

High-yield intracellular production of an exo-polygalacturonase enzyme via heterologous expression of *Penicillium notatum* gene in *Saccharomyces cerevisiae*

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Article history

Received: 21 August 2020

Received in revised form:

10 November 2020

Accepted:

14 December 2020

Abstract

Exo-polygalacturonase (Exo-PG) is one of the most important members of the pectinolytic group of enzymes with immense applications in the food industry. The present work was undertaken to investigate the cloning, expression, and transformation of an Exo-PG gene in yeast *Saccharomyces cerevisiae* to achieve the high titre of Exo-PG from *Penicillium notatum*. For this, the Exo-PG gene from *P. notatum* was cloned into BamHI and XbaI digested pYES2 plasmid with GAL1 promoter, and heterologously expressed in *S. cerevisiae*. The recombinant yeast cells were cultivated at 30°C in shake flask fermentation using minimal media without uracil, in the presence of ampicillin (100 µg/mL), following the addition of 2.0% galactose as an expression inducer. Results revealed that the yeast was a good expression host, and successfully produced 6.67 U/mL of the recombinant enzyme into the culture media after 24 h of induction; under longer induction time, the activity was decreased. The secreted Exo-PG exhibited two strong bands with an approximate molecular weight of 20 - 25 kDa and 70 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, thus indicating a dimeric protein. In conclusion, the results demonstrated that the gene was successfully expressed, thus resulting in high-yield intracellular production of Exo-PG.

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Keywords

exo-polygalacturonase, heterologous expression, *Penicillium notatum*, *Saccharomyces cerevisiae*, cloning, enzyme activity

Introduction

Pectinases are a unique group of pectinolytic enzymes that mediate the catalytic depolymerisation and degradation of pectinaceous materials by hydrolases, lyases (depolymerisation reaction), or esterases (de-esterification reaction) (Amin *et al.*, 2019). Based on their approach towards pectin-containing substances, they are classified as (1) esterases, and (2) depolymerases. Depolymerases show catalytic action on pectin, polygalacturonic acid, and rhamnogalacturonan. Depolymerases can be further divided into two groups; one acts on pectin and the other one acts on polygalacturonic acid. The first group can be further subcategorised into (i) polymethylgalacturonase (PMG, E.C. 3.2.1.1), which cleaves pectin exolytically (Exo-PMG) or endolytically (Endo-PMG); and (ii) pectin lyase (PL, E.C. 4.2.2.10), which carries out the trans-elimination reaction of pectin (Endo-PL) endolytically only, thus resulting in an unsaturated oligosaccharides. Depolymerases acting on polygalacturonic acid can be

further divided into two groups based on their mode of action; (i) polygalacturonase (PG), which exolytically carries out the hydrolysis of polygalacturonic acid (Exo-PG1, E.C. 3.2.1.67 and Exo-PG1, E.C. 3.2.1.82), or endolytically (Endo-PG, E.C.3.2.1.15), and (ii) pectate lyase (PGL), which causes trans-elimination of polygalacturonic acid by its exolytic (Exo-PGL, E.C. 4.2.2.9) and endolytic (Endo-PGL, E.C. 4.2.2.2) catalysis (Lu *et al.*, 2016; Patidar *et al.*, 2018). Depolymerases that show action by taking rhamnogalacturonan as a substrate are additionally categorised into (i) rhamnogalacturonan-hydrolysing enzyme rhamnogalacturonase (RG, E.C.3.2.1.171), and (ii) rhamnogalacturonan endolyase (RGL, E.C. 4.2.2.23), which cause trans-elimination reaction to depolymerise rhamnogalacturonan. Esterases also show action on pectinaceous materials, and are classified into (i) pectin acetyl esterase (E.C. 3.1.1.6), and (ii) pectin methylesterase (E.C. 3.1.1.11) (Singh *et al.*, 2012; Amin *et al.*, 2017a; 2020).

Pectinases are extensively found in fruits and vegetables, where they play a vital contribution to the

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natural ripening process. However, pectinases of microbial origins have advantages such as large-scale production is feasible under controlled environments using cheaper resources, and ease of multiplication and maintenance (Amin *et al.*, 2017b). Yeast, bacterial, and fungal strains have been employed for the biosynthesis of pectinases. Among the fungal strains, various species of *Aspergillus* and *Penicillium* are the powerful houses for pectinase production. Among the aspergilli, *A. fumigatus* (Phutela *et al.*, 2005; Wong *et al.*, 2017), *A. kawachii* (Byrne *et al.*, 2017), *A. niger* (Khatri *et al.*, 2015; Beulah *et al.*, 2015), and *A. giganteus* (Pedrolli and Carmona, 2010) have been reported to be the most efficient pectinase producers, while among the penicilli, *P. notatum* (Amin *et al.*, 2017b), *P. occitanis* (Damak *et al.*, 2011), and *P. janczewskii* (Amin *et al.*, 2020) are also potent pectinase producer. Many other fungal species also exhibit the ability to synthesise a significant titre of pectinases such as *Aureobasidium pullulans* (Bennamoun *et al.*, 2016), *Thermoascus indicaeseudaticae* (Martins *et al.*, 2010), and *T. aurantiacus* (Martins *et al.*, 2002). Bacterial species such as *Chryseobacterium indologenes* (Roy *et al.*, 2018), *Bacillus subtilis* (Mercimek Takci and Turkmen, 2016; Yu *et al.*, 2017), *Pectobacterium carotovorum* (Maisuria and Nerurkar, 2012), and *Sclerotium rolfsii* (Schnitzhofer *et al.*, 2007) are also competent alkaline pectinase producers.

Pectinases produced via fermentation from microbial sources usually face some problems in industrial implementation such as less bioactivity, lower half-life, and lower level of tolerance towards extreme environments, *i.e.*, acidic, alkaline, and high-temperature ranges. In addition, most of the microorganisms produce a complex of enzymes which are responsible for the contamination of the enzyme of interest. Although this consortium could be employed in several applications with promising outcomes, many drawbacks are associated with the utilisation of the complex mixture of enzymes such as (1) difficult to screen explicit enzyme for specific function, (2) cost-expensive production due to elaborate optimisation without appropriate acquaintance of particular gene, and (3) challenges in augmenting the enzyme titre without the examination of a particular enzyme. As a result, researchers and scientists started using gene-cloning methods for the study, function, and expression purposes in yeast host systems. Gene cloning studies are not only important in characterising specific genes of interest, but are being conducted in this era to achieve the over-expression of a target protein with industrial importance (Alimardani-Theuil *et al.*, 2011). These technologies

can yield highly pure enzymes with high yields for functional studies (Dalboge, 1997). Therefore, due to the increased economic interests and demands, extensive research has been devoted to the selection of natural producers, construction of Exo-PG producers, and process optimisation. Recombinant pectinases are being explored from numerous novel microbial sources due to their promising catalytic performance, half-life, and tolerance to large temperature and pH ranges to work under restrictive environments. *Bacillus clausii* (Zhou *et al.*, 2017), *Aspergillus aculeatus* (Abdulrachman *et al.*, 2017), *Paenibacillus* sp. (Ko *et al.*, 2011), *Bacillus halodurans* (Mei *et al.*, 2013), *Bacillus licheniformis* (Singh *et al.*, 2012), and *Bispora* sp. (Yang *et al.*, 2011) are amongst the few potential recombinant pectinolytic enzyme-synthesising species. The present work aimed to investigate the cloning, expression, and transformation of a gene in a yeast host system, *Saccharomyces cerevisiae*, to achieve the high titre values of Exo-PG from *P. notatum*.

Material and methods

Strains, plasmids, and growth conditions

Saccharomyces cerevisiae strain selected for the expression of the pectinolytic gene was maintained in YPD media (bactopeptone, 20 g/L; yeast extract, 10 g/L; and a final concentration of 2% glucose) at 30°C. A shuttle plasmid pYES2 with GAL1 promoter provided by the Division of Molecular Biology, Molecular and Cellular Biology (MCB), University of Minnesota, USA was used for cloning and expression purposes (Figure 1). The competent *E. coli* cells (XL10) used for the cloning were maintained on Luria-Bertani (LB) broth at 37°C. The recombinant construct of *S. cerevisiae* harbouring the pYES2-Exo-PG gene was proliferated, maintained, and cultured on minimal media without uracil with the composition of 6.7 g/L yeast nitrogen base without amino acids, 1.92 g/L yeast synthetic drop-out medium supplement without uracil, and a final 2.0% glucose concentration with 100 µg/mL ampicillin. The pH of the medium was maintained at 6.5 - 7.0, and 2% galactose was used for protein induction.

Expression of *P. notatum* Exo-PG gene in *S. cerevisiae* system

Penicillium notatum Exo-PG gene was expressed into the yeast host system with the aim of getting the overexpression of the fungal gene in *S. cerevisiae* in a shorter span of time with less energy input. *P. notatum* gene sequence was found on

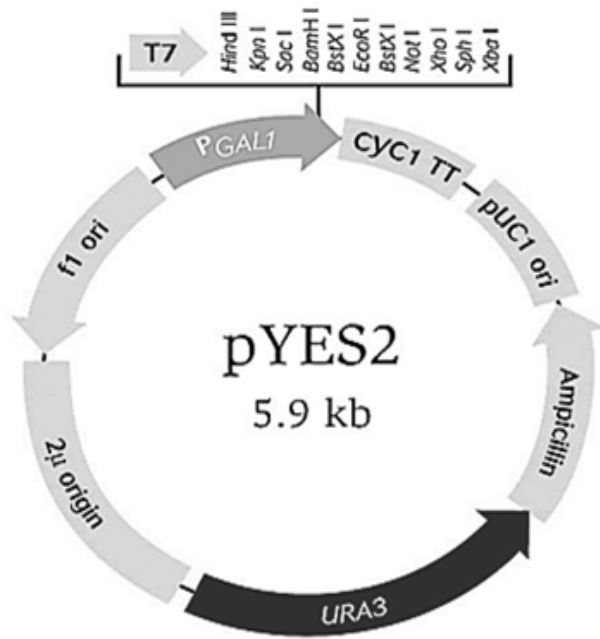


Figure 1. Map of the pYES2 vector used in the present work; the expression system of this yeast was driven by the strong GAL1 promoter in the pYES2 vector, which was induced by inexpensive galactose. An important advantage of this system is its ability to achieve extremely high cell densities and efficient protein production.

<http://www.ncbi.nlm.nih.gov> and <http://fungi-cyc.broadinstitute.org:1555/PCWC541255/NEW-IMAGE?type=LocusPosition&object=G53-1206&detaillevel=2&chromosome=NIL&show-tracks=on> from the whole genome sequence (8,770 bp) of *P. chrysogenum* Wisconsin 54-1255, and blasted on <http://www.ncbi.nlm.nih.gov/BLAST> against many sequences of polygalacturonase genes from other fungal sources. After confirming its identity by BLAST analysis, the 1,278 bp gene sequence was ordered for synthesis/manufacturing with added restriction enzyme cut sites of BamHI and XbaI synthesised by Eurofins MWG Operon (USA).

Cloning of gene insert into competent cells

The Exo-PG gene constructs obtained after synthesis from Eurofins MWG Operon (USA) were gel-purified using DNA purification kit (Zymo Research, USA). The gel-purified insert DNA was ligated to BamHI and XbaI digested pYES2 vector using the DNA ligase at room temperature, and then subsequently cloned into *E. coli* XL10 cells by thawing the cells on ice for 10 - 20 min. The cloned mixture was plated on LB agar plates with ampicillin (100 µg/mL), and incubated overnight at 37°C. The next day, 4 - 5 colonies from cloned *E. coli* XL10 cells

were picked and cultured separately in 1 - 2 mL LB media with 100 µg/mL ampicillin overnight at 37°C at 150 rpm. After overnight culturing, the plasmid DNA was isolated, purified, and run on 1% agarose gel electrophoresis after restriction digestion with BamHI and XbaI in 1X TAE buffer at 250 volts using 1 kb plus DNA ladder. The gel was then examined under UV-light for the insert DNA and plasmid DNA band, and then transformed into yeast cells.

Transformation of plasmid DNA into yeast

The plasmid DNA was transformed into *S. cerevisiae* cells, and freshly cultured in YPD media with 2% glucose as carbon source using the following protocol: yeast cells were centrifuged and washed twice with DNA-free water, centrifuged, and then 240 µL polyethylene glycol (50%), 36 µL lithium acetate (1 M), 50 µL boiled ssDNA (2 µg/µL), 29 µL DNA-free water, and 5 µL insert DNA were added to make a total volume of 360 µL. The sample was vortexed for 2 - 3 s after mixing, and incubated in a water bath at 42°C for 40 min. After the transformation, the cells were centrifuged at 15,000 rpm for 30 s, washed twice with sterile ddH₂O, followed by centrifugation at 15,000 rpm for 1 min, dissolved in 100 µL of DNA-free water, and finally plated on a minimal media agar plate with 2.0% glucose as carbon source with 100 µg/mL ampicillin. The inoculated plates were then incubated at 30°C for 2 d.

Culturing of the recombinant construct

The intracellular expression of the pectinolytic gene was carried out in shake flask fermentation in minimal media without uracil with 100 µg/mL ampicillin. The recombinant construct of *S. cerevisiae* was cultivated in 5 mL of minimal media without uracil using 2.0% glucose as carbon source and cultured overnight at 30°C followed by centrifugation (10,000 g, 10 min) and washing the cells several times with sterile ddH₂O. The cells were then grown in 100 mL of minimal media without uracil with 2.0% glucose as a carbon source at 30°C until OD₆₆₀ reached 2.0. After proliferation, the cells were centrifuged, washed several times with sterile ddH₂O, transferred to minimal media without uracil using 2% galactose as an inducer, and cultured at 30°C at 150 rpm for different periods. Wild yeast cells were also cultured side by side to check the background noise. Different aliquots of cells were taken at different time intervals (0, 7, 14, and 24 h), centrifuged, washed with sterile ddH₂O several times, and dissolved in Tris-HCl lysis buffer (pH 6.8). Glass beads of 250 µm size were added in the cells suspended in Tris-HCl lysis buffer followed by vortexing for 5 min at 1 min

interval by keeping the cells on ice. After vortexing, the cells were subjected to sonication for 5 min at 1 min interval by keeping the cells on ice. After the sonication, the lysed cells were centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant obtained after centrifugation (10,000 rpm, 20 min) was subjected to protein estimation and enzyme activity. The protein production after each induction time was further confirmed by the separation of the expressed protein by SDS-PAGE; 4% stacking gel, and 12% separating gel by monitoring the flow with 10 - 250 kDa protein ladder.

Determination of Exo-PG enzyme activity

The Exo-PG enzyme activity was measured by determining the reducing groups liberated from the pectin mixture using 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). D-Galacturonic acid monohydrate was used as the standard. An appropriately diluted enzyme solution (0.1 mL) was mixed with the reaction combination comprising a 1% pectin solution and acetate buffer (50 mM, pH 5.5). The resultant reaction mixture was allowed to incubate at 40°C for 30 min, and ended by incorporating 1 mL of DNS reagent, followed by placing in a boiling water bath for 5 min. The reducing sugars formed in the solution were measured at 535 nm, and the activity units were calculated using Eq. 1.

$$\text{Exo-PG activity} = \frac{\text{Abs. of sample} \times \text{standard factor}}{\text{Time of reaction} \times \text{enzyme extract in mL}}$$

Eq. 1

One unit (U) of Exo-PG represents the enzyme quantity liberating one micromole of galacturonic acid per min at 40°C and pH 5.5. The enzyme produced in SSF was denoted in U/gds.

Protein quantification

The Bradford method (Bradford, 1976) using BSA as a standard was performed for total protein estimation. In a test tube comprising an appropriate amount of enzyme *i.e.*, 100 µL, 0.9 mL of Bradford reagent was added, and absorbance was measured at 595 nm on a spectrophotometer after 10 - 15 min incubation. Control blank was maintained using an equal volume of distilled water in lieu of the enzyme.

Results and discussion

Gene sequence and synthesis

The gene of pectinase enzyme from *P. notatum* was found on <http://www.ncbi.nlm.nih.gov> and <http://fungicyc.broadinstitute.org:1555/P>

CWC541255/NEW-IMAGE?type=LOCUS-POSITION and object = G53-1206 and detail-level=2 and chromosome=NIL and show-tracks=on from the whole genome sequence (8,770 bp) of *P. chrysogenum* Wisconsin 54-1255 (NCBI accession number AM920416). The locus tag of this pectinase gene in the whole genome on NCBI was Pc22g22930, and blasted (<http://www.ncbi.nlm.nih.gov/BLAST>) against many sequences of polygalacturonase genes from other fungal sources to further confirm its identity. It showed a high similarity index with the maximum up to 84% when blasted against *P. griseoroseum* CCT polygalacturonase gene (*pgg1*) sequence (NCBI accession number AF085238). It also showed a 79% similarity with *P. digitatum* polygalacturonase gene (NCBI accession number AB015286), and 82% similarity with *P. olsonii* *pgl* gene (NCBI accession number AJ243521). However, no significant similarity was found when blasted against *P. janthinellum* DNA for polygalacturonase (accession number D79980), and *P. expansum* polygalacturonase (*pepg1*) gene (accession number AF047713). After identification, the nucleotide sequence (Pc22g22930) with 1,278 bp coding for the Exo-PG enzyme with 425 amino acid residues was ordered from Eurofins MWG Operon (USA), which is a gene synthesis company. The gene was ordered (without introns) with added restriction enzyme cut sites BamHI and XbaI. The following is the sequence of the pectinase gene which was ordered and delivered by the company ligated into the pEX-K vector (2,507 bp):

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ATGAAGATTTCAAATACCTT-
GACCCAGGCCTTTGGGCTTTTGTACCCT-
C A G C G C C G -
CAGTTCAGGCCAAGGAATACTCCCGAAGCG
AGGTGTGCCAGCCGAACCATCCGTTCAAGC
CCCTTCCGGGCAGCCAAGCCAGAACTCGCA
CCTGTCATGTAGCTAACCATGGCGATGGCCG
CGACGATTCAGCCAATGTTCTTAGCGCATTG
AAGCAATGCAACAATGGAGGAAAGGTCGTC
TTCGATGCCGACAAGAAGTATACGATTGGG
AAGGCACTTGACATGACTTTTTTGAAGCATG
TGGACCTCGAAATCCAAGGACATGTCCAAT
TTAGCGATGACACGGATTATTGGCAAGAAA
ATTCATTCAAGCAAATCTTCCAAAATGCCAC
AACATTCTTCCAGATCGGTGGTGAGGATGTC
AATGTCTACGGCGAGGGCACCTGGATGGT
AATGGTCAGGTCTGGTATGACTTATATGCTG
AGGACCCCTCATCTTGCGCCAATTCTGGT
TGGTATAGTTGGGCTAAATGGTGGTACCATT
GGACCTCTCAACCTTCGTTATTTCGCTCAGT
GGTATCAATTCGTGGCAAACCTCATCGAACGT
CCTTTTCGACGGAATTGATATCAGTGGGTAC
AGTAGCAGTGAGAATGAGGCCAAGAACC
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GACGGATGGGACCTTTATCGTTCAACGAAT-
 GTTGTTATCCAAAACCTCTGTGATCAACAAT-
 G
 GAGATGACTGCGTCTCCTTCAAGCCCAATGT
 TACCGACATTCTGGTCCAGAACCTCCATTGC
 AACGGCTCGCACGGTATCTCTGTTGGCTCTC
 TGGGCAATACCCGGGCGAGGTTGATATCG
 TGCAGAACGTGCTCGTGTACAATATTTCCAT
 GTTTAAACGCATCGGATGGTGC GCGTATCAA
 GGTTTGGCCTGGAGGTTCCGCTGCGATGTCG
 ACCGATCTACAGGGCGGCGGCGGATCAGGT
 CTGGTAAAGAACATTACTTACGATGGCATG
 ACCATTGATAATGTTGACTATGCCATCGAAG
 TGACCCAGTGCTATGGCCAGAAGAATTTGA
 CTCTCTGCAACGAGTTCCTAGCAAACCTTGC
 CATTGAAGATATCGTCTTCAAGAATTTCAAG
 GGCGTCACCTCTGGTAAACGTGACCCTGATG
 TGGGAATGATTGTCTGCTCAAGTCCCGATGT
 CTGCTCAAATATCAAGGCTAGTAACATCGA
 CGTTGTTAGCCCCGATGGGGATGATCAGTTC
 ACTTGCACAAATGTAAGTTTACTTTAA.

Cloning of plasmid DNA into *E. coli* XL10 competent cells and transformation into *S. cerevisiae*

After the gel purification of the Pc22g22930-pEX-K delivered by the company, we ligated the 1,278 bp sequence of Exo-PG gene (Pc22g22930) with BamHI and XbaI digested pYES2 vector (5.2 kb), and then cloned the resulting plasmid into the *E. coli* XL10 by thawing the cells on ice. The cells were then grown in LB agar plates in an incubator overnight at 37°C. Successful clones were checked by running 1% agarose gel electrophoresis after restriction digestion of the purified plasmid, and the results are shown in Figure 2. The insert-DNA was then successfully transformed into the freshly cultured *S. cerevisiae* cells, and proliferated on minimal media plate with 100 µg/mL ampicillin.

Gene expression and enzyme activity

Initially, we expressed this gene in *E. coli* BL21 host cell system using pZElac vector with IPTG (Isopropyl β-D-1-thiogalactopyranoside) induction, but the experiments did not give productive results, and pectinolytic activity was not detected in the cell lysates. Therefore, the fungal pectinolytic gene was expressed in a yeast host system, and *S. cerevisiae* was selected for expression purposes. Transformed yeast cells were grown at 30°C in shake flask fermentation followed by a maximum of 48 h cultivation in minimal media without uracil but with ampicillin (100 µg/mL) at the same temperature after the addition of 2% galactose to induce the expression of a functionally active enzyme. This yeast proved to be a good host

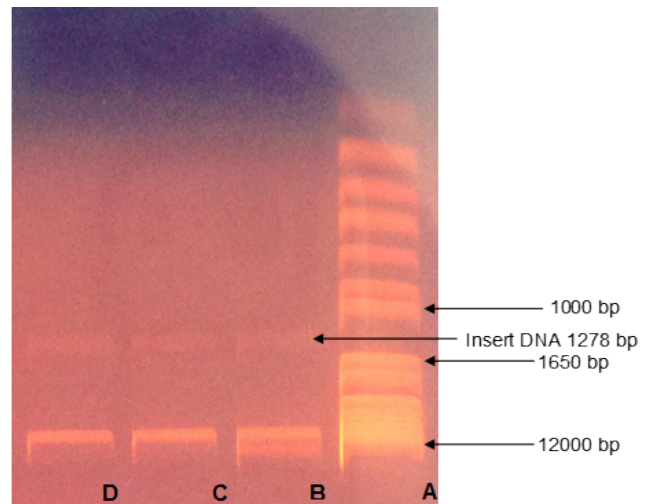


Figure 2. Agarose gel electrophoresis of successful clones after restriction digestion: 1% agarose gel electrophoresis in 1X TAE buffer, with run time of 30 min at 250 volts. (A) = 1 kb plus DNA ladder; and (B), (C), and (D) = restriction digested plasmid DNA samples after cloning in *E. coli* XL10.

for expression purposes of the fungal gene as compared to the *E. coli* expression system. The gene was expressed at its maximum level in 24 h of induction, and thereafter, the induction decreased, leading to low enzyme activity. The enzyme titres obtained after the gene expression are presented in Figure 3. The enzyme assay was conducted using the DNS method, and the enzyme encoded by the gene was further confirmed by the SDS-PAGE experiment (Figure 4). The wild yeast and control were also cultured in parallel to collect the background noise, which showed little enzyme activity. While in the engineered strain, the activity peaked at 24 h of fermentation, and after prolonged time, the activity decreased.

From the SDS-PAGE separation analysis of the expressed Exo-PG, it seemed that this might be a dimeric protein as we observed two strong protein bands after the electrophoretic run. As it is clear from lane C (maximum induction) of Figure 4, one band was observed at 20 - 25 kDa, and the other was observed at 70 kDa. This flow was monitored by 10 - 250 kDa pre-stained protein ladder. The intensity of the bands observed in SDS-PAGE further confirmed that gene expression gradually increased with increasing induction time, and was at its peak after 24 h of induction, and later on decreased. Previously, many authors have worked on gene expression of pectinolytic enzymes, and successfully achieved high expression levels. Yang *et al.* (2011) gained 50 U/mL of an Endo-PG after methanol induction of 72 h in shake flask fermentation. Zou *et al.* (2013)

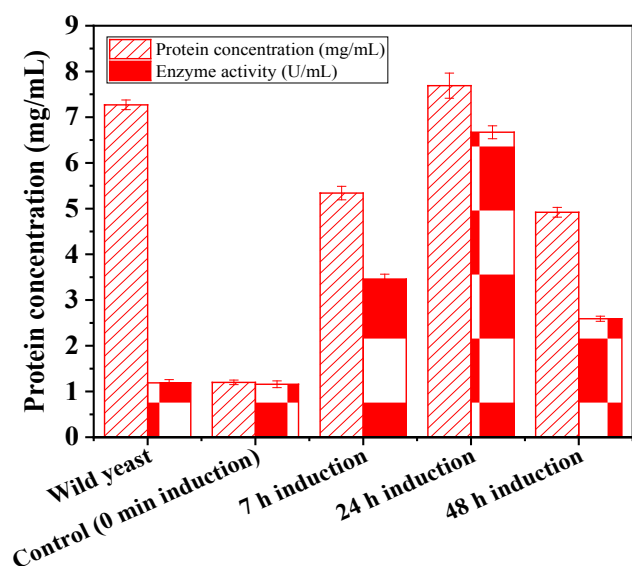


Figure 3. Exo-polygalacturonase gene expression after various induction times.

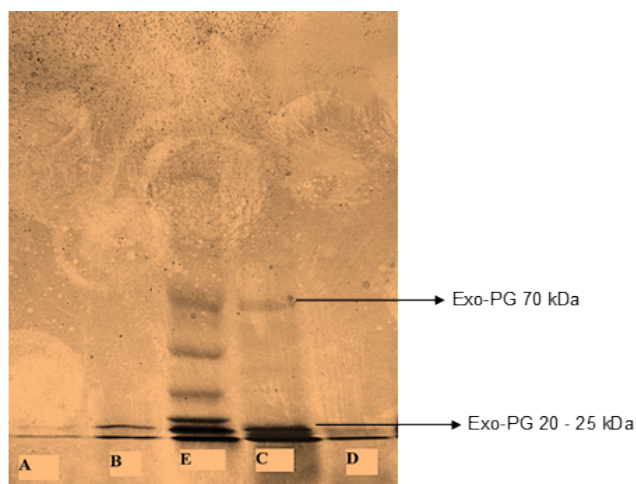


Figure 4. SDS-PAGE of the Exo-PG enzyme expressed in *Saccharomyces cerevisiae*; (A) = control - no induction; (B) = 7 h induction; (C) = 24 h induction; (D) = 48 h induction; and (E) = molecular weight marker 10 - 250 kDa (Fermentas pre-stained protein ladder).

homologously overexpressed the polygalacturonate lyase (PGL) gene in *B. subtilis* 7-3-3 to increase the gene copies and improve the PGL production. The resultant PGL titre was 2,138 U/mL at 44 h, which reached approximately 48.58 U (mL/h) in 7.5 L fermenter. Zhang *et al.* (2013) optimised several regulatory elements to achieve the production of alkaline polygalacturonate lyase (PGL), and successfully obtained 313.7 U/mL of PGL. After the optimisation and application of strong promoter P43 and Shine-Dalgarno sequence, the titre reached 446.3 U/mL of PGL. The present work was conducted to improve the activity of Exo-PG by gene

overexpression to get higher titres of Exo-PG in shorter time. The results demonstrated that the gene was successfully expressed, resulting in 4.323 U/mL (by subtracting the background noise) in 24 h of induction.

Conclusion

Exo-polygalacturonase is an important member of the pectinolytic group of enzymes with immense applications in the food industry. Herein, a high titre of Exo-PG was obtained from *P. notatum* by cloning, expression, and transformation of the Exo-PG gene in yeast *S. cerevisiae*. The Exo-PG gene from *P. notatum* was cloned into pYES2 plasmid, expressed in *S. cerevisiae* system, and allowed to cultivate at 30°C in minimal media. Results evidenced that the yeast was demonstrated to be an adequate expression host, and successfully produced a notable amount of recombinant enzyme into the culture media after 24 h of induction. The secreted Exo-PG enzyme exhibited two strong bands with an approximate molecular weight of 20 - 25 kDa and 70 kDa by SDS-PAGE, indicating a dimeric protein. In conclusion, the results demonstrated that the gene was successfully expressed, thus resulting in high-yield intracellular production of Exo-PG.

Acknowledgement

The authors are grateful to the Higher Education Commission, Pakistan for the financial support awarded under the International Research Support Initiative Program (IRSIP) program to complete the present work.

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