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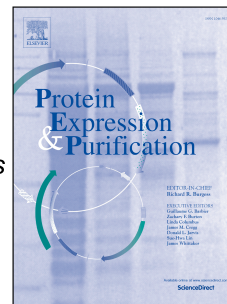

Modified chemical method for efficient transformation and diagnosis in *Pichia pastoris*

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Author statement

Sandeep Kumar: Methodology, Validation, Investigation, Writing-Original draft preparation. **Aruna Mannil:** Methodology. **Sarma Mutturi:** Conceptualization, Supervision, Formal analysis, Writing-Reviewing and Editing.

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1 **Modified chemical method for efficient transformation and diagnosis in *Pichia pastoris***

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3

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24 **Abstract**

25 In the present study, green fluorescence protein (GFP) was used as a candidate protein to test
26 and optimize a robust chemical transformation procedure in *P. pastoris*. Towards this, it was
27 adjudged that pretreatment of *P. pastoris* cells with lithium chloride (LiCl) and its optimal
28 concentration is critical for efficient transformation. Using three different methods (M1: 100
29 mM LiCl, 10 min, M2: 1 M LiCl, 10 min and M3: 1 M LiCl, 1 h), it was found that
30 concentration and incubation time for LiCl treatment significantly affects the transformation
31 efficiency. The transformation efficiency (transformants/µg DNA) was observed to be 1.01 x
32 10^2, 5.07 x 10^3 and 6.52 x 10^3 using methods M1, M2 and M3, respectively, indicating the
33 superiority of M3. Moreover, presence of the GFP gene in the positive transformants was
34 confirmed using a novel colony PCR method where the colonies were treated with LiCl prior
35 to GFP specific amplification. Also, it was established using fluorescence microscopy and
36 western blot analysis that increasing zeocin concentration as a post transformational vector
37 amplification (PTVA) strategy increased the fluorescence and gene expression, respectively.
38 Further, RT-qPCR revealed that the gene copy number using methods M1, M2 and M3 were
39 2.9, 5.29 and 7.16, respectively, when 500 µg/ml zecocin was used for selection, thus
40 corroborating western blot results. In conclusion, we demonstrate a cheap and robust
41 chemical method for achieving higher transformation efficiency in *P. pastoris* and a simple
42 procedure for colony-PCR based- diagnosis alleviating the need for enzymatic treatment.

43 **Keywords: *Pichia pastoris*; LiCl; Transformation; Copy number; RT-qPCR; Methods**

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47 **1. Introduction**

48 *Pichia pastoris*, which has been reclassified as *Komagataella pastoris*, is a methylotrophic
49 yeast which is popularly used for the large-scale production of recombinant proteins [1]. Over
50 the years *P. pastoris* has been successfully used as an expression system for the production of
51 a wide range of heterologous proteins of both eukaryotic and prokaryotic origin [1]. These
52 proteins are either secreted or present intracellularly in the host cells [1]. The widespread
53 popularity of *P. pastoris* for recombinant protein synthesis is attributed to its tightly
54 regulated, strong methanol inducible promoters (AOX1, AOX2) that allows it to utilize
55 methanol as a sole carbon source when no other carbon supplements are available [2-6].
56 Although *P. pastoris* is widely used as the cell factory for protein expression studies using
57 commercial vectors such as pAO815, pGAPZ/α, pPICZ/α etc. the transformation is generally
58 carried according to the supplier's protocol which requires standardization for a given case.
59 Moreover, there exists no generic transformation protocol which can be adapted to given
60 protein expression. As the transformation efficiency is governed by several factors, there is a
61 need to optimize these conditions in order to achieve consistent results. For the DNA
62 mediated transformation in *P. pastoris*, many methods are available which depend on several
63 factors such as size of the DNA to be transformed, cost, convenience etc. The most popular
64 methods used are electroporation, spheroplast-generation, LiCl and PEG1000- based
65 transformations [7]. Since the transformation of yeast cells by spheroplast preparation is a
66 tedious one and less effective, other alternatives were sought after. Usage of alkali metal ions
67 and some detergents are widely used in order to prepare competent *P. pastoris* cells for
68 transformation [7]. It has been found that the alkali metal ions such as lithium and sodium
69 were effective for rendering competent cells, whereas the detergents could not attribute
70 competency [7]. The transformation efficiency, using these cations were very much
71 comparable to that obtained from the spheroplast method [7]. Cregg & Barringer [8] were the

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72 first to use lithium chloride for the preparation of competent cells of *P. pastoris* for
73 transformation. Their study resulted in the discovery of a novel method for making the whole
74 cells of the *P. pastoris* competent. They also established that the transformation efficiency
75 was higher in linearized plasmid DNA in comparison to the circular plasmid. The other
76 commonly used method for *P. pastoris* transformation is electroporation. In the studies where
77 electroporation method was used, transformation efficiency was around 10^3-10^4 /µg of DNA
78 [9]. For increasing the transformation efficiency using electroporation method, Wu and
79 Letchworth [10], suggested pretreating the *P. pastoris* cells with lithium acetate and DTT.
80 They reported an increase in the transformation efficiency by 150 folds by using 100 mM
81 lithium acetate and 10 mM DTT. Moreover, it has been also reported that the electroporation
82 is highly efficient in comparison to the chemical method using lithium chloride [10].
83 Generating multi-copy strains is one of the methods for optimizing the protein expression in
84 *P. pastoris*. Designing an experiment leading to high transformation efficiency and high
85 throughput screening of robust positive clones during protein expression in *P. pastoris* is a
86 crucial step. Although electroporation has been well established for high transformation
87 efficiency, it also requires optimization of the conditions and replacement of cuvettes after
88 each run. Moreover, the chemical methods for transformation in yeast are always considered
89 to yield very low transformants in comparison to electroporation [11].
90 Here we report a modified chemical method using lithium chloride based competent cell
91 preparation, which is not only simple and cost effective in comparison to electroporation-
92 based technique but also yields high transformation efficiency. Also, a non-enzymatic
93 methodology for carrying colony PCR for positive transformants has been standardized for
94 the first time. Furthermore, in this study, the effect of increasing zeocin concentration
95 (selection marker) on the gene copy number as a post transformational vector amplification

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96 (PTVA) strategy has been investigated. All the protein expression were carried using
97 pPICZαA plasmid (Invitrogen Inc.) and green fluorescent protein (GFP) as the candidate.
98
99 **2. Materials and Methods**
100 **2.1 Strains, vectors and media**
101 For the transformation studies, GFP was used as the reporter gene, and was isolated from
102 pGREG599 (Euroscarf, SRD GmbH) which contained the nucleotide sequence for GFP. *E.*
103 *coli* DH5α was used as the bacterial host for the library preparation, and the *P. pastoris* X-
104 33 strain was selected as the expression host. Plasmid pPICZαA (Invitrogen, USA) was
105 used as the shuttle vector for carrying the insert gene into the host cells. *P. pastoris* cells
106 were grown in YPD medium (1% yeast extract, 2% peptone and 2% dextrose), and the LB
107 medium (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl) was used for *E. coli* DH5α
108 cultivation. The media was supplemented with Zeocin™ (Thermo Fisher Scientific, India).
109 In the case of *E. coli* selection, zeocin concentration was fixed at 25 μg/ml, whereas in case
110 of *P. pastoris* selection, the concentrations varied in the range 50-500 μg/ml.
111
112 **2.2 PCR amplification of GFP**
113 Plasmid pGREG599 was isolated using GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich,
114 USA); gene specific primers were designed with the help of OligoAnalyzer v3.1. 100 ng of
115 this isolated plasmid served as a template for the PCR amplification of GFP gene. Phire
116 polymerase (Thermo Fisher Scientific, USA) was used for PCR. Conditions for PCR are as
117 follows: initial denaturation at 98°C for 4 minutes, denaturation at 95°C for 30 s,
118 annealing at 55°C for 30 s extension at 72°C for 1 min, and a final extension at 72°C for 10
119 min. After the reaction, the end product was analysed using 1% agarose gel
120 electrophoresis.

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121

122 **2.3 Vector construction for GFP**

123 Isolation of the plasmid pPICZαA was done using GenElute™ Plasmid Miniprep Kit (Sigma-
124 Aldrich, USA). The PCR amplified gene was purified using Column-Pure PCR Clean-Up Kit
125 (abm, Canada), and the purified gene and the vector pPICZαA were used for the double
126 digestion. Digestion was carried using the enzymes XbaI and NotI (NEB, USA) in a reaction
127 mixture of 50 µl and incubating at 37 ^{o}C for 1 h. The double digested gene and the vector
128 were purified using abm™ Column-Pure PCR Clean-up Kit. The purified gene and vector
129 were ligated using T4 DNA ligase (NEB, USA) for obtaining the final construct pPICZαA-
130 GFP. The constructed plasmid containing both insert gene was transformed into freshly
131 prepared competent cells of *E. coli* DH5α using the heat shock method. 25 µg/ml zeocin was
132 used for the selection of the positive clones. Later, cloning was confirmed by colony PCR
133 using GFP specific primers and insert release using XbaI and NotI enzymes.

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134

135 **2.4 Transformation in *P. pastoris***

136 For transformation in *P. pastoris,* pPICZαA-GFP was isolated from *E. coli* DH5α library
137 using GenElute™ Plasmid Miniprep Kit. The plasmid was then linearized using PmeI (NEB,
138 USA) restriction enzyme, by carrying single digestion in the AOX promoter region. The
139 linearized plasmid was later transformed into *P. pastoris* cells using the heat shock method. 1
140 M LiCl and 100 mM LiCl were used for the competent cell preparation using following three
141 methods: (M1, Method-1)- *P. pastoris* cells were grown in YPD broth medium overnight at
142 28°C and were later sub-cultured into 50 ml YPD medium with starting OD_{600} of 0.1. Cells
143 were grown to an OD in the range 0.8-1.0, later harvested and washed thrice with 25 ml of
144 autoclaved distilled water. An aliquot of cells was later transferred to 2.0 ml microcentrifuge
145 tube to wash again with 1 ml of distilled water followed by 1 ml of 100 mM LiCl. Cells were

146 later kept in 100 mM LiCl for 10 min at 30°C. This method is according to the Invitrogen's
147 pPICZα user manual. The remaining two methods are modifications of method-1 and are
148 described briefly as follows: (M2, Method-2)- In this method, cells were washed with 1 M
149 LiCl followed by incubation in 1 M LiCl for 10 min at 30°C. (M3, Method-3)- In this method,
150 cells were washed with 1 M LiCl were incubated in 1 M LiCl for 1 h at 30°C.
151 In all the three methods, after the LiCl incubation, cells were centrifuged and the pellet was
152 re-suspended in 400 µl of 100 mM LiCl in the case of M1 and 400 µl of 1 M LiCl in case of
153 M2 and M3. Later, 50 µl of the prepared competent cells were transferred into a 1.7 ml tube
154 and centrifuged. To the obtained pellet, 240 µl of 50% PEG, 36 µl of LiCl, 25 µl of 2 mg/ml
155 SS DNA and 1 µg of plasmid pPICZαA DNA were added according to the Invitrogen manual.
156 The mixture was incubated for 20-30 min at 30°C, and later heat shock was provided by
157 keeping the tubes at 42°C for 20 min. The pellet was collected and re-suspended in 1 ml YPD
158 broth. It was allowed to grow for 2-4 h as described in Gietz and Woods [12]. After the brief
159 growth phase 50, 100 and 200 µl cell broth were spread onto YPDA plate having 50 µg/ml
160 zeocin from all the three methods of competent cell preparations. The plates were incubated at
161 30°C for 2 to 4 days until colonies were formed. Transformation efficiency was then
162 calculated by counting the number of colonies per ml, and was expressed as a number of
163 transformants per microgram DNA.
164

165 **2.5 Screening of transformants on zeocin selection pressure**

166 Around 20-30 colonies were sub-cultured from parent plate (50 µg/ml zeocin) to YPD plates
167 with 100 µg/ml zeocin concentration and incubated for 24 h at 30°C temperature. The
168 colonies that grew on 100 µg/ml were again sub-cultured onto 250 µg/ml zeocin
169 concentration and finally at a concentration of 500 µg/ml. For proteins expression studies,
170 colonies formed on the plate with 500 µg/ml zeocin concentrations were selected.

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171

172 **2.6 Colony PCR of *P. pastoris***

173 To confirm integration of gene cassette, three colonies from 500 µg/l zeocin plate were
174 selected for the colony PCR from each method. Briefly, a loop-full of colony was taken from
175 the plate and were resuspended in 200 µl of following three solvents: (i) water (ii) 100 mM
176 LiCl and (iii) 1 M LiCl. Again, all three solvents were incubated for 10, 30 and 60 min prior
177 to colony PCR. After incubation, cells were washed three times with sterile distilled water and
178 the pellet was re-suspended in 200 µl of distilled water. 5 µl of this cell mixture was used for
179 a colony PCR reaction. Taq DNA polymerase master mix (abm, Canada) was used for
180 performing PCR. For all the selected colonies, colony PCR was carried using GFP specific
181 primers as well as AOX specific primers. The primers for GFP are forward 5'-
182 GCGCGCGCGGCCGCATGTCTAAAGGTGAAGAATTATTCACTGGTGT-3' and reverse
183 5'-GCGCGCTCTAGACCTTTGTACAATTCATCCATACCATGGGT-3', and for AOX are
184 forward 5'-GCCATCCGACATCCACAGG-3' and reverse 5'-
185 GCCCAATAACTGGGCTGGTT-3'. The amplification was confirmed by running the product
186 in 1% gel electrophoresis.

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187

188 **2.7 Expression study of GFP using fluorescence microscopy**

189 Single colonies from transformation plates using M1, M2 and M3 were grown in 25 ml of
190 BMGY medium and shaken (150 rpm) at 30 °C until OD_{600} of 5.0-6.0 was reached. The cells
191 were collected by centrifugation at 6000 rpm for 5 min at 4 °C; sufficient cells were
192 resuspended in 50 ml of BMMY to reach initial OD_{600} 1.0. To induce the expression,
193 methanol was added every 12 h to a final concentration of 1.5% (v/v). After 72 h induction, 1
194 ml cells were collected by centrifugation, washed and resuspended in 1 ml distilled water. 50

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195 µl cells were taken on glass slide and examined using fluorescence microscopy (Olympus,
196 USA).
197

198 **2.8 Western blot analysis**

199 Protein was collected as supernatant from 96 h methanol induced cultures and were
200 concentrated using vacuum dryer (Biotron, South Korea). Sodium dodecyl sulphate-
201 polyacrylamide (gradient 4-20%) gel electrophoresis (SDS-PAGE) was performed using a
202 Mini-Protean II unit (Bio-Rad, CA, USA). Western blot was carried out after protein was
203 transferred from SDS-PAGE gel onto a PVDF membrane using a Mini Trans-Blot
204 Electrophoretic Transfer Cell (BioRad, CA, USA) following manufacturer's instructions. As
205 the GFP expression was in-frame with 6X-his, its detection was carried out using his-tag
206 mouse mAb (Cell Signaling Technology, USA) with a dilution 1:1000. Anti-mouse IgG
207 conjugate (HRP-linked Antibody (Cell Signaling Technology, USA)) was used to a 1:3000
208 dilution as secondary antibody for GFP detection. Detection was carried out using
209 chemiluminescent substrate SignalFire™ ECL Reagent (Cell Signaling Technology, USA)
210 and the signal was collected by a photographic film.
211

212 **2.9 Genomic DNA extraction and RT-qPCR for copy number estimation**

213 After the confirmation of gene insertion by colony PCR, the DNA was isolated using the
214 protocol given by Tomita et al. [13]. Briefly, one colony from the three methods growing on
215 different zeocin concentrations was selected. Here the wild-type strain X-33 served as a
216 negative control. The purity of the extracted DNA was confirmed by running on 0.8%
217 agarose gel electrophoresis and concentration of the DNA was determined by the Nanodrop
218 at 260 nm. Prior to RT-qPCR, isolated DNA was subjected to normal PCR using AOX
219 specific primers for the confirmation of insert gene. Later, RT PCR was carried using AOX

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220 specific primers, forward (5'-GCCATCCGACATCCACAGG-3') and reverse
221 (GCCCAATAACTGGGCTGGTT) to determine the insert copy number present in the
222 genome. Six different concentrations of plasmid viz, 0.25 , 0.5 , 0.75 , 1 , 1.25 and 1.5 ng/µl
223 served for calibration of standard. The reaction mixture of 10 µl was prepared using these
224 different concentrations of plasmid, and SYBR green master mix (Bio-Rad, USA) was used
225 for RT-qPCR. Similarly, 20 ng of the isolated genomic DNA from different zeocin
226 concentrations were used for the reaction. For the qualitative analysis of the amplified
227 products, gel electrophoresis was done using 1% agarose and the calculation of the insert
228 gene number was done according to Abad et al. [14].

229

230 **3. Results**

231 **3.1 Transformation in *P. pastoris***

232 The plasmid pPICZαA-GFP was constructed as described in the methods and was later
233 used for transformation in *P. pastoris*. The schematic representation of work flow is
234 provided in Fig. S1. The transformation of *P. pastoris* was carried out using the heat shock
235 method. Competent cells of *P. pastoris* were prepared using three different methods using
236 different concentrations of lithium chloride and different times of incubation in lithium
237 chloride. For each method, three different volumes of transformation mixtures viz., 50 µl,
238 100 µl, and 200 µl were added to the YPD zeocin containing plates. Colonies were
239 observed after two days of incubation at 30˚C. Both LiCl concentration and time of
240 incubating competent cells were found to have a significant effect on transformation and
241 the transformation efficiency (cf. Fig. 1(A-C)). It was clearly visible that the transformed
242 colonies from M2 (Fig. 1B) and M3 (Fig. 1C) were higher in comparison to M1 (Fig. 1A),
243 indicating that higher concentration of LiCl improves the transformation efficiency. The
244 transformation efficiency results are provided in Fig. 2D&E. There is a net improvement of

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245 50.19- and 64.55- fold increase in the transformation efficiency using M2 and M3,
246 respectively, when compared to M1. In the case of M3 the fold improvement over M2 was
247 found to be 1.28. These results suggest that increased LiCl concentration and the time of
248 incubation positively affect transformation efficiency. Overall, it was found that the
249 method M3 yielded the highest transformation efficiency with optimal conditions of
250 washing the cells with 1 M LiCl and later incubating for 1 h in the same solution prior to
251 the actual transformation. Also, higher incubation times, viz., 2 h and 6 h were also tested,
252 however the number of transformant colonies observed to be lesser in comparison to 1 h
253 case (data not shown).

254

255 **3.2 Screening for positive transformants using colony PCR**

256 Colony PCR is a common strategy to diagnose the success of the transformation. Unlike
257 bacterial cells, *P. pastoris* cells requires a pretreatment step to carry colony PCR. Here, in
258 the present study, the colonies were treated again with 100 mM-1 M LiCl similar to
259 competent cell preparation as a pretreatment procedure for colony PCR, thus alleviating
260 the usage of expensive enzymatic treatments or harsh heat treatments. Total three different
261 pretreatments were carried for the cells before carrying the colony PCR as described in
262 methods. Colony PCR result revealed that resuspending the loop-full colony in 1 M LiCl
263 and incubating for 30 -60 min was highly effective for the colony PCR screening (Fig. S2).
264 No amplification was observed in water and 100 mM LiCl treated cells. Both AOX
265 specific primers and GFP specific primers were used for colony PCR- based screening.
266 Initially, for colony PCR, five colonies were selected from each transformation methods
267 (M1, M2 and M3) and AOX specific primers were used. It was observed that all colonies
268 showed positive amplification (Fig. 2A). Subsequently, colony PCR was carried again for
269 the confirmation of the presence of GFP cassette from these positive colonies using GFP

270 specific primers. In method-1 (M1), out of five colonies only three colonies were positive.
271 And in the other two methods all selected colonies were positive (Fig. 2B). It was found
272 that in the negative control no amplification was observed (cf. Fig. 2B). According to these
273 results, it was clearly visible that transformation efficiency was less in method M1,
274 whereas both M2 and M3 were highly effective for the transformation of the linear plasmid
275 into *P. pastoris*.

276

277 **3.3 Screening for multiple copy transformants**

278 Increased resistance to the selection marker (zeocin, in case of present study) indicates the
279 enrichment of the *P. pastoris* population by multiple copies of the insert [15]. Hence, the
280 selection of the colonies growing in high dosages of the antibiotic is a straightforward
281 strategy to screen colonies possessing multiple gene insertions. Therefore, the motive here is
282 to scout for high copy insertion candidate which is resistant to high dosages of antibiotic.
283 However, it was observed that only 5% of such robust high antibiotic resistance colonies
284 contain multiple gene insertions [16]. Moreover, such screening procedures are time
285 consuming and tedious. Sunga et al. [17] introduced a novel *in-vivo* strategy termed as post-
286 transformational vector amplification (PTVA) where the antibiotic resistance is gradually
287 increased in linear/gradient fashion thus enriching the strain with increased number of vector
288 copies. Here too, we applied the same technique to the transformed colonies obtained from all
289 the three methods and understand the role of PTVA on GFP expression.

290 Twenty to thirty colonies were randomly selected from fresh transformants grown on 50
291 µg/ml for each method and were subjected to PTVA by increasing the zeocin
292 concentrations every 24 h of incubation. The transformant colonies were first plated onto
293 YPD plates having 100 µg/ml zeocin. All the colonies showed growth after 24 h and hence
294 were then sub-cultured onto YPD plates having zeocin concentration of 250 µg/ml. The

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295 following day positive colonies were sub-cultured onto fresh YPD plates having 500 µg/ml
296 of zeocin.

297 Post methanol induction as described in methods, *P. pastoris* X-33 cells across three different
298 zeocin concentrations (100, 250 and 500 µg/ml) and methods (M1, M2 and M3) were
299 examined using fluorescence microscopy (cf. Fig. 3). It can be observed that the fluorescence
300 intensity of GFP using M1 at 100 µg/ml zeocin is lowest, and using M3 at 500 µg/ml is
301 highest (Fig. 3). It can be seen that the fluorescence varied not only across the methods but
302 also with varying zeocin concentration. Although, PTVA has been proved to improve the
303 copy numbers of the expressed gene [16], it is interesting to observe that the increased LiCl
304 (1 M) using method M3 also increased fluorescence. To confirm these qualitative results,
305 western blot analysis was carried for protein samples as described in methods and the results
306 are provided in Fig. 4A. It was observed that intensity of band was highest in lane 3 where
307 method-3 and 500 µg/ml zeocin were used for transformation and PTVA selection,
308 respectively. The intensity lowered at 250 µg/ml (lane 2) and was not observed at 100 µg/ml
309 (lane 1) for method-3 transformation procedure. This shows that gradual increase in zeocin
310 concentration during PTVA positively affects the protein expression (probably due to
311 increase in gene copies). Similarly, when western blot results were compared across methods
312 M1, M2 and M3 at 500 µg/ml, no band was seen in M1 (lane 4), low intensity band was seen
313 in M2 (lane 5) and highest intensity in M3 (lane 3). These results indicates that there is
314 significant variation among the transformation methods used. And method-3 transformation
315 procedure synergistically affects PTVA implementation. Therefore, it is demonstrated that
316 both LiCl concentration and incubation time during processing of *P. pastoris* cells affects
317 chemical transformation.

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319 In order to determine quantitatively whether increase in zeocin selection pressure enriched
320 the *P. pastoris* cells by increasing insert copy number in the genome, RT-qPCR was
321 carried.

322 For the insert copy number determination, genomic DNA was isolated from positive
323 transformant colonies from the three methods at different zeocin concentrations, whereas, the
324 wild type strain *P. pastoris* X-33 served as negative control. For the confirmation of the
325 presence of GFP in the isolated genomic DNA, normal PCR was done using GFP specific
326 primers. The amplified product was run in 1% gel electrophoresis and the band corresponded
327 to 717 bp, which confirmed the amplification (results not shown).

328 The analysis of RT-qPCR results was done as described by Abad et al. [14]. For preparing the
329 calibration curve in order to quantify, the vector construct pPICzαA-GFP was linearized with
330 Xba1 enzyme. Different plasmid concentrations 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 ng for 10
331 µl reaction were selected for the RT-qPCR reaction. Copy number of the gene in ng plasmid
332 DNA was calculated using Avogadro's number. Further, RT-qPCR, standard curve was made
333 by plotting the mean Cq value of the plasmid dilutions with the log of plasmid copy number.
334 Using this standard curve (cf. Fig. 4B), the insert copy number in the samples was
335 determined.

336 The gene copy number of the control samples was determined by the equation below [14].

337 $$\text{Gene copy number} = \frac{\text{Target gene copy number after RT-PCR}}{\text{Control gene copy number after RT-PCR}}$$

338 The results from the RT-qPCR experiments are shown in Table 1. Gene copy number of 7.19,
339 which is highest amongst all the treatments was observed in the colonies growing on 500
340 µg/ml of zeocin using M3 procedure. In the case of M1, M2 and M3 methods the copy
341 number in 500 µg/ml zeocin plates increased by 3.33, 3.95 and 2.19- folds over 100 µg/ml
342 zeocin plates, respectively. M2 based transformation showed lower copy number than M3;

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343 and M1 yielded least amongst three methods. However, in all the three methods the
344 increasing zeocin concentration has resulted in increased copies of the gene.

345

346 **4. Discussion**

347 The transformation efficiency is usually in the range of 10^3-10^4 transformants/µg DNA
348 when electroporation or spheroplast procedures are adopted in the case of *P. pastoris* [10,
349 18]. Transformation efficiency in *P. pastoris* using chemical method is lower when
350 compared to electroporation (Invitrogen manual). Some improvements were made by
351 combining both chemical treatment and electroporation. The results showed around 140-
352 fold increase in transformation efficiency in such methods [10]. The present study is a
353 combination of chemical treatment and heat shock method. Here the method-1 is according
354 to the conditions as described in the Invitrogen manual, however by modifying the LiCl
355 concentrations and incubation time we could achieve a significant increase in the
356 transformation efficiency.

357 LiCl is a chaotropic ion (those ions which favour the transfer of polar groups to water)
358 which is well known to destroy plasma membrane protein and lipids [19-22]. LiCl
359 treatment releases the protein elements from the outer surface of the cell, and helps in
360 genetic transformation [19]. It was found that the viability of the cell is altered if the LiCl
361 concentration increased from 0.1 M to 4 M and transformability was completely inhibited
362 at 2 M LiCl concentration. Increment in the LiCl concentration destroyed the cell structure
363 and it was clearly observed that more than 1 M LiCl concentration reduced the
364 transformability [19]. Therefore, in this study, 0.1 M to 1 M LiCl was used for competent
365 cell preparation.

366 Earlier, Ito et al. [7] observed that intact yeast cells when treated with alkali metal ions
367 such as Li^+, Cs^+, Rb^+, K^+, and Na^+ induced competency to take up plasmid DNA thereby

368 eliminating the tedious protoplast-based transformation. It was also observed in this earlier
369 study that the salts of lithium yielded several fold higher transformation efficiencies in
370 comparison to other metal ions. However, uptake of plasmid DNA using metal ions was
371 observed only in the presence of polyethylene glycol [7]. The Invitrogen protocol (protocol
372 available at http://www.invitrogen.com) strongly recommends to use LiCl and not LiAc for
373 transformation in *Pichia pastoris* when the chemical method is adopted. Thompson et al.
374 [11] and Wu & Letchworth [10] have established that pretreatment of competent yeast
375 cells prior to electroporation improves transformation efficiency by several folds. In the
376 present study, we too observed that pretreatment step with increasing the LiCl
377 concentration and incubation time has improved the transformation by several folds when
378 compared to Invitrogen protocol. The optimal conditions concluded in the present study
379 using GFP expression could also be valid for other protein expression studies in *P.*
380 *pastoris*.
381
382 Colony PCR is a high-throughput and rapid initial screening procedure to find positive
383 transformants. In brief, the colonies from the selection plate are picked and the PCR is carried
384 using insert specific primers. However, in the case of *P. pastoris* colonies, unlike bacterial
385 cells, colony PCR requires pretreatment of colonies in order to achieve reasonably positive
386 results. This is majorly due to the hindrance of the tougher cell wall [8]. Hence pretreatment
387 procedures such as usage of lytic enzymes (eg. zymolyase, Zymo Research) and heat
388 treatments such as boiling or microwave procedures were carried for the *P. pastoris* cells.
389 The other procedure for screening colonies could be elaborate genomic DNA isolation from
390 each colony and insert specific PCR, which certainly consumes more time. There is a need
391 for simple, robust and quick screening of positive transformants. The present study

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392 demonstrates that the treatment of positive colonies with LiCl could alleviate the costly
393 enzymatic and harsher heat treatments for efficient colony PCR.

394 Linearized DNA once transformed into competent cells can generate stable insertions in
395 *Pichia pastoris* using the homologous recombination strategy [23]. There could one or more
396 copies of the integrated cassette based on how many recombinations it undergoes. However,
397 it was observed that such multiple recombinations are both stochastic as well as low
398 occurrence events [24]. Moreover, head to tail *in-vitro* preparation of the integration cassette
399 with multiple copies of the gene of interest in the backbone prior to transformation has
400 resulted in improved protein expression [15]. The relationship between copy number and
401 protein expression is highly variable. In some cases, it has been reported that increasing copy
402 numbers increases the expression of proteins such as EGF and human TNF [25]. In rather
403 rare scenarios this direct correlation between copy number and protein expression was proven
404 wrong [26]. There exists direct interconnection between the number of integrations and the
405 manifestation of antibiotic resistance (by antibiotic selection marker). The results from RT-
406 qPCR (cf. Table 1) indicate that PTVA is an efficient *in-vivo* strategy to increase the copy
407 number. Similar results were observed in Marx et al. [27] where two different proteins,
408 human serum albumin (for secretory expression) and human superoxide dismutase (for
409 cytoplasmic accumulation) were studied for PTVA strategy. Also, Soboleski et al. [28]
410 observed an increase in GFP fluorescence intensity when the copy number of GFP was
411 increased. Moreover, the results from RT-qPCR have corroborated the transformation
412 efficiency results obtained from western blot analysis.

413 Some of the transformation procedures in *P. pastoris* using both electroporation and chemical
414 methods are summarized in Table 2. The modified chemical method proposed in the present
415 study is on par with the some of the electroporation- based methods with lesser standard
416 deviation in transformation efficiency. In order to achieve optimal transformation efficiency

417 using the proposed strategy, it is recommended to adopt following steps: (i) cells should be
418 harvested once the OD_{600} is in the range of 0.8-1.0, (ii) cells should be washed thoroughly
419 using 1 M LiCl before incubating for 1 h in the same solution, (iii) PEG 3350 should be
420 preferred over others, (iii) initial selection of transformants should be at 50 µg/L of zeocin
421 concentration, and (iv) during PTVA procedure, cells should be gradually subjected to higher
422 zeocin concentration in the range of 50-500 µg/ml.

423

424 In conclusion, we propose a simple modified chemical method using LiCl for efficient
425 transformation in *P. pastoris*. This strategy is cheap and highly reproducible, and it can be
426 easily adapted for all routine protein expression experiments using *P. pastoris* cell factory. A
427 novel LiCl pretreatment for colony PCR- based selection of positive transformants has been
428 established. Finally, the PTVA strategy has been validated for the improvement of copy
429 numbers in the *P. pastoris* genome which also corroborated the enrichment of transformation
430 efficiency.

431

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437

438 **Conflicts of Interest**

439 The authors declare that they have no conflict of interest.

440

441 **References**

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520 **Figure Captions**

521 **Fig. 1:** Positive transformant colonies seen on YPD plates containing 50 μg/ml zeocin using
522 different competent cell preparation methods (A) 100 mM LiCl for 10 min, method 1 (B) 1 M
523 LiCl for 10 min, method 2, and (C) 1 M LiCl for 1 h, method 3. (D) Number of transformants
524 and (E) Transformation efficiency (transformants/μg DNA) across these three methods. The
525 values on bars indicate the quantitative value of respective treatment. The columns labelled
526 with the same letter represent not-significantly different means, according to Duncan's
527 multiple range test ($P<0.05$), after ANOVA. The values shown are mean ± SD (n=4).

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528 **Fig. 2:** Colony PCR with (A) AOX specific primers and (B) GFP specific primers. PC
529 (Positive control X-33), X-33 wild strain 1A, 1B, 1C, 1D, and 1E colonies of method-1. 2A,
530 2B, 2C, 2D, and 2E colonies of Method-2. 3A, 3B, 3C, 3D and 3E colonies from Method-3

531 **Fig. 3:** Fluorescence microscopy of *P. pastoris* cells from 100 µg/ml zeocin (M1, M2 and
532 M3), 250 µg/ml zeocin (M1, M2 and M3) and *P. pastoris* cells from 500 µg/ml zeocin (M1,
533 M2 and M3)

534 **Fig. 4**: (A) Cell-free supernatants from different zeocin (µg/ml) treatments were analysed by
535 western blot analysis using His-Tag (27E8) Mouse mAb (primary) and Anti-mouse IgG,
536 HRP-linked Antibody (secondary). M represents marker, lane 1: Method-3 using 100 µg/ml;
537 lane 2: Method-3 using 250 µg/ml; lane 3: Method-3 using 500 µg/ml; lane B: blank (buffer);
538 lane 4: Method-1 using 500 µg/ml; lane 5: Method-2 using 500 µg/ml; and lane 6:
539 untransformed X-33. (B) Standard graph of AOX gene using pPICzαA vector for the
540 determination of copy number in *P. pastoris* GFP-Zeocin strain, SYBR Green detection in
541 RT-qPCR. Linear fit regression is Cq value = [-4.1655×log copy number] + 44.413 with
542 0.963 correlation coefficient (R^2).
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Table 1: Relative copy number integrated gene for different transformation strategies using RT-PCR

Zeocin (µg/mL)	Method	Log copy number (LCN)	Gene copy number (GCN) = 10^LCN	Relative copy number RCN=GCN_S/GCN_C
0	CS (X-33)	5.50	3.16E+05	-
	M1	5.45	2.83E+05	0.89
100	M2	5.62	4.22E+05	1.33
	M3	6.02	1.04E+06	3.28
	M1	5.82	6.66E+05	2.10
250	M2	5.96	9.21E+05	2.91
	M3	6.10	1.26E+06	3.99
	M1	5.97	9.40E+05	2.97
500	M2	6.22	1.67E+06	5.26
	M3	6.36	2.27E+06	7.19

CS (Control strain *Pichia pastoris* X-33), GCN_C and GCN_S are control and sample gene copy numbers, respectively.

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Table 2: Comparison of transformation efficiency using different transformation procedures in *P. pastoris*

Method	*P. pastoris* strain	Transformation efficiency (transformants/µg DNA)	Reference
LiAc Electroporation	GS115	$2.00 \times 10^{4} \pm 1.02 \times 10^{4}$	[10]
Electroporation	GS200, GS115, PPY12h PPY12m	$1.30 \times 10^{3} \pm 1.23 \times 10^{2}$	[18]
LiCl	X-33	1.00×10^{2} to 1.00×10^{3}	Invitrogen
TE-buffer LiCl	PPF1	2.29×10^{2}	[8]
TE-buffer LiCl	GS115	5.19×10^{3}	[8]
Electroporation	---	$1.10 \times 10^{5} \pm 2.07 \times 10^{2}$	[29]
Electroporation	JC100 GS115	2.00×10^{3}	[30]
Modified LiCl method*	X-33	$6.52 \times 10^{3} \pm 1.05 \times 10^{1}$	Present study

*Results are the mean and standard deviation ($\bar{y} \pm SD$) of four experiments

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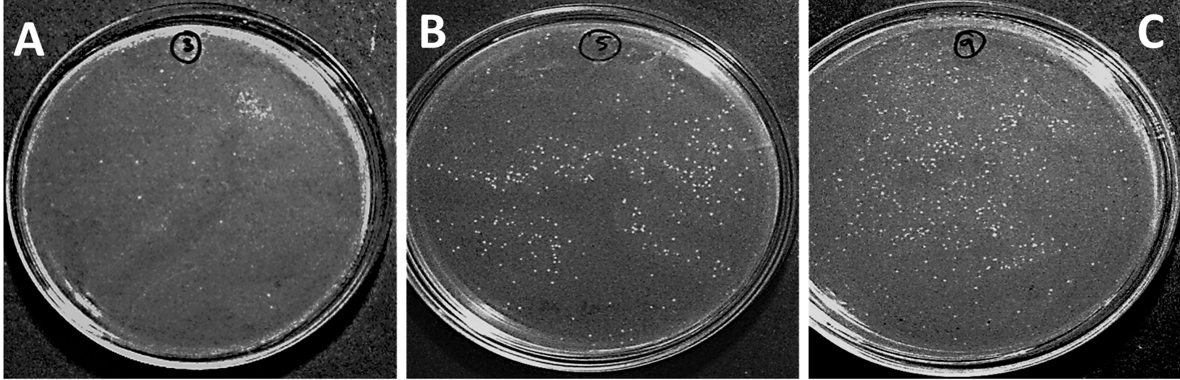

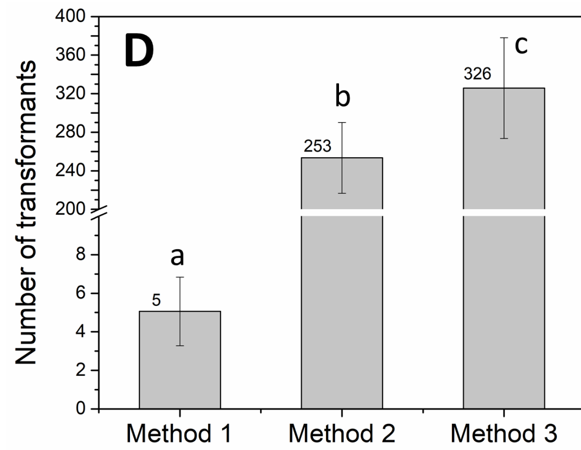

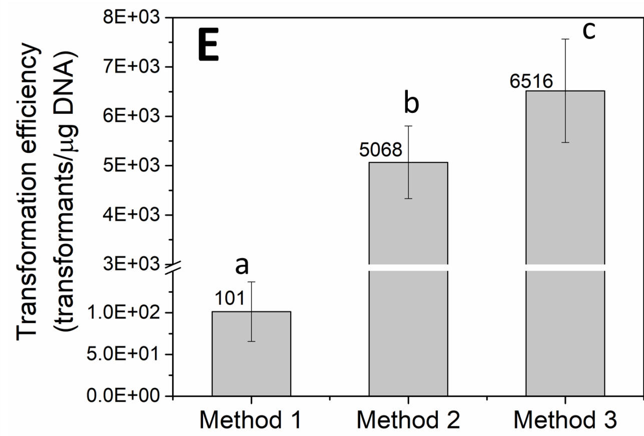

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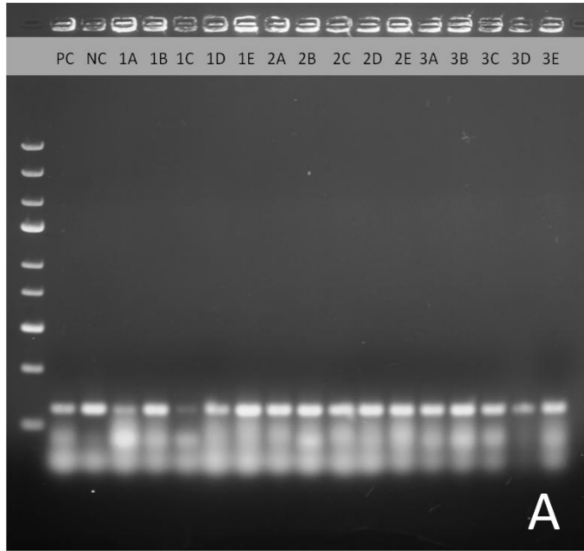

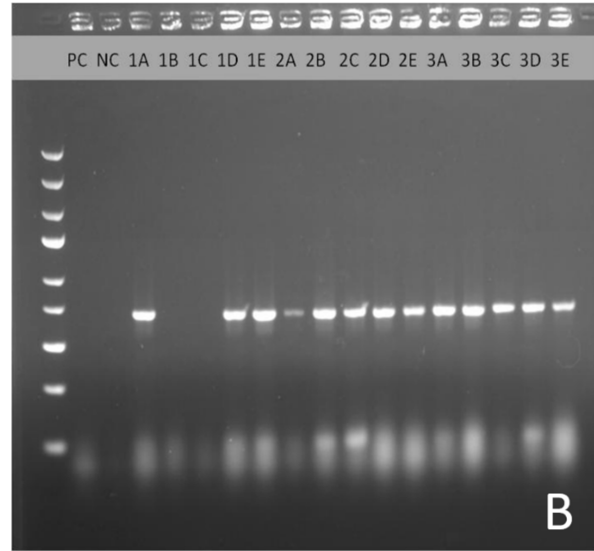

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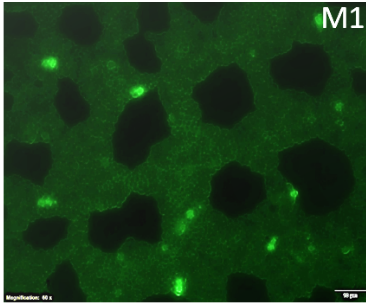
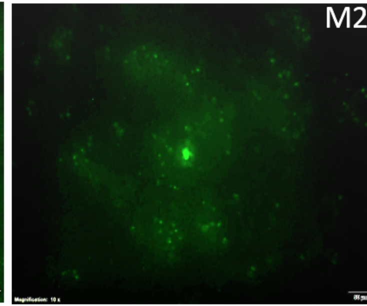
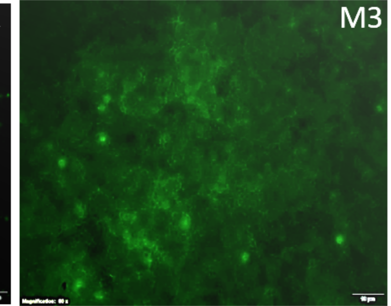
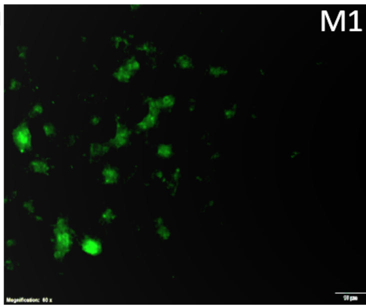
100 µg/ml zeocin

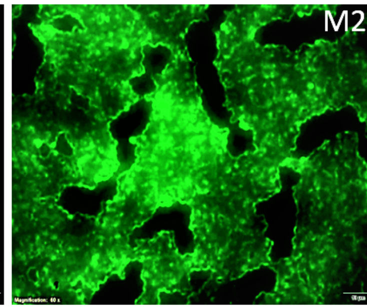


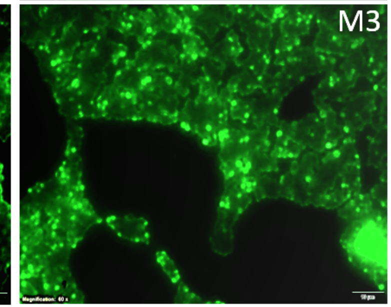




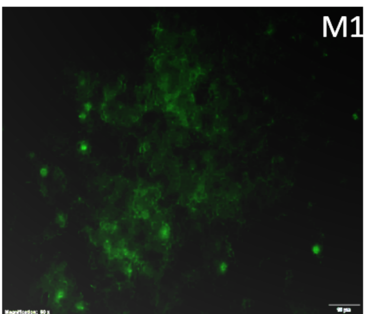
250 µg/ml zeocin

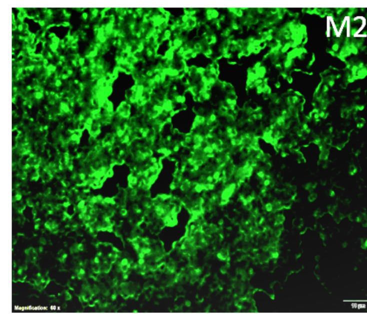


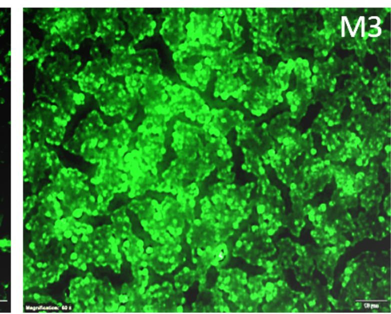




500 µg/ml zeocin







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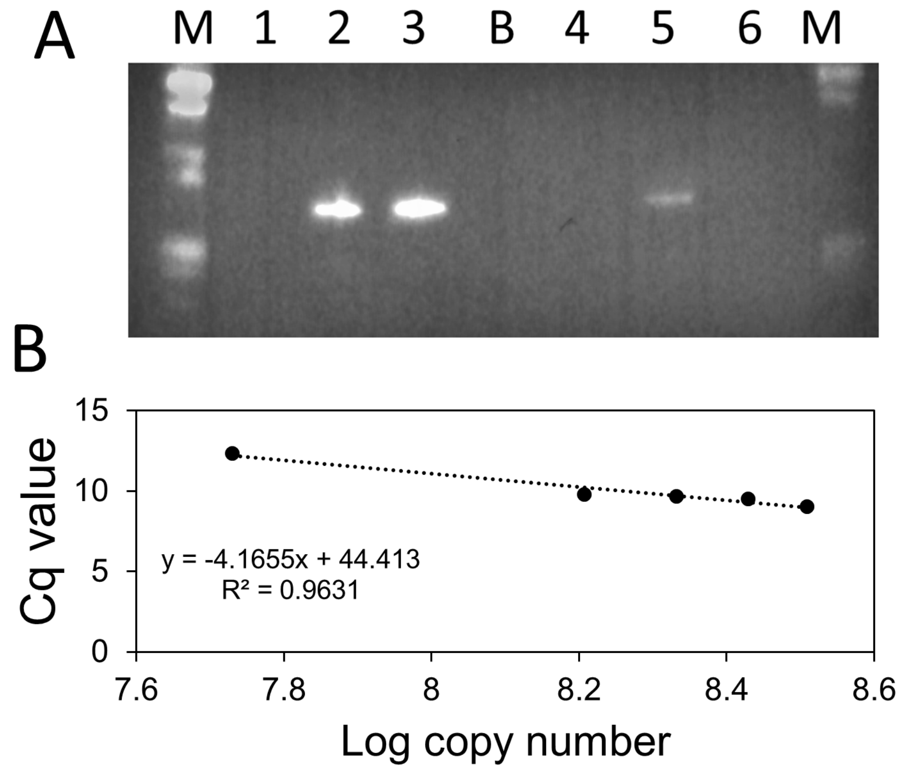

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Highlights

- Incubation time and LiCl concentration affects *P. pastoris* transformation efficiency
- Establishment of colony-PCR method for diagnosis of successful transformants
- PTVA coupled to proposed transformation strategy improves protein expression
- PTVA coupled to proposed transformation strategy improves gene copies

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