Original Research Paper

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Microbiology

EXPRESSION AND CHARACTERIZATION IN PICHIA PASTORIS BY CLONING OF DELTA 4 DESATURASE FROM ISOCHRYSIS GALBANA.

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KEYWORDS :

INTRODUCTION

Docosahexaenoic acid (DHA,C22:6n3) is a type of polyunsaturated fatty acids in omega 3 group that found in lipid of marine fish, fresh-water fish and some grains. Briefly, The DHA is produced by altering of alpha-linolenic acid (ALA, C18:3n3) to docosapentaenoic acid (DPA, C22:5n3) using delta 2 Desaturase (Δ2) and delta 6 Elongase (E6) enzymes. Thereafter, the DPA is changed to DHA by 2 pathways (i) beta-oxidation pathway or (ii) delta 4 desaturase (Δ4) pathway. Previous results from Gosalawit 2013 have shown that the β-oxidation pathway is not efficient to produce DHA in P. pastoris. Therefore, Δ4 pathway was tried to observe if it is more productive for DHA production.

Objectives

The objective of this project are to identify Δ4 gene derived from a marine microalgae, Isochrysis galbana and clone into P. pastoris that has previously been engineered to contain Δ2 and E6. The recombinant P. pastoris was cultured in YPD (4% glucose) and then the fatty acids were extracted. Fatty acids were esterified and determined by using GC.

Methodology

Part 1

[1.] Total RNA was extracted from I. galbana then converted to cDNA.
[2.] The Δ4 gene was amplified by PCR then purified. The Δ4 gene was ligated with pTG191cloning1vector.
[3.]The pTG19:Δ4 vector was transformed into E. coli (DH5α) competent cells. The transformed cells were spread on LB plate that contained ampicillin, X-Gal, and IPTG. White colonies were chosen for colony PCR.
[4.] The corrected pTG19:Δ4 vector was extracted from E. coli then sequenced. The obtained Δ4 sequence was compared with the NCBI database.

Part 2

[5.] The Δ4 gene was cloned into pGAPZ vector and confirmed by colony PCR.

Part 3

[6.] The pGAPZ:Δ4 was digested with BglII and BamHI and pGAPZ:Δ2:E6 vector (Gosalawit, 2013) was cut with BglII. Both fragments were ligated together then transformed into E. coli. The cells were spread on LB plate that contained Zeocin. The corrected clones were screened by colony PCR.
[7.] The pGAPZ:Δ2:E6:Δ4 vector was extracted and digested with AvrII to generate linear form of the recombinant vector.
[8.] The linearized pGAPZ:Δ2:E6:Δ4 vector was transformed into P. pastoris (SMD1168H) competent cells. Afterward, the transformed cells were spread on YPD contained Zeocin. Cells were screened by colony PCR. **[**
9.] All recombinant P. pastoris colonies were re-streaked on YPD agar contained 500 μg/ml Zeocin to select high copy number of vector integrated into the genome of P. pastoris.
[10.] The recombinant P. pastoris that could grow
on YPD plate containing high concentration of Zeocin was cultured in YPD (4% glucose) for 4 days at 30°C with 200 rpm.
[11.] The cells were harvested and fatty acids were extracted and the DHA level was determined by GC.

RESULTS AND DISCUSSION

Part 1

RNA was extracted from I. galbana then converted to cDNA. The cDNA was used as a template for Δ4 gene amplification then this gene was cloned into pTG19 cloning vector and transformed into E. coli. The pTG19:Δ4 vector was confirmed by colony PCR technique. The result showed that 8 clones exhibited target size of the Δ4 gene, which was about 1,200-1,300 bp. The vector were extracted and sequenced. The result from the sequencing indicated that clone 31 showed 99% nucleotide identity to I. galbana (JQ791105.1) (figure 1).

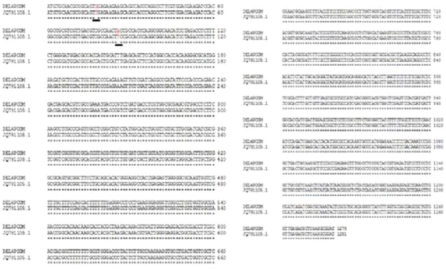


Figure 1 Alignment between Δ4 of I. galbana (JQ791105.1) from the database and Δ4 from pTG19:Δ4 clone 31. "*" identical amino acid and "." semi-conserved substitutions.

Part 2

Thereafter, the Δ4 gene was cloned into pGAPZ vector to construct pGAPZ:Δ4. This recombinant vector was transformed into E. coli then confirmed using colony PCR technique. The result showed that 14 clones exhibited target size about 1,500 bp (figure 2). The length of Δ4 gene (JQ791105.1) was 1,284 bp. In this work (figure 2), the bans of approximately 1,500 bp were seen because the primers used in this case also amplify part of the multiple cloning site of the vector

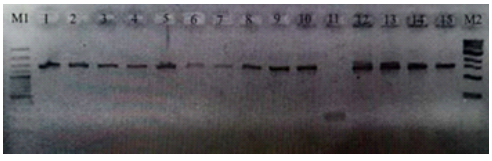
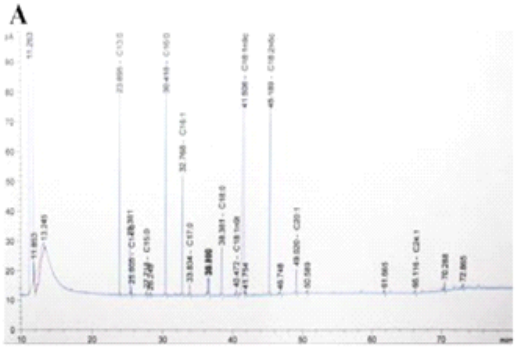


Figure 2 Colony PCR of pGAPZ:Δ4 using pGAPZ forward and reverse primers. Lane M1: 100 bps marker Lane 1-15: clone 1-15; Lane M2: 1 kb marker

Part 3

The recombinant P. pastoris clone 4 that had been selected from high concentration Zeocin (500 μg/ml) was cultured in YPD (4% glucose) for 4 days at 30°C with 200 rpm. then the fatty acids were measured using GC and the fatty acid profiles were compared among wild type P. pastoris, P. pastoris that containing each of empty pGAPZ vector, pGAP:Δ2:E6 vector, and pGAP:Δ2:E6:Δ4. The results displayed that the P. pastoris containing pGAPZ:Δ2:E6:Δ4 presented efficiency to produce DHA when compared with other groups (Figure 3).



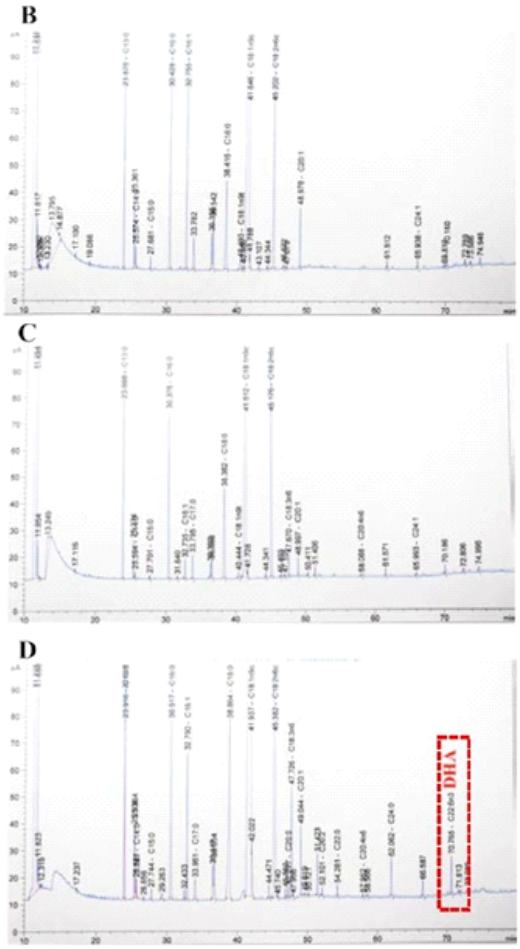


Figure 3 Fatty acid profiles. A: Wild type P. pastoris; B: P. pastoris containing empty pGAPZ vector; C; P. pastoris containing pGAPZ:Δ2:E6; D: P. pastoris containing pGAPZ:Δ2:E6:Δ4.

CONCLUSION

In this work, the Δ4 gene was isolated from I. galbana then cloned and sequenced. The results presented that this Δ4 gene exhibited high percent nucleotide identity with Δ4 genes from database. Consequently, this Δ4 gene was expressed in P. pastoris that has been contained Δ2 and E6. This recombinant P. pastoris was cultured then the fatty acid profiles were determined using GC. The results presented that P. pastoris that contained 3 genes exhibited DHA accumulation when compared with other yeasts. This result indicated that the corporation of 3 genes for DHA production was successfully constructed in this work.

REFERENCES

1. Gosalawit, C. The Construction of Pichia pastoris to Produce Polyunsaturated Fatty Acids (PUFAs). Master's Thesis,Biotechnology Suranaree University of Technology, 2013.