Recombinant Moringa Oleifera Lectin produced in Pichia Pastoris is a Potential Natural Coagulant

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Recombinant *Moringa Oleifera* Lectin produced in *Pichia Pastoris* is a Potential Natural Coagulant

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The natural coagulant *Moringa oleifera* lectin (MoL) has coagulation efficiency comparable to that of aluminum sulfate and is more environmentally sustainable than the latter. The coagulation efficiencies of culture supernatants from 50 MoL-secreting colonies and a control lacking MoL secretion were compared by observing turbidity removal from a 600 NTU kaolin suspension. Remarkably, 80% of the tested supernatants showed more than 80% turbidity removal, down to less than 100 NTU, the level achieved with the control supernatant. The coagulation efficiency of the four colonies achieving the highest levels of turbidity removal (colonies 2, 29, 31, and 49) was tested in triplicate alongside a control sample using 200 NTU turbid water. The mean difference in turbidity removal between the four colonies and the control was found to be significant using Dunnett's test ($P < 0.01$). The secreted MoL from GS115/pPIC9/*MoL* colony 2, the most efficient MoL-producing colony (mean difference compared to control, 42%; $P = 0.001$), was purified using an N-terminal hexa-His-tag and Ni-NTA spin columns. The expression of MoL was confirmed by the presence of a single 8 kDa band using SDS-PAGE analysis. The recombinant MoL produced by *Pichia pastoris* was secreted, and possessed a coagulation capacity of 83%, as determined based on the reduction in turbidity of 400 NTU water.

Keywords: Lectin, *Moringa oleifera*, Natural coagulant, *Pichia pastoris*, Turbidity.

1. Introduction

Every year, reports show an increase in human population, urbanization, and industrialization, all of which affect the global demand for clean water [1]. Although water is present in vast quantities on earth, the total volume that can be directly utilized by humans is only around 0.7% [2]. More than four billion people live in parts of the world where the scarcity of freshwater directly threatens human water security or river biodiversity.

Traditional water treatment systems generally begin with coagulation, followed by settling or sedimentation, gravity or granular filtration, and finally, chemical disinfection [3]. The first step in water processing by water treatment plants is usually the removal of colloidal particles through coagulation. The efficiency of this process, and thus the extent of colloidal particle removal, directly affects the final water quality [4]. The large surface area-to-volume ratio of colloids means that they are naturally resistant to sedimentation under gravity. However, the colloidal surface has a net negative charge, and neutralization of this charge by interactions with particles carrying a net positive charge is a potential strategy for colloid removal. Aluminum sulfate (alum) has a charge as high as 4^+ in water, although it is more typically bivalent, with a charge of 2^+. Alum is therefore a more effective coagulating, charge-neutralizing particle than a monovalent ion [5].

In Malaysia, alum is commonly used as coagulants in water treatment plants. However, studies performed prior to 2001 on the relationship between aluminum in drinking water and Alzheimer's Disease (AD), revealed a significant association between aluminum concentrations at or exceeding 100 g/L in drinking water and the risk of developing AD [6]. Additionally, aluminum is not biodegradable, and therefore the disposal of the sludge that is generated is problematic [7].

Throughout the world, various natural coagulants, produced and extracted from plants such as *Moringa oleifera*, *Cactus opuntia*, *Cactus latifaria*, *Strychnos potatorum*, *Prosopis juliflora*, *Fabaceae*, and *Jatropha curcas* have been utilized in water treatment [8]. In a previous study, the performance of three natural coagulants, extracted from *M. oleifera*, *S. potatorum*, and *Phaseolus vulgaris* seeds, were compared, and the *M.*

oleifera seed extracts were found to be the most effective coagulant [9]. Of all the natural coagulants that have been studied, *M. oleifera* seed extract is of particular interest, as it possesses both antimicrobial properties and efficient coagulation potential [10]. Therefore, lectin, an active compound from *M. oleifera*, has been the focus of recent efforts to replace alum. *M. oleifera* lectin is a cationic protein with a molecular weight of 6.5 kDa and an isoelectric point in the range of 10–11 [5].

All organisms produce lectins, also known as hemagglutinating protein. In plants, lectin allows seeds to act as sites of deposition. In general, the structure and carbohydrate specificity of lectin in seeds shares no similarity to the lectins found in other plant tissues [11].

Instead of obtaining *M. oleifera* seed lectin by conventional means, this study uses a novel recombinant protein engineering approach to sustainably produce the lectin coagulant.

2. Materials and methods

The *M. oleifera* lectin (*MoL*) gene sequence, represented by the mRNA sequence for *M. oleifera* protein 2.1 ($MO_{2.1}$), was obtained from the NCBI database (GenBank: AJ345072.1). The gene sequence was synthesized by Invitrogen, and inserted into the plasmid pPIC9, which was cloned in *Escherichia coli* K12 cells.

The pPIC9 plasmid carrying *MoL* (pPIC9/*MoL*) was purified and used to transform into *P. pastoris* strain GS115, giving GS115/pPIC9/*MoL*. The transformation and *MoL* expression were performed using an EasySelect *Pichia* Expression Kit according to the manufacturer's instructions [12]. *E. coli*/pPIC9/*MoL* was cultured on Luria-Bertani (LB) agar, and *Pichia pastoris* GS115 was cultured in yeast extract-peptone-dextrose medium (YPD). Following transformation with pPIC9/*MoL*, *P. pastoris* transformants were screened by culturing on selective minimal dextrose (MD) and minimal methanol (MM) agar, simultaneously. MoL expression was induced by culturing GS115/pPIC9/*MoL* in buffered minimal methanol (BMM) medium.

Analysis of the coagulation efficiency of the different *P. pastoris* colonies was performed using a 2100Q turbidimeter (Hach, USA). A kaolin suspension was used as synthetic turbid water; it was prepared by mixing 10 g of light kaolin powder (R & M, Essex, UK) in 1 L of ultrapure water, stirring for 1 h, and then incubating at room temperature for 24 h to complete particle hydration. Turbid waters of 600 NTU and 200 NTU were prepared by diluting the stock solution with ultrapure water to give a final volume of 500 mL.

The 50 colonies of transformed *P. pastoris*, grown on MM agar, were picked and cultured in BMM medium, and their supernatants were harvested. Colonies were labeled sequentially from GS115/pPIC9/*MoL* colony 1 to GS115/pPIC9/*MoL* colony 50. Next, 10 mL of supernatant from either *P. pastoris* GS115/pPIC9/*MoL* and *P. pastoris* GS115/his$^-$ negative control were added to a beaker containing a 600 NTU kaolin suspension.

Jar tests were performed on the supernatants from the 50 cultured colonies, using a PB 900 series programmable jar tester (Phipps and Bird, USA), with 2 min of rapid mixing at 100 rpm, followed by 30 min of slow mixing at 30 rpm and settling for 30 min. The jar testing is shown in Fig. 1.

The four supernatants that gave the highest turbidity removal (2, 29, 31, and 49), along with a control sample, were selected for further investigations into turbidity removal, using a 200 NTU kaolin suspension. The statistical significance of the differences in turbidity removal between the samples and the control was determined using the Dunnett's test at a confidence level of 99% ($P < 0.01$), using SPSS software.

The *MoL* gene from GS115/pPIC9/*MoL* colony 2 was amplified by polymerase chain reaction (PCR), using MoL-specific Forward (5'-CAGGGACCTGGTCGGCAGCC-3') and Reverse (5'-GGTGCTAGGTATATTGGATG-3') primers, to give an amplicon of 224 bp. A second confirmation screening with region containing the alcohol dehydrogenase 1 (*AOX1*) and *MoL* gene was also amplified using universal AOX1 forward primer (5'-GACTGGTTCCAATTGACAAGC-3') and AOX1 reverse primer (5'-GCAAATGGCATTCTGACATCC-3') (Invitrogen).

PCR was carried out in a 20 µL reaction mixture, containing 5 µL 5 × reaction buffer, 1.2 µL 25 mM $MgCl_2$, 0.4 µL 25 mM dNTPs, 0.4 µL forward primer (10 ρmol/µL), 0.4 µL reverse primer (10 ρmol/µL), and 12.5 µL *P. pastoris* cell suspension, added in this order, using a GoTaq® DNA Polymerase kit (Promega Corporation). The PCR was initiated with 0.1 µL Taq polymerase, and run for 30 cycles with the following steps: denaturation at 95°C for 1 min, annealing at either 51°C (MoL primer) or 54°C (AOX1 primer) for 1 min, extension at 72°C for 1 min. A final extension step at 72°C for 7 min was carried out in all cases. The controls for PCR were *P. pastoris* colonies lacking the additional *MoL* gene, and the PCR mixture without *P. pastoris* cell. Finally, the amplified products were separated by 1% agarose gel electrophoresis, and visualized.

The supernatant of GS115/pPIC9/*MoL* colony 2 was concentrated 10-fold, the resulting protein was quantified using a Qubit® 2.0 Fluorometer (Invitrogen) and was purified using a Ni-NTA spin kit according to the manufacturer's instructions (Qiagen, USA). A 600 µL aliquot of the concentrated MoL (pH 7.5) was bound to the Ni-NTA spin column and eluted in buffer containing 500 mM imidazole, 300 mM NaCl, 200 mM KCl, and 50 mM phosphate. The purity of MoL was verified by SDS-PAGE; 10 µL of eluate containing the purified MoL, flow through, and concentrated sample were mixed with 10 µL of tricine-SDS loading buffer and loaded into an 18% SDS-PAGE (Bio-Rad, USA). The coagulation activity of the purified MoL was determined using a

spectrophotometer as described previously, using turbid water of approximately 400 NTU [13].

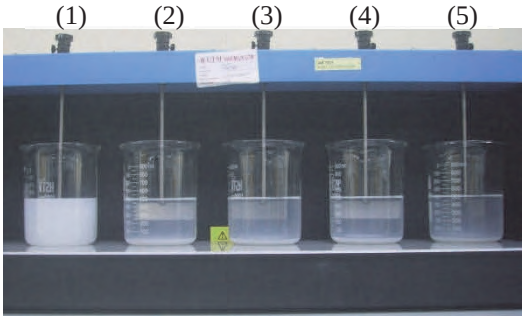

Fig. 1. Jar test for turbidity removal, using 500 mL of kaolin suspension (600 NTU) and 10 mL of GS115/pPIC9/*MoL* supernatant. (1) no coagulant (control); (2) colony 2; (3) colony 29; (4) colony 31; (5) colony 49.

3. Results and Discussion

3.1 Coagulation performance of culture supernatants from 50 transformed *P. pastoris* colonies

The cultured supernatants from transformed *P. pastoris* colonies contain native protein as well as recombinant MoL. Supernatant from colony 2 achieved a 96% reduction in turbidity, from 600 to 20 NTU, the highest of all the samples tested (Fig. 2). Moreover, the MoL in the *P. pastoris* supernatants displayed coagulation activity that was similar to that of *M. oleifera* lectin extracted from seeds [14]. Over 80% of the cultured colonies expressed high concentrations of MoL in their supernatants, giving a significant reduction in kaolin suspension turbidity. The remaining colonies exhibited a less than 70% reduction in turbidity relative to the control, indicating lower levels of secreted MoL. The lowest turbidity reduction was seen with colonies 5, 7, 17, 46, and 47; no MoL and low native protein was secreted in their supernatants. In the present study, the integration of the pPIC9 plasmid into the genomes of the *P. pastoris* was confirmed by growing transformed colonies on selective regeneration dextrose (RD) agar. However, the RD agar screen only verifies the expression of the *HIS4* in pPIC9, and it is possible that the *MoL* is not expressed, perhaps because of incomplete insertion or subsequent deletion.

In over 80% of colonies displaying efficient turbidity removal, variations can be attributed to the efficiency of *MoL* integration into the *P. pastoris* genome. Previously, the efficiency of heterologous protein expression improved significantly when multiple copies of the expression plasmid were integrated into host cells [14]. The integration of multiple pPIC9/*MoL* plasmids into the *P. pastoris* genome, through tandem insertions at the restriction enzyme *Sal*I site within *his4*, was reported to substantially increase protein yield [15]. The four colonies inducing the highest reduction in turbidity, colonies 2, 29, 31, and 49, were selected for further analysis.

Section : 3.1 Coagulation performance of culture supernatants from 50 transformed *P. pastoris* colonies

Fig. 2. (a)

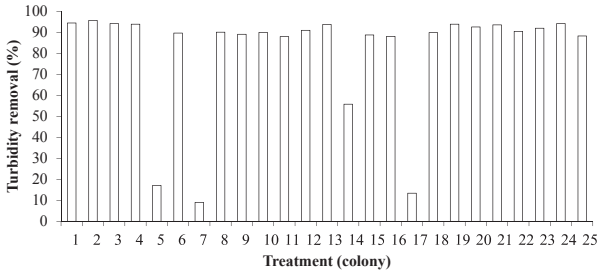

Fig. 2. (b)

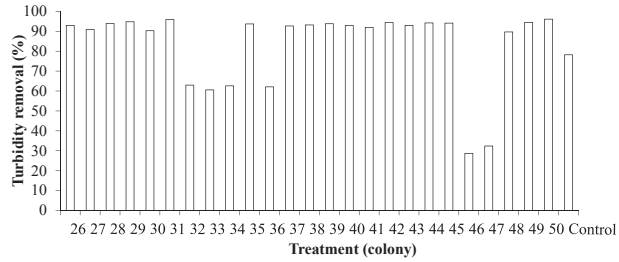

Fig. 2. Turbidity removal by cultured supernatant of transformed *P. pastoris* colonies and a control. A kaolin suspension was used at an initial turbidity of 600 NTU. (a) Results with supernatants from GS115/pPIC9/*MoL* colonies 1 through 25; (b) Results with supernatants from GS115/pPIC9/*MoL* colonies 26 through 50, as well as the GS115/his$^-$ control.

The neutralization observed during turbidity removal may be attributable to the coagulant properties of MoL, which has a net positive charge on its surface. The interaction of the positively charged MoL and the negatively charged kaolin is expected to form flocs, leading to coagulation [16]. Natural coagulants extracted from organisms, such as the MoL used here, are viable alternatives to currently used coagulants, as they are known to be biodegradable and safe for human health. Additionally, they are effective flocculating agents for various colloidal suspensions, with a wide range of effective doses [16].

3.2 Statistical analysis of the four supernatants with highest turbidity removal

Turbidity removal was examined statistically, as follows. Triplicate samples of the four colonies that gave the highest levels of turbidity removal with the 600 NTU kaolin suspension were examined using a 200 NTU suspension, and compared with the control. The results confirm the highly effective removal of turbidity, indicating the presence of MoL in the respective supernatants (Table 1).

The colony 2 supernatant achieved significantly higher turbidity removal than did the control (42%), suggesting that MoL was produced at highest level compared to other three colonies (colony 29, 31 and 49). Overall, all four of the colony supernatants exhibited significantly higher turbidity removal than that of the control.

Table. 1. Statistical analysis of the difference in turbidity removal achieved by the control and colony supernatants, using an initial turbidity of 200 NTU.

Supernatants		Mean Difference	Sig. (P < 0.01)
2	Control	42.03*	0.001
29		41.28*	0.001
31		39.23*	0.002
49		40.13*	0.001

* The mean difference is significant at < 0.01 level.

3.3 PCR analysis of GS115/pPIC9/*MoL* colony 2

The *MoL* gene inserted into the *P. pastoris* genome was investigated further using universal AOX1 and specific MoL primers, respectively. As shown in Fig. 3, in lane 2, a clear 724 bp band was seen corresponding to 500 bp of *AOX1* fused to 224 bp of *MoL*, indicating that *MoL* was properly inserted into the pPIC9 plasmid. The presence of *MoL* within the 724 bp amplicon was then confirmed using *MoL*-specific primers and the colony 2 genome as a template. The *MoL*-specific primers were designed to be complimentary to the 22 bp sequences at either end of the *MoL* sequence. As shown in Fig. 3, a 224 bp *MoL* band was seen as expected (lane 3), and no band was seen in the negative control lacking template DNA (lane 4). The observed molecular weight of *MoL* is consistent with the amplicon product of $MO_{2.1}$ [17]. The insertion of *MoL* after AOX1 promoter could induce high levels of MoL production.

Two different genes in the *P. pastoris* genome, *AOX1* and *AOX2*, are expressed when the carbon source is methanol. These genes are transcribed at different levels; *AOX1*, which possesses a strong promoter (PAOX1), generates the heterologous protein more efficiently than *AOX2*. The combination of PAOX1-mediated transcriptional activation and the secretion of recombinant proteins by *P. pastoris* is extremely useful, and impressive protein yields can be achieved (up to 22 g/L of intracellular protein and 15 g/L of secreted protein) [18].

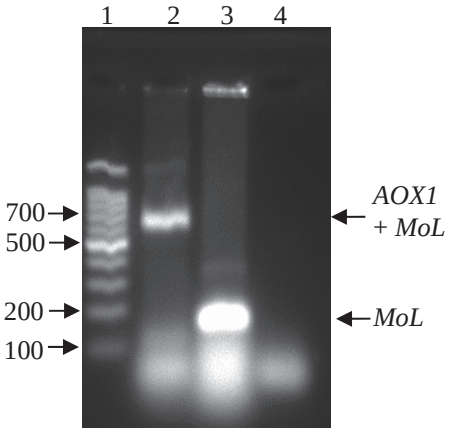

Fig 3. PCR analysis of GS115/pPIC9/*MoL* colony 2. Lane 1: 100 bp DNA marker (Promega); lane 2: PCR product amplified with AOX1 primers (724 bp); lane 3: PCR product amplified with MoL primers; lane 4: negative control (no colony. The arrows on the left indicate molecular weight.

3.4 SDS-PAGE evaluation of MoL secreted from *P. pastoris*

The increased expression of heterologous MoL compared to that of the native *P. pastoris* protein was determined by SDS-PAGE, and a more intense 8 kDa band was seen in the concentrated supernatant (Fig. 4, lane 3). Following protein purification, the presence of secreted MoL in the culture supernatant from GS115/pPIC9/*MoL* colony 2 was confirmed by SDS-PAGE, and a single 8 kDa band was observed (Fig. 4, lane 1). However, this band was of a higher molecular weight than the previously reported 6.5 kDa for MoL, indicating that the protein has undergone post-translational modification. Glycosylation of MoL following extracellular secretion by *P. pastoris* increased its molecular weight by ~1.5 kDa, consistent with a study describing the production of the 40.3 kDa recombinant *Triticum aestivum* xylanase inhibitor I (rTAXI-I) protein in *P. pastoris*, which showed a 1732 Da increase in mass due to N-glycosylation [19]. Like all eukaryotic organisms, but unlike bacterial expression systems, *P. pastoris* is able to perform glycosylation, although the glycosyl chains it generates are much shorter than those produced by *S. cerevisiae* [20].

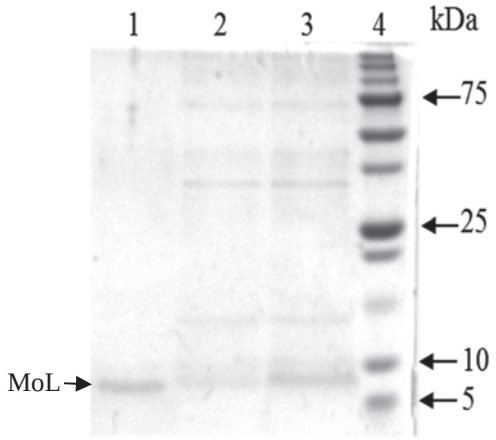

Fig. 4. SDS-PAGE using an 18% tricine buffer. Lane 1: purified MoL (314 µg/mL); lane 2: sample flow-through; lane 3: concentrated supernatant from GS115/pPIC9/*MoL* colony 2; lane 4: precision plus protein dual extra markers (Bio-Rad). The arrows on the right indicate molecular weight.

3.5 Coagulation test for purified MoL

Turbidity removal was investigated at increasing concentrations of purified MoL. As shown in Fig. 5, the most effective purified MoL concentration was the lowest, 2 µg/mL, which achieved 83% turbidity removal, with 4, 6, 8, and 10 µg/mL of purified MoL being progressively less effective. However, at the highest protein concentration tested, 10 µg/mL, the coagulant activity achieved by the purified MoL, defined as the percentage of turbidity removal, was still 13.86% better than that of the control. At MoL concentrations of 6 µg/mL and above, there is an excess of MoL over suspended kaolin, and the occurrence of a charge reversal effect is possible. This is consistent with a previous report on the chemical coagulant aluminum, wherein the charge reversal effect began to dominate at higher concentrations due to the restabilization of negative particles [21].

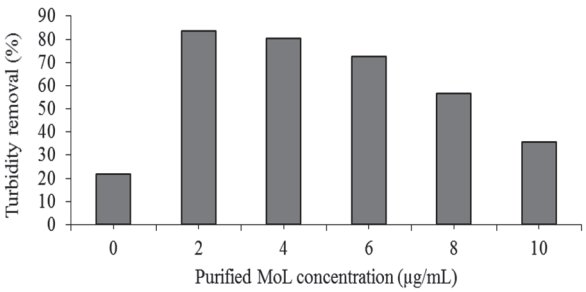

Fig. 5. Percentage turbidity removal by increasing concentrations of purified MoL.

4. Conclusion

M. oleifera seed extract contains an active coagulant protein. In methods using this seed extract to treat turbid water, the entire extract is mixed with either distilled water or salt water, and then added directly to turbid water to study the turbidity removal. Although some of the extraction methods previously used with *M. oleifera* seeds have been applied in this study, we have also shown that *P. pastoris* supernatant containing MoL is highly effective in reducing the turbidity of water, from 600 to 20 NTU.

Recombinant MoL, produced by *P. pastoris*, is a promising coagulant for water treatment. Molecular screening of the transformed colonies to identify those that are producing high levels of MoL is very expensive, and so a direct screening using protein activity as an indicator of MoL expression has been described in this study. The highest level of MoL production, indicated by the reduction in turbidity, was seen with GS115/pPIC9/*MoL* colony 2.

Secreted MoL in colony supernatants was active in highly turbid waters of 600 and 200 NTU. The high turbidity removal activity was confirmed using purified MoL at 400 NTU kaolin suspension. However, in order to reduce the cost of water purification, the supernatant could be used directly in water treatment. The MoL-containing *P. pastoris* supernatant could also potentially be used to treat turbid water from reservoirs, with water quality being monitored thereafter. As a natural coagulant, we believe the sludge production from MoL-containing supernatants will be much lower than that of conventional coagulants, and its storage in landfill much safer [1].

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References

1) E.E.L. Muller, A.R. Sheik and P. Wilmes, *Current Opinion in Biotechnology*, **30**, 9-16 (2014).
2) M.A. Oturan, and A. Jean-Jacques, *A review, Environmental Science and Technology*, **44**, 2577-2641 (2014).
3) H. Xua, F. Xiaoa, D. Wanga and C. Yeb, *Colloids and Surfaces A: Physicochem. Eng. Aspects*, **456**, 211-221 (2014).
4) M. Zainal-Abideen, A. Aris, F. Yusof, Z. Abdul-Majid, A. Selamat and S.I. Omar, *Water Science & Technology*, **65**, 496-503 (2012).

5) A.W.M. Azhar, H. Hara and M.N.M. Johari, *Malaysian Journal of Civil Engineering*, **28**, 26-34 (2016).
6) J.R. Walton, *Journal of Alzheimer's Disease*, **40**, 765-838 (2014).
7) K.C. Valverde, L.C.K. Moraes, M.C. Bongiovani, F.P. Camacho and R. Bergamasco, *Acta Scientiarum*, **5**, 485-489 (2013).
8) C.Y. Yin, *Process Biochem.*, **45,** 1437–1444 (2010).
9) G. Muthuraman and S. Sasikala, Journal of Industrial and Engineering Chemistry, **20**, 1727–1731 (2014).
10) S.K. Kansal and A. Kumari, *Chem. Rev.*, **114**, 4993-5010 (2014).
11) A.F.S. Santos, L.A. Luz, A.C.C. Argolo, J.A. Teixeira, P.M.G. Paiva and L.C.B.B. Coelho, *Process Biochemistry*, **44,** 504–508 (2009).
12) Invitrogen, *Life Technologies Corporation*, (2014).
13) R.S. Ferreira, T.H. Napolea ˜o, A.F.S. Santos, R.A. Sa, M.G. Carneiro-da-Cunha, M.M.C. Morais, R.A. Silva-Lucca, M.L.V. Oliva, L.C.B.B. Coelho, and P.M.G. Paiva, *Lett. Appl. Microbiol.*, **53**, 186–192 (2011).
14) S. Kelle, K. Zelena, U. Krings, D. Linke and R.G. Berger, *Protein Expression and Purification*, **95**, 233-239 (2014).
15) R.M. Bill, *Methods and Protocols, Methods in Molecular Biology*, **866**, Springer (2012).
16) B.S. Deshmukh, S.N. Pimpalkar, R.M. Rakhunde and V.A. Joshi, *International Journal of Innovative Research in Science,Engineering and Technology*, **2(11)**, 6183-6189 (2013).
17) A.R. Pavankumar, J. Noren, L. Singh and N.K.C. Gowda, *RSC Adv.*, **4**, 7136–7141 (2014).
18) T. Vogl and A. Glieder, *New Biotechnology*, **30**, 386-401 (2013).
19) K. Fierens, N. Geudens, , K. Brijs, C.M. Courtin, K. Gebruers, J.Robben, S.V. Campenhout, G. Volckaert and J.A. Delcour, *Protein Expr. Purif.*, **37**, 39–46 (2004).
20) S. Macauley-Patrick, M.L. Fazenda, B. McNeil and L.M. Harvey, *Yeast*, **22**, 249-270 (2005).
21) J. Duan and J. Gregory, *Advances in Colloid and Interface Science*, **100-102**, 475–502 (2003).