin/Cumming, 1998.
12. Molina, S.M.G., Pelissari, F.A., and Vitorello, C.B.M., *Braz. J. Microbiol*., 2001, vol. 32, pp. 320–326.
13. Laemmli, U.K., *Nature*, 1970, vol. 227, pp. 680–685.
14. Switzer, R.C., Merril, C.R., and Shiprin, S., 1979, *Anal. Biochem.,* vol. 98, p. 231.
15. Engel, P.C., *Enzyme Kinetics – A Steady State Approach*, London: Chapman and Hall, 1977.
16. Zhang, J., Henriksson, H., Szabo, I.J., Henriksson, G., and Johansson, G., *J. Ind. Microbiol. Biotechnol*., 2005, vol. 32, pp. 431–438.
17. Semenova, M.V., Grishutin, S.G., Gusakov, A.V., Okunev, O.N., and Sinitsyna, A.P., *Biochemistry (Moscow),* 2003, vol. 68. no. 5, pp. 559–569.

18. Jong-Chon, K., Kim, H.Y., and Choi, Y., *J. Microbiol. Biotechnol.*, 1998, vol. 8, pp. 353–360.

19. Sinitsyna, O.A., Fedorova, E.A., Semenova, M.V., Gusakov, A.V., Sokolova, L.M., Bubnova, T.M., Okunev, O.N., Chulkin, A.M., Vavilova, E.A., Venetsky, Y.P., and Sinitsyn, A.P., *Biochemistry (Moscow)*, 2007, vol. 72, no. 5, pp. 565–571.

20. Silva, D.O., Attwod, M.M., and Tempest, D.W., *World J. Microbiol. Biotechnol.*, 1993, vol. 9, pp. 574–578.

21. Guevara, M.A., Gonzalez-Jaen, M.T., and Estevez, P., *Prog. Biotechnol.*, 1996, vol. 14, pp. 747–760.

22. Ishii, S. and Yokotsuka, T., *Agric. Biol. Chem,,* 1975, vol. 39, pp. 313–321.

23. Alana, A., Llama, M.J., and Serra, J.L., *FEBS Letters*, 1991, vol. 280, no. 2, pp. 335–340.

24. Yadav, S., Yadav, P.K., Yadav, D., and Yadav, K.D.S., *Process Biochem.*, 2008, vol. 43, pp. 547–552.

25. Hamdy H.S., *Annals Microbiol.*, 2005, vol. 55, no. 3, pp. 205–211.

26. Sakiyama, C.C.H., Paula, E.M., Pereira, P.C., Borges, A.C., and Silva, D.O., *Lett. Appl. Microbiol.*, 2001, vol. 33, pp. 117–121.

27. Linhardt, R.J., Galliher, P.M., and Cooney, C.L., *Appl. Biochem. Biotechnol.*, 1986, vol. 12, pp. 35–176.

28. Mayans, O., Scott, M., Connerton, I., Gravesen, T., Benen, J., and Visser, J., *Structure*, 1997, vol. 5, pp. 677–689.

29. Chen, W.C., Hsieh, H.J., and Tseng, T.C. *Bot. Bull. Acad. Sin.*, 1998, vol. 38, pp. 181–186.

30. Yadav, S., Yadav, P.K., Yadav, D., and Yadav, K.D.S., *Applied Biochem. Biotechnol.*, 2009, vol. 159, pp. 270–283.

31. Yadav, S., Yadav, P.K., Yadav, D., and Yadav, K.D.S., *Biochemistry (Moscow)*, 2009, vol. 74, no. 7, pp. 800–806.

32. Rihouey, C., Jauneau, A., Cabin-Flaman, A., Demarty, M., Lefevre, F., and Morvan, C., *Plant Physiol. Biochem.,* 1995, vol. 33, pp. 497–508.

33. Bratt, R.P., Brown, A.E., and Black, D.L. R., *Rec. Agric Res.*, 1986, vol. 34, pp. 9–15.

34. Henriksson, G., Akin, D.E., Slomczynski, D., and Eriksson, K.E.L., *J. Biotechnol.*, 1999, vol. 68, pp. 115–123.

35. Akin, D.E., Dodd, R.B., Perkins, W., Henriksson, G., and Eriksson, K.E.L., *Text. Res. J.*, 2000, vol. 70, pp. 486–494.