Selecting *Pichia pastoris* recombinant clones for higher secretion of human insulin precursor into the culture supernatant

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**Abstract.** The methylotrophic yeast, *Pichia pastoris*, is one of the preferred yeast hosts for recombinant protein expression. It has been developed as a potential host to express a high level of recombinant proteins, and to achieve efficient secretion as well as growth to very high cell densities. Previously, we have obtained 19 *P. pastoris* recombinant clones harboring synthetic insulin precursor (IP) expression cassette integrated into their genomes through homologous recombination. To select *P. pastoris* recombinant clones which exhibit high levels of protein expression, we conducted secreted expressions of IP protein in shake flasks. The secretion of IP into the culture supernatants was verified by SDS-PAGE. IP protein concentrations were estimated using ImageJ by applying lysozyme as standard. All of the 19 *P. pastoris* recombinant clones were confirmed to secrete the IP protein into their culture supernatants, and a single protein band with a molecular size of approximately 7 kDa was found in the SDS-PAGE gel. The six highest IP-expressing clones were selected for second screening in shake flasks. We selected three recombinant clones (CL-3, CL-4, and CL-18), which secreted the highest levels of IP proteins compared to the other clones. The secreted IP concentrations estimated by ImageJ for clones CL-3, CL-4, and CL-18 were 1230, 1143, and 1010 mg/L, respectively.

1. Introduction

Large scale human insulin recombinant production majorly employs *Escherichia coli* and yeast-based expression systems (mainly *Saccharomyces cerevisiae*) [1]. In *E. coli* system, the insulin precursor (IP) is overexpressed in the form inclusion bodies which required solubilization and oxidative refolding [2], whereas, in yeast-based expression system, the IP is directly secreted into culture supernatant and converted into human insulin through enzymatic reactions. Although half of the world insulin demand is covered by the *S. cerevisiae* production system [3], human insulin produced in *S. cerevisiae* need to go through multiple steps of purification processes [4].

In the past few decades, *P. pastoris* has appeared as a promising alternate yeast recombinant expression system exceeding *S. cerevisiae* due to its ability to produce high titers, and it does not glycosylate the heterologous protein as extensively as *S. cerevisiae*. Thus, it can lessen the immune activation risk in human. Besides, it can reach high cell densities by avoiding sugar fermentation to eliminate the toxic fermentative product, ethanol [5]. Furthermore, *P. pastoris* as an expression system of heterologous proteins has another excellent qualifications, such as the powerful and methanol regulated *AOX1* promoter, the stable expression of the integrated target gene, the high secretory ability, the low amount of proteins other than the expression product in the cell culture and inexpensive culture medium requirement [6].

The methylotrophic yeast *P. pastoris* is a potential expression system with high capability to secrete recombinant proteins to the medium [7]. Several studies have reported the secretory expression of IP protein in *P. pastoris* [8 - 12]. The highest reported secreted IP protein employing the *P. pastoris* system in the fermentor scale reached up to 4.51 g/L [13].
In our previous study, we introduced a synthetic IP encoding gene constructed in a pD902 expression vector into P. pastoris X33 strain, and 19 recombinant clones have been obtained. These 19 recombinant clones have been screened in YPD plate with increasing antibiotic concentrations to obtain multicopy clones. Since all 19 recombinant clones were able to recover up to 2000 µg/L of the antibiotic, in this study, we aimed to screen all of the 19 recombinant clones to obtain best IP-expressing clones for subsequent expression study in fermentor scale.

2. Materials and Methods

2.1. Strain
P. pastoris recombinant clones No. 1-19 used in this study harbor IP cassette were established in our previous study [14] and derived from P. pastoris X33 strain (Invitrogen, Carlsbad, CA).

2.2. Expression of IP in P. pastoris recombinant clones
A single colony of each P. pastoris recombinant clone from Yeast Extract peptone dextrose (YPD) agar plate [1% (w/v) yeast extract, 2% (w/v) peptone, 2% w/v glucose, 2% (v/v) agar] supplemented with Zeocin 100 µg/mL, was inoculated into 2 mL YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% w/v glucose] supplemented with 100 µg/mL Zeocin and shaken at 250-300 rpm and 30°C for 48 h. Cells were harvested by centrifuging at 1,500-3,000 × g for 5 min at room temperature. The cell pellet was then inoculated into 10 mL BMGY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6.0, 1.34% (w/v) YNB, 4 x 10⁻⁵% (w/v) biotin, 1% (v/v) glycerol) in a 100 mL flask and grown at 30°C in a shaking incubator (250 rpm) for 24 h. The cells were harvested by centrifuging at 3,000 x g for 5 min at room temperature. Cell pellet was resuspended to an OD₆₀₀ of 10 in 10 mL BMMY medium (1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6.0, 1.34% (w/v) YNB, 4 x 10⁻⁵% (w/v) biotin, 2% (v/v) methanol) to induce expression. Culture was placed in a 100 mL flask. A 100% methanol was added to a final concentration of 2% methanol every 24 h to maintain induction. After 3 d of methanol induction, supernatant was transferred to a separate tube and stored at −20°C until ready to assay.

2.3. Genetic validation of the six selected P. pastoris recombinant clones
Previously, we have identified the methanol utilization (Mut) phenotypes of all 19 recombinant clones. Here, we validated the integrant, IP cassette constructed in the pD902 vector, in the genome of the six selected recombinant clones and confirmed their Mut phenotype using an AOX1 specific primer set consisted of AOX1F (5'-GACTGGTTCCAATTGACAAGC-3') and AOX1R (5'-GCAAATGGCATTCTGACATCC-3'). The primers are listed in the EasySelect™ Pichia Expression Kit manual (Invitrogen 2010). Genomic DNAs of 6 recombinant clones were extracted from the cell pellet of 72 h post methanol induction cultures using YeaStar Genomic DNA Kit (Zymo Research Corp., Irvine, USA) following the manufacturer’s instructions. The isolated genomic DNAs were then used as the template for PCR amplification using Dream Taq DNA polymerase (Thermo Fisher Scientific, Lithuania). PCR amplification with AOX1 primer set was carried out in a 25 μL mixture (total volume) containing ~50 ng of P. pastoris genomic DNA, 2.5 μL 10-fold reaction Dream Taq buffer, 2 mM dNTPs, 0.5 μM of each primer, and 1.25 U Dream Taq DNA polymerase. We used the following PCR conditions: 95°C for 3 min; then 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by the last cycle at 72°C for 5 min. The amplicon of the predicted IP cassette was then sequenced (1st Base DNA sequencing service, Malaysia). Clustal multiple sequence alignment was carried out using DnaMan1.

2.4. Protein analysis and quantification
Protein analysis was conducted by SDS-PAGE analysis. The supernatant of culture broth samples was analyzed by denaturing 15% polyacrylamide gel electrophoresis using the Tricine buffer system [15].
Samples (20 μL) were mixed with an equal volume of Tricine sample buffer, mixed and boiled for 15 min. Samples were loaded on to the gel (20 μL per lane) and electrophoresed. The separated polypeptides were stained using Coomassie Brilliant Blue Solution (BioRad) for SDS-PAGE analysis. Each gel was destained in 15 mL destaining solution I [methanol 40%, acetic acid 7%, water 53%] for 30 minutes and in 20 mL destaining solution II [methanol 5%, acetic acid 7%, water 88%] for about 3 hours. Protein concentration in Tricine SDS-PAGE was quantified using ImageJ software by applying lysozyme as standard ranging from 0.03 to 2 mg/mL.

3. Results and Discussion

3.1. IP expression of 19 P. pastoris recombinant clones

The 19 P. pastoris recombinant clones were confirmed previously by PCR to harbor the fragment of IP expression cassette. The IP encoding gene was constructed in the expression cassette in the following composition: truncated α-factor signal peptide-spacer peptide (EEAEAEAEPK)-insulin B chain (1-29)-short connecting peptide (DGK)-insulin A chain (1-21). The IP expression cassette was constructed in a Pichia integrative vector pD902-IP (3921 bp). This vector was linearized by SacI enzyme to facilitate homologous recombination of the vector into the Pichia genome. One of the recombinant clones (CL-4) was confirmed to secrete IP protein in the culture supernatant, which has a

![Figure 1](image_url)

**Figure 1.** A) SDS-PAGE result for culture supernatants of 19 recombinant clones 72 h post-methanol induction in 15% SDS-PAGE gel (WT= wild type, C1-19= recombinant clones No. 1-19); B) Expression levels analyzed by ImageJ.
The predicted molecular weight size of secreted IP is 7053 Da, comprising of 63 amino acids [14]. Here, we screened all of the 19 recombinant clones to obtain high IP-expresser candidates among these recombinant clones. The *P. pastoris* recombinants and wild-type were cultivated in BMMY medium with 100% methanol added to a final concentration of 2% methanol every 24 h to maintain induction. The supernatant was collected from the culture 72 h post-methanol induction. The culture supernatant of 19 recombinant clones was analyzed for IP secretion using SDS-PAGE. Figure 1A shows that the IP bands with a molecular weight size of ~7 kDa were detected in all 19 clones culture supernatant. The band was not found in the culture supernatant of wild-type. To identify high IP-expresser clones, we conducted an ImageJ analysis of the IP bands detected in SDS-PAGE. Six recombinant clones, i.e., CL-3, CL-4, CL-10, CL-12, CL-13, and CL-18, exhibited a higher expression level compared to other clones (Figure 1B). The six selected clones were then subjected to the genetic validation after the cultivation in BMMY medium for 3 days. The genetic validation was confirmed by PCR using specific primer pair of *AOX1* gene (*AOX1F/AOX1R*). The PCR of six selected clones resulted in two fragments of the *AOX1* gene (~2000 bp) and IP cassette (~500 bp) (Figure 2A). It confirmed that all six selected clones identified as *Mut* phenotype and the recombinant clones maintain the integrated foreign pD902-IP in their genomes after 4 days cultivation in BMGY and BMMY medium without selectable marker selection.

*Mut* phenotype strain grows normally on methanol since the integrative vector was linearized using *Sac*I, which facilitates integration by single crossover into the chromosomal *AOX1* locus; therefore the *AOX1* gene will not be disrupted [16]. Sequencing analysis was conducted for the confirmation of the 500 bp fragments of six selected clones. The 511 bp PCR fragments of all selected clones have 100% identity to the 849-1359 bp position of pD902-PI. Figure 2B shows multiple sequence alignment of the 511 bp of PCR products amplified from PCR confirmation of the selected clones against the 511 bp (849-1359 bp) of the pD902-PI sequence. The IP cassette position in the pD902-PI vector is from 944 to 1314 bp. It revealed that all selected clones harbor correct IP cassette in their genomes.

### 3.2. Screening of best IP-expressing clones

The second expression screening was conducted to obtain best IP-expressing clone, which is going to be used for a fed-batch high-cell-density fermentation. The six selected *P. pastoris* recombinant clones and wild-type were again grown in BMMY medium with 100% methanol added to a final concentration of 2% methanol every 24 h to maintain induction. The supernatant was collected from the culture after 72 h of methanol induction. The SDS-PAGE analysis of the culture supernatants consistently revealed a single band of IP protein with a molecular weight size of ~7 kDa (Figure 3A).

Three highest expression level was achieved by CL-3, CL-4, and CL-18, with the estimated amount
B) CLUSTAL multiple sequence alignment

IP-ref  GACGTGGTCCAAATGACGCTTTTGGATTTTAACGCACTTTGACAAGCAGG
CL-3   GACGTGGTCCAAATGACGCTTTTGGATTTTAACGCACTTTGACAAGCAGG
CL-4   GACGTGGTCCAAATGACGCTTTTGGATTTTAACGCACTTTGACAAGCAGG
CL-10  GACGTGGTCCAAATGACGCTTTTGGATTTTAACGCACTTTGACAAGCAGG
CL-12  GACGTGGTCCAAATGACGCTTTTGGATTTTAACGCACTTTGACAAGCAGG
CL-13  GACGTGGTCCAAATGACGCTTTTGGATTTTAACGCACTTTGACAAGCAGG
CL-18  GACGTGGTCCAAATGACGCTTTTGGATTTTAACGCACTTTGACAAGCAGG

IP-ref  CAAAACACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-3   CAAAACACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-4   CAAAACACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-10  CAAAACACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-12  CAAAACACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-13  CAAAACACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-18  CAAAACACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG

IP-ref  CTGTTTTCTCGACGCATTCCTGCGCTACTGCGCTTTGCAAAAGCGCACTTTGACAAGCAGG
CL-3   CTGTTTTCTCGACGCATTCCTGCGCTACTGCGCTTTGCAAAAGCGCACTTTGACAAGCAGG
CL-4   CTGTTTTCTCGACGCATTCCTGCGCTACTGCGCTTTGCAAAAGCGCACTTTGACAAGCAGG
CL-10  CTGTTTTCTCGACGCATTCCTGCGCTACTGCGCTTTGCAAAAGCGCACTTTGACAAGCAGG
CL-12  CTGTTTTCTCGACGCATTCCTGCGCTACTGCGCTTTGCAAAAGCGCACTTTGACAAGCAGG
CL-13  CTGTTTTCTCGACGCATTCCTGCGCTACTGCGCTTTGCAAAAGCGCACTTTGACAAGCAGG
CL-18  CTGTTTTCTCGACGCATTCCTGCGCTACTGCGCTTTGCAAAAGCGCACTTTGACAAGCAGG

IP-ref  AATTAGAGGGAGTACACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-3   AATTAGAGGGAGTACACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-4   AATTAGAGGGAGTACACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-10  AATTAGAGGGAGTACACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-12  AATTAGAGGGAGTACACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-13  AATTAGAGGGAGTACACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-18  AATTAGAGGGAGTACACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA

IP-ref  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-3   GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-4   GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-10  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-12  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-13  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-18  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG

IP-ref  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-3   CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-4   CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-10  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-12  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-13  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-18  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG

IP-ref  TGGAGAATTACTGCAACTAAGCGGCCGCGGTTAAAGGGGCGG
CL-3   TGGAGAATTACTGCAACTAAGCGGCCGCGGTTAAAGGGGCGG
CL-4   TGGAGAATTACTGCAACTAAGCGGCCGCGGTTAAAGGGGCGG
CL-10  TGGAGAATTACTGCAACTAAGCGGCCGCGGTTAAAGGGGCGG
CL-12  TGGAGAATTACTGCAACTAAGCGGCCGCGGTTAAAGGGGCGG
CL-13  TGGAGAATTACTGCAACTAAGCGGCCGCGGTTAAAGGGGCGG
CL-18  TGGAGAATTACTGCAACTAAGCGGCCGCGGTTAAAGGGGCGG

IP-ref  ATGCTGTACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-3   ATGCTGTACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-4   ATGCTGTACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-10  ATGCTGTACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-12  ATGCTGTACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-13  ATGCTGTACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA
CL-18  ATGCTGTACCTCGAAGAGAGAGAGCGAGAAGAAACTCAGA

IP-ref  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-3   GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-4   GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-10  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-12  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-13  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-18  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG

IP-ref  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-3   CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-4   CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-10  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-12  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-13  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-18  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG

IP-ref  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-3   GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-4   GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-10  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-12  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-13  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-18  GACTGGTTCCAATTGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG

IP-ref  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-3   CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-4   CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-10  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-12  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-13  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG
CL-18  CAAAAAACACCAATTATGGAAGATTCAAAACGACGATTCCACAAACCAACACAGG

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Figure 2. Genetic validation of the six highest IP-expressing clones. A) PCR confirmation of six recombinant clones [M = Marker 1 kb DNA ladder; lane 1-7= recombinant clones CL-3, CL-4, CL-9, CL-10, CL-12, CL-13, CL-18; W = control negative (water); P = control plasmid pD902-IP; WT = control wild type;], B) Clustal multiple sequence alignment of IP-cassette sequences of CL-3, CL-4, CL-10, CL-12, CL-13, CL-18 compared to the synthetic IP sequence harbored by pD902-IP expression vector.

of secreted IP of around 1 g/L (Figure 3B, Table 1). Although both the highest IP expression (1230 mg/L) and cell biomass (OD600= 21.14) were observed in CL-3, the productivity of clones CL-3, CL-4 and CL-18 were almost similar as shown by the yield of IP of these clones which was around 0.1 mg/10^8 cells (Table 1). The best IP producing clones have almost similar starting (OD600 ≈ 15) and final cell densities (OD600 ≈ 20) to the wild type indicating that the IP expression had no harmful effect on the Pichia cells. It was similarly shown for the streptokinase (SK) production in *P. pastoris* where the SK-producing clones had a similar growth rate to their wild type [17].

Figure 3. A) SDS-PAGE of culture supernatants of 6 recombinant clones 72 h post-methanol induction in 15% SDS-PAGE gel (M= molecular weight polypeptide standards, lane 1= wild type, lane 2-7= recombinant clones No. 3, 4, 10, 12, 13, and 18); B) Expression levels analyzed by imageJ.
Table 1. The amount of secreted IP (mg/L) in the culture supernatant of selected recombinant clones

| Strains | Starting cell density (OD<sub>600</sub>) | Final cell density (OD<sub>600</sub>) | Final cell density (cells/mL x 10<sup>8</sup>)<sup>a</sup> | Secreted IP (mg/L) | Yield of IP (mg/10<sup>8</sup> cells) |
|---------|---------------------------------|---------------------------------|---------------------------------|-----------------|-------------------------------|
| WT      | 15.21                           | 20.57                           | 10.3                            | -338            | -                             |
| CL-3    | 15.10                           | 21.14                           | 10.6                            | 1230            | 0.12                          |
| CL-4    | 15.44                           | 20.81                           | 10.4                            | 1142            | 0.11                          |
| CL-10   | 14.21                           | 20.33                           | 10.1                            | 832             | 0.08                          |
| CL-12   | 13.84                           | 20.00                           | 10.0                            | 769             | 0.08                          |
| CL-13   | 13.64                           | 20.88                           | 10.4                            | 868             | 0.08                          |
| CL-18   | 14.81                           | 20.51                           | 10.3                            | 1010            | 0.10                          |

<sup>a</sup>The final cell density (cells/mL x 10<sup>8</sup>) was calculated from the amount of the final cell densities (OD<sub>600</sub>) where one OD<sub>600</sub> = ~5 x 10<sup>7</sup> cells/ml

4. Conclusions
Three of 19 <i>P. pastoris</i> recombinants clones, CL-3, CL-4, and CL-18, had higher IP expression level compared to other 16 clones with secreted IP of 1230, 1142, and 1010 mg/L, respectively. They can be employed as potential candidates in the fermentor scale.

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