ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

UP-REGULATION OF CELLOBIASE SECRETION BY THERMAL STRESS IN THE FILAMENTOUS FUNGUS *PENICILLIUM CHRYSOGENUM*

Ahana Das[1], Maitree Bhattacharya[2], Samudra Prosad Banik*[1]

[1]*Maulana Azad College, Department of Microbiology, 8, Rafi Ahmed Kidwai Road, Taltala, Kolkata, West Bengal, India*

[2]*Jagadis Bose National Science Talent Search, 1300, Rajdanga Main Road, Sector C, East Kolkata Township, Kolkata, West Bengal, India*

**Corresponding author: samudraprosad.banik@maulanaazadcollegekolkata.ac.in*

ABSTRACT

Cellobiase (E.C. 3.2.1.21) controls the rate limiting step of cellulose hydrolysis in the production of cellulosic ethanol chiefly from agricultural biomass. The effect of thermal stress on secretion of cellobiase by the filamentous fungus *Penicillium chrysogenum* was studied. Exposure of 40 hours old mycelial culture to 42°C temperature reduced mycelial growth by 10% and resulted in constricted hyphal morphology. The intracellular elucidation of heat stress was also confirmed by upregulation of specific activity of the stress marker protein disulphide isomerase. Concomitantly, heat stress also resulted in increased specific activity of extracellular cellobiase by 50% from 0.5 U/ml to 1 U/ml which was also confirmed through zymography after Native PAGE. The cellobiase retained in intracellular fraction also had higher specific activity indicating that more cellobiase was synthesized under thermal stress. Trehalose is synthesized as a protective osmolyte under physiological stress. However, it is enzymatically degraded as soon as stress subsides. Specific activity of intracellular trehalase slowly increased post stress which indicated gradual dissipation of stress induced trehalose accumulation. The results collectively suggested that the fungus responds to heat stress by resorting to increased production and secretion of cellobiase to mobilize more nutrients from the extracellular environment. The findings provided valuable insights for industrial upregulation of synthesis and secretion of cellobiose.

Keywords: Cellulosic ethanol, Extracellular cellobiase, Stress induced trehalose, Stress biotechnology.

1. INTRODUCTION

The global transition to a sustainable biobased economy has seen the emergence of the filamentous fungi as organisms of high industrial importance due to their supreme ability to convert plant biomass to a wide range of products. Fungal fermentations have the potential to replace many chemical processes that are based on fossil resources [1]. Their ability to grow on a wide variety of inexpensive substrates with concomitant production of a rich repository of interesting metabolites and enzymes has made them the undisputable leaders of the biotechnology industry. Industrially used filamentous fungi are often superior to bacterial and yeast based production systems, in terms of versatility and secretory capacity [2]. Over the years, one of the critical bottlenecks in achieving enhanced secretion of these enzymes has been the lack of a thorough knowledge of the regulation of fungal secretion. Very few studies have reported the effect of stress on secretion of proteins and enzymes in these organisms [3, 4]. However, scientists are yet to find answers to all bottlenecks of production and secretion of enzymes by these organisms.

Environmental changes influence functionality and morphology of any organism. Excessive high temperature is a significant environmental challenge. This alteration in temperature can be associated with their surrounding climate change and in industrial scenario, it could also be due to instrumental malfunctioning inside fermenters or other factors. It is commonly reported that thermal stress affects the physiological processes which results in the collective general heat-shock response as is known in lower eukaryotes [5, 6].While some studies report the molecular basis of various stress response pathways in filamentous fungi, little is known about the effect of heat stress on enzymatic secretion from fungi.

Cellobiase, a secreted hydrolytic β-glucosidase (E.C. 3.2.1.21) plays important role in conversion of cellobiose to glucose, thus controlling the rate limiting step of cellulose hydrolysis. Therefore, most of the

current technological advancements involved in boosting production of the Biofuel industry are aimed towards manipulation of this step to increase glucose yield. The fungal cellulolytic enzyme has biotechnological applications in several other industries including food, animal feed, biomass refining [1]. In the past, secretion of cellobiase enzyme from *Termitomyces clypeatus* has been studied to illuminate the regulatory aspects of the enzyme secretion [7]. The pathway on which the secretion of cellobiase could be influenced had also been observed [8, 9]. In some yeast strains, elevated temperature induced secretion of this β-glucosidase [3,10]. Similarly other cellulolytic enzymes have also been employed to study the kinetics of enzyme secretion in filamentous fungi [11].

In addition to elucidation of heat shock response, almost all prokaryotes and lower eukaryotes synthesizes certain small molecule osmolytes as part of their defense response against stress. One such stabilizing osmolyte is trehalose, an alpha-alpha 1, 1 non-reducing disaccharide of glucose which binds to proteins and prevents their misfolding and simultaneous aggregation during cellular stress. Trehalose has been found to accumulate in these organisms both during conidial germination as a reserve source of energy [12], and during induction of heat shock response [13]. But no definitive information is available on the role of trehalose in regulating the secretion of native extracellular enzyme aggregates during induction of stress.

In the present studies, the effect of elevated temperature was investigated on the secretion of cellobiase from *Penicillium chrysogenum*. Parallely, the correlation between cellobiose and intracellular trehalose levels was also studied to understand the role of trehalose on secretion during stress.

2. MATERIAL AND METHODS

2.1. Growth conditions

The filamentous fungus *Penicillium chrysogenum* BF02 (GenBank Accession No.KC469896) was used in the present studies as the producer organism of cellobiase (E.C.3.2.1.21). The strain was maintained in PDA or CMC-agar slants supplemented with salt solution [14]. For the heat stress experiment, spore suspensions (1 x 106 cellsml-1) of the organism in sterile water was inoculated in 20 ml of liquid medium with salt solution, 1% glucose as carbon source and 0.2% mycological peptone in 100 ml shake flasks and grown at 28°C for 40 hrs (late log phase). Subsequently, four replicate flasks were subjected to heat shock at 42°C for one hour. Two of them were terminated immediately by separating the mycelia from the culture medium whereas the other two were put back for normal growth to assess if the heat shock was lethal. In order to induce oxidative stress, filter sterilized (0.22μm, Millipore) 10-8 M hydrogen peroxide was added to replicate 16 hr cultures and terminated after three days. Proper control flasks (in duplicate) were maintained in all cases. The mycelia were separated from the culture medium by filtration through nylon cloth and subsequent centrifugation at 8,000 rpm for 10 minutes. In order to ascertain growth, 3 day old mycelia were pressed in tissue paper and subsequently air dried in order to obtain the dry weight. Intracellular extract was prepared by macerating the dried mycelia with acid washed sand in 0.1 M acetate buffer with fungal protease inhibitor cocktail (Roche) and subsequent centrifugation at 8,000 rpm for 10 minutes 0.2% respective nitrogen sources. Media components were purchased from Sigma & Himedia whereas chemicals unless otherwise mentioned were of analytical grade obtained from SRL.

2.2. Hyphal imaging

Phase contrast images of washed live mycelia were acquired in a Zeiss Axiocam microscope under 40 X objective. The acquired images were processed using the associated software Zen, 2011.

2.3. Enzyme and protein assays

Cellobiase (EC 3.2.1.21) assay was carried out spectrophotometrically by using *p*NPG Sigma as substrate [15]. The reaction mixture (1 ml) contained 4 mM *p*NPG in 0.1 Msodium acetate buffer, pH 5.0 and an appropriate amount of the enzyme. Incubation was carried out at 50°C for 30min. Reaction was terminated by the addition of 0.25 ml Na_2CO_3 (0.5 M). Intensity of the yellow colour developed by liberation of *p*NP was measured at 400 nm. One unit (U) of enzyme activity was expressed by the enzyme that produced 1 μmole of *p*NP per min under the assay conditions. Protein content in the crude culture filtrate and intracellular extract was ascertained using Bradford reagent after extensive dialysis to remove the interfering sugars. Protein di-sulphide isomerase assay was carried out using insulin turbidity assay as prescribed by Sigma. Briefly, 0.75 ml of the reaction cocktail containing 10 mg/ml insulin in 50mMTris-HCl buffer pH 7.5, 100

mM DTT, 100 mM Sodium EDTA pH 7.0 and 100 mM Sodium phosphate Buffer pH 7.0 was incubated with 0.25ml of intracellular extract containing 25 µg protein in a cuvette thermostatted at 298 K. The reaction was initiated by addition of 10 µl of DTT and turbidity of the sample was noted at 650 nm in 5 min intervals. One unit of pdi is defined as that amount which causes a change of A_{650nm} of 0.01 per minute at 25°C at pH 7.5 of a 1mg/ml solution of insulin in presence of DTT.

The assay for neutral trehalase contained 50 µl cell-free extract (25-100µg protein) and 200µl 200 mM trehalose prepared in 25 mM MES pH 7±1, 125µM $CaCl_2$. The reactions were incubated at 37°C for 15-30 min and stopped by heating in a water bath at 100°C for 5 min. The glucose release was determined using the glucose oxidase-peroxidase method. One unit of trehalase is defined as the amount of enzyme that hydrolyses 1 µmol trehalose (2 µmol glucose) per min.

2.4. Native PAGE Zymography

Zymography or activity staining for in gel detection of cellobiase activity was performed as reported in Kwon et al. [16]. Briefly, after electrophoresis (5% Native PAGE), the gel was soaked in a 100 mM sodium citrate buffer (pH 5.0) containing esculin ferric chloride for 5min. It was then incubated at 50°C for several minutes until a black band appeared.

3. RESULTS AND DISCUSSION

3.1. Mycelial growth and hyphal morphology

The effect of elevated thermal stress was investigated on the secretion and synthesis of cellobiase from *Penicillium chrysogenum*. In order to determine the effect of heat stress at 42°C, the morphology of the hypha were investigated by phase contrast microscopy. The mycelia subjected to heat shock and immediately imaged showed deflated hypha and less prominent "Spitzenkorper" (fig. 1B) as compared to the control hyphae (fig. 1A). It may be due to the induction of stress granules in response to heat stress as a result of which inhibition of translation occurs in the cell of filamentous fungi *Aspergillus oryzae* [5]. The pruning like appearance of mycelia was observed in the cells exposed to heat stress. Similar result has also been observed previously in other fungal cells under thermal stress [17, 18] and is thought to be a part of adaptive response of the fungus. Parallelly, mycelial growth was evaluated by taking the dry weight of individual cultures. Growth decreased by 10% in heat exposed mycelia from 1.32 gm to 1.18 gm (fig. 2).

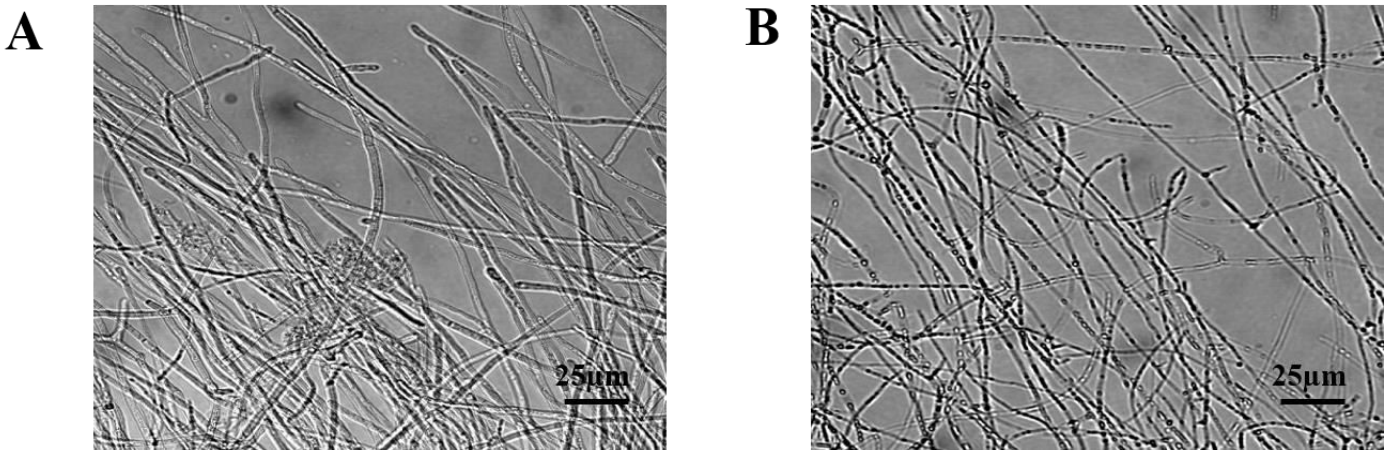


Fig. 1: Hyphal morphology of *Penicillium chrysogenum* under phase contrast microscope (40X), A) control, B) mycelia subjected to heat stress at 42°C.

3.2. Assessment of intracellular stress marker

Protein disulphide isomerase (Pdi) is an intracellular chaperone which catalyzes the formation of native disulfides of peptide chains from either the reduced form or randomly joined disulfides. It is upregulated in response to stress which may result in protein misfolding[19]. Thus, intracellular level of pdi was monitored to get remark of the degree of stress induced within the cell. The level of this chaperone was determined in mycelia exposed to thermal stress. Pdi specific activity increased from 0.436 U/mg (control) to 2.376 U/mg (heat stressed) (Fig 3, Table 1) and subsequently came back again to the basal level as stress subsided.

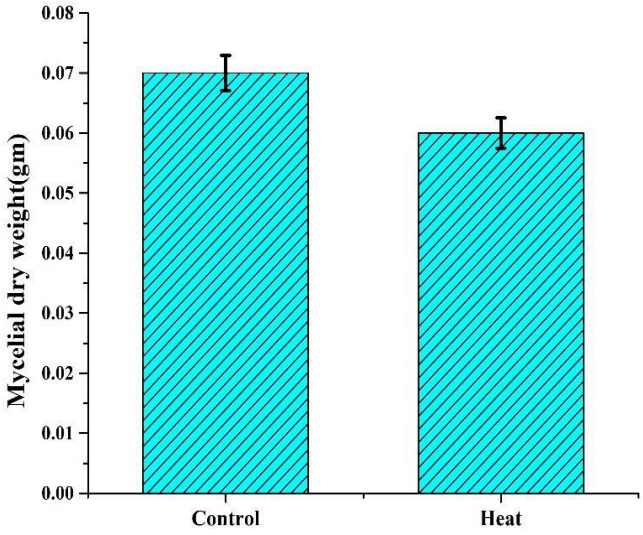
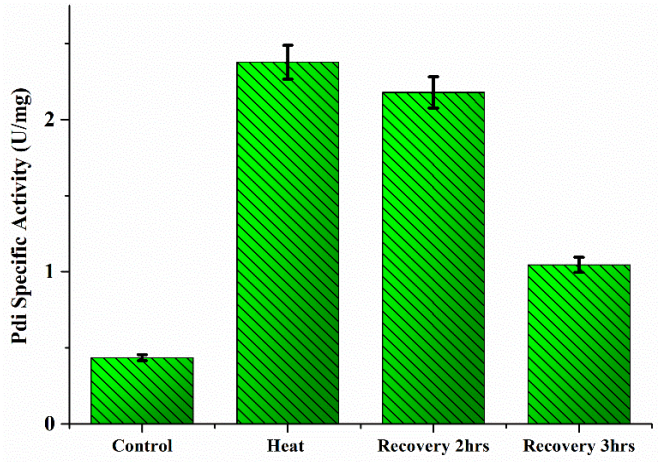


Fig. 2: Effect heat stress on mycelial growth as hyphal dry-weight. Error bars represent deviation from three replicate experiments



Error bars were drawn from data obtained from three replicate experiments

Fig. 3: Protein disulphide isomerase activity from intracellular extracts. Enzyme activity was calculated after the saturation in absorbance increase in each case

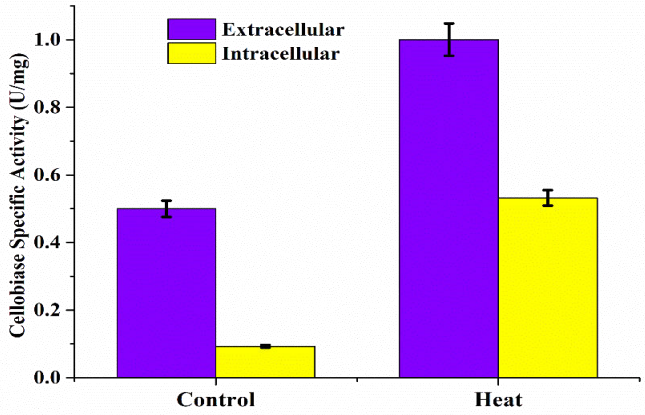
Table 1: Distribution of cellobiase, trehalase and protein disulphide isomerase in 30 hrs. old mycelia cultures during heat stress and post heat stress

Sample	Specific activity (U/mg)			
	Extracellular cellobiase	Intracellular cellobiase	Trehalase	Pdi (Protein disulfide isomerase)
Control	0.5±0.04	0.093±0.012	12.133±1.42	0.436±0.0
Heat stress	1±0.07	0.532±0.061	29.306±3.1	2.376±0.243
Recovery(2 hours)	0.513±0.042	0.206±0.018	36.113±3.34	2.178±0.31
Recovery(3 hours)	0.5±0.04	0.732±0.06	22.472±2.81	1.045±0.098

Experimental data sets were obtained in triplicate and ± indicates S.D. for each value

3.3. Secretion and synthesis of cellobiase under stress

In order to study the effect of stress on the secretory mechanism of *Penicillium chrysogenum,* cellobiase assay was carried out to determine the level of the enzyme under heat stress condition. Extracellular cellobiase activity doubled from 0.5 U/mg to 1 U/mg (fig. 4). Correspondingly, the intracellular cellobiose level also increased five times. This was indicative of enhanced secretion of cellobiase induced by heat stress with simultaneous increase in cellobiase synthesis in the mycelia. In order to ascertain whether the heat shock was lethal or permanently altered the cellular secretion machinery, replicate cultures were also put back to normal growth conditions after the heat shock and both extracellular along with intracellular cellobiase activity were compared with that of control cultures. The activities of extracellular cellobiase of the recovered cells became almost similar like that of control mycelia, suggesting that enhanced titer due to heat shock was only transient (table 1).



Error bars represent deviations from three replicate experiments

Fig. 4: Specific activities of extracellular and intracellular cellobiase in control and heat exposed mycelia

The significant increase in extracellular titer of cellobiose was also confirmed through native PAGE zymography (fig. 5). In presence of thermal stress, extracellular cellobiase from heat exposed mycelia showed much more intense band (lane 2), as compared to that of control (lane 1).

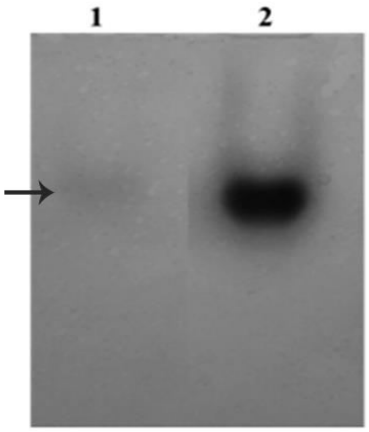


Fig. 5: Native PAGE zymography of extracellular cellobiase

3.4. Intracellular trehalose synthesis under stress

Trehalose is produced intracellularly as the precursor trehalose-6-phosphate by the enzyme trehalose-6-phosphate synthase (TPS) from UDP-Glucose. At times of stress, it is converted to trehalose by trehalose-6-phosphate phosphatase (TPP). However, immediately as stress subsides, trehalose is degraded by a neutral trehalase (TN) since presence of trehalose causes disruption in functioning of proteins as well as cellular chaperones. Neutral trehalase levels were assessed in heat induced fungal mycelial extracts as well as in cells recovered from the stress. Trehalase specific activity was highest (36.11 U/mg) within 2 hours after heat stress had subsided supporting the notion that post stress trehalose must be degraded.

4. CONCLUSION

Although substantial insights into the secretory apparatus of most of the higher eukaryotes have been attained, we are yet to completely delineate the secretory blackbox of the most powerful secretion machineries. Keeping in mind the ever increasing demand of glycosidases in the commercial market for industrial applications, it will be extremely convenient to understand the regulatory pathways controlling secretion in these organisms. Inhibition of secretion and other ER stresses has been shown to elicit Unfolded Protein Response (UPR) in most eukaryotes [20, 21]. In filamentous fungi, the trademark of UPR induction is upregulation of hac1 transcript, a transcription factor of UPR genes and Bip, an ER resident chaperone [22]. However, no systemic attempt has yet been taken to connect the secretion stress and the concomitant enzyme synthesis and secretion. Attending to these issues is crucial in order to fully realize and exploit the potential of these organisms. The present studies provided important insights into the new emerging field of stress biotechnology and established that controlled exposure to heat stress can boost the enzyme titre significantly to cater industrial demands. Our other previous studies and results [23] have reported for the first time the role of trehalose on stabilization of aggregation prone glycoproteins such as cellobiase. Trehalose stabilizes cellobiose aggregates by preventing their water induced dissociation. This provides a cross-linker independent solution for stabilizing these big fungal glycosidase aggregates for enhanced thermo-stability and catalysis. Trehalose is synthesized intra-cellularly in this β-glucosidase producing filamentous fungus during cellular stress concomitant with enhanced production and secretion of cellobiase. However, post stress, trehalose is degraded justifying the notion of interference caused by trehalose towards normal functioning of proteins. However, further work remains to be carried out to understand the molecular crosstalk between various physiological stresses and the secretion machineries involved in these high market value glycosidases.

5. ACKNOWLEDGEMENT

The work was carried out with partial funding from University Grants Commission, Govt. of India. Fellowship to AD was provided by UGC. The authors express their sincere thanks to the Principal, Maulana Azad College for providing all infrastructural support.

Conflict of interest

All authors have seen and agreed upon the final version of the manuscript. There is no conflict of interest with any authority.

6. REFERENCES

1. Srivastava N, Rathour R, Jha S, Pandey K, Srivastava M, Thakur VK, et al. *Biomolecules*, 2019; **9(6)**:220.

2. Meyer V. *Biotechnology advances*, 2008; **26(2)**:177-185.
3. Smith JD, Richardson NE, Robinson AS, *Biochim. Biophys.Acta (BBA)-Proteins and Proteom.*, 2005; **1752 (1)**:18-25.
4. Pakula TM, Laxell M, Huuskonen A, Uusitalo J, Saloheimo M, Penttilä M. *J. Biol. Chem.*, 2003; **278 (45)**:45011-45020.
5. Huang HT, Maruyama J, Kitamoto K. *PLoS One*, 2013, **8(8)**:e72209.
6. Chen Q. Li, Q. Wang, D Lu, Zhang H, Wang J, Fu R. *Sci. Rep.*, 2017; **7**:1-14.
7. Pal S, Banik SP, Ghorai S, Chowdhury S, Khowala S. *Carbohydr. Res.*, 2011; **346 (15)**:2426-2431.
8. Banik SP, Pal S, Chowdhury S, Ghorai S, Khowala S. *J. Microbiol. Biotechnol.*, 2011; **21(4)**:412-420.
9. Mukherjee S, Khowala S. *Biotechnol. Prog,*, 2002; **18**:1195-1200.
10. Genç, TT. *SAR J. Sci. Res.*, 2020; **3**:10-16.
11. Pakula TM, Uusitalo J, Saloheimo M, Salonen K, Aarts RJ, Penttilä M. *Microbiology*, 2000; **146(1)**:223-232.
12. d'Enfert C, Bonini BM, Zapella PD, Fontaine T, Da Silva AM,Terenzi HF. *Mol. Microbiol.*, 1999; **32(3)**:471-483.
13. Fillinger S, Chaveroche MK, Van Dijck P, de Vries R, Ruijter G, Thevelein J, d'Enfert C. *Microbiology*, 2001; **147**:1851-1862.
14. Banik SP, Bhattacharyya S, Ghorai S. *J. Microbiol., Biotechnol., Food Sci.*, 2014; **3(4)**:322-328.
15. Mukherjee S, Chowdhury S, Ghorai S, Pal S, Khowala S. *Biotechnol. Lett.*, 2006; **28**:1773-1778.
16. Kwon KS, Lee J, Kang HG, Hah YC, *Applied and Environmental Microbiology*, 1994; **60(12)**:4584-4586.
17. Zhang X, Ren A, Li MJ, Cao PF, Chen TX, Zhang G, Shi L, Jiang AL, Zhao MW. *Appl. Environ. Microbiol.*, 2016; **82(14)**:4112-4125.
18. Ordaz A, Favela E, Meneses M, Mendoza G,Loera O. *J. Basic Microbiol.*, 2012; **52(2)**:167-174.
19. Saloheimo M, Lund M, Penttilä M, *Mol. Gen. Genetics MGG*, 1999; **262**:35-45.
20. Hetz C. *Nature reviews Mol. Cell Biol.*, 2012; **13**:89-102.
21. Travers KJ, Patil CK, Wodicka L, Lockhart DJ, Weissman JS, Walter P. *Cell*, 2000; **101(3)**:249-258.
22. Saloheimo M, Valkonen M, Penttilä M. *Mol. Microbiol.*, 2003; **47(4)**:1149-1161.
23. Das A, Basak P, Pramanick A, Majumder R, Pal D, Ghosh A, et al. *Int. J. Biol. Macromol.*, 2019; **127**:365-375.