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Harnessing pichia pastoris for efficient co-expression of ACC deaminase and PMEI fusion protein

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Abstract
The post-harvest losses are mainly due to over-ripening of fruits or vegetables. The main hormone responsible for fruit ripening is ethylene, which is also produced in excess under stress conditions. ACC (1-aminocyclopropane-1-carboxylic acid) is a precursor of ethylene. Enzyme ACC deaminase breaks ACC and thus helps reduce ethylene levels in plants. Pectin is a major part of the plant cell wall, and enzyme pectin methylesterase (PME) breaks the pectin, ultimately loosening the cell wall, which greatly affects physiochemical plant activities. Enzyme pectin methylesterase inhibitor (PMEI) inhibits PME and reduces cell wall breakage. Both genes were isolated, fused together, and transformed into *Pichia pastoris*. Gene ACC deaminase was isolated from the fungus *Trichoderma asperellum*, and gene *PMEI* was isolated from *Solanum lycopersicum* (tomato), which were 1118 bp and 555 bp in size, respectively. Both genes were linked with YFP and GFP and cloned into the pET101 vector, then fused together using a 2A peptide linker. The fused construct was cloned into the pPIC9 vector and transformed into *Pichia pastoris*. From transformed cells, single-colony PCR results showed the amplification of both genes. The biochemical tests of both genes further demonstrated protein expression in the host. This study ultimately helps in increasing fruit shelf life and reducing post-harvest losses by mitigating abiotic stress.

Keywords: ACC deaminase, PMEI fusion protein, harnessing pichia pastoris, co-expression

Introduction
The methylotrophic yeast *Pichia pastoris* is widely recognized as a robust expression system for heterologous proteins due to its ease of genetic manipulation, high protein yield, and capability to perform post-translational modifications such as glycosylation and disulfide bond formation. These features, combined with its GRAS (Generally Recognized as Safe) status awarded by the FDA, make it an ideal candidate for industrial and pharmaceutical applications.

Ethylene, a key plant hormone, regulates various physiological processes, including senescence and stress responses. Overproduction of ethylene under stress conditions can lead to detrimental effects on plant growth and yield. ACC deaminase (*ACCD*) catalyzes the breakdown of ACC, the precursor of ethylene, thereby mitigating stress-induced ethylene production and promoting plant growth. On the other hand, Pectin Methylesterase Inhibitor (*PMEI*) plays a vital role in maintaining cell wall integrity by inhibiting Pectin Methylesterase (*PME*), an enzyme responsible for pectin de-esterification. This study aims to coexpress *ACCD* and *PMEI* as a self-processing fusion protein in *Pichia pastoris*. The approach involves linking the two genes using a 2A peptide sequence to achieve efficient coexpression and simultaneous functional protein production.

Materials and Methods
Strains
- *Trichoderma asperellum* T203 (for *ACCD* gene)
- *Solanum lycopersicum* (for *PMEI* gene), (Cregg *et al*., 1993) [2]
- *Escherichia coli* DH10α (for cloning), (Sambrook & Russell, 2001) [9]
- *Pichia pastoris* GS115 (for expression), (Invitrogen Corporation, 2010) [8].

Vectors

- PET101 TOPO: Used for cloning in *E. coli*. (Tuite & Cregg, 1999) [14].
- pPIC9: Used for expression in *Pichia pastoris*. (Cregg *et al.*, 1993) [2].

Media

Standard media such as LB, YPD, and Minimal Dextrose (MD) were prepared following standard protocols.

Gene Cloning and Construct Design

The genes encoding ACCD and PMEI were amplified using specific primers with overhangs for fusion and restriction enzyme sites. The ACCD gene was linked to a yellow fluorescent protein (YFP) tag using a flexible $(G4S)_3$ linker, while PMEI was linked to a green fluorescent protein (GFP) tag. The constructs were fused using overlapping PCR and cloned into pET101 TOPO for validation before subcloning into the pPIC9 expression vector as per in Figure 1.

Transformation and Expression in *Pichia pastoris*

The recombinant pPIC9 constructs were linearized and transformed into *Pichia pastoris* GS115 via electroporation. Positive transform ants were selected on MD plates and further screened for protein expression using SDS-PAGE and fluorescence analysis. (Invitrogen Corporation, 2010) [8].

Protein Purification and Characterization

The expressed fusion proteins were purified using hydrophobic interaction chromatography and analyzed for activity. Functional assays were performed to evaluate the *ACCD* activity (ACC degradation) and *PMEI* functionality (PME inhibition). (Gietz & Schiestl, 1995) [4].

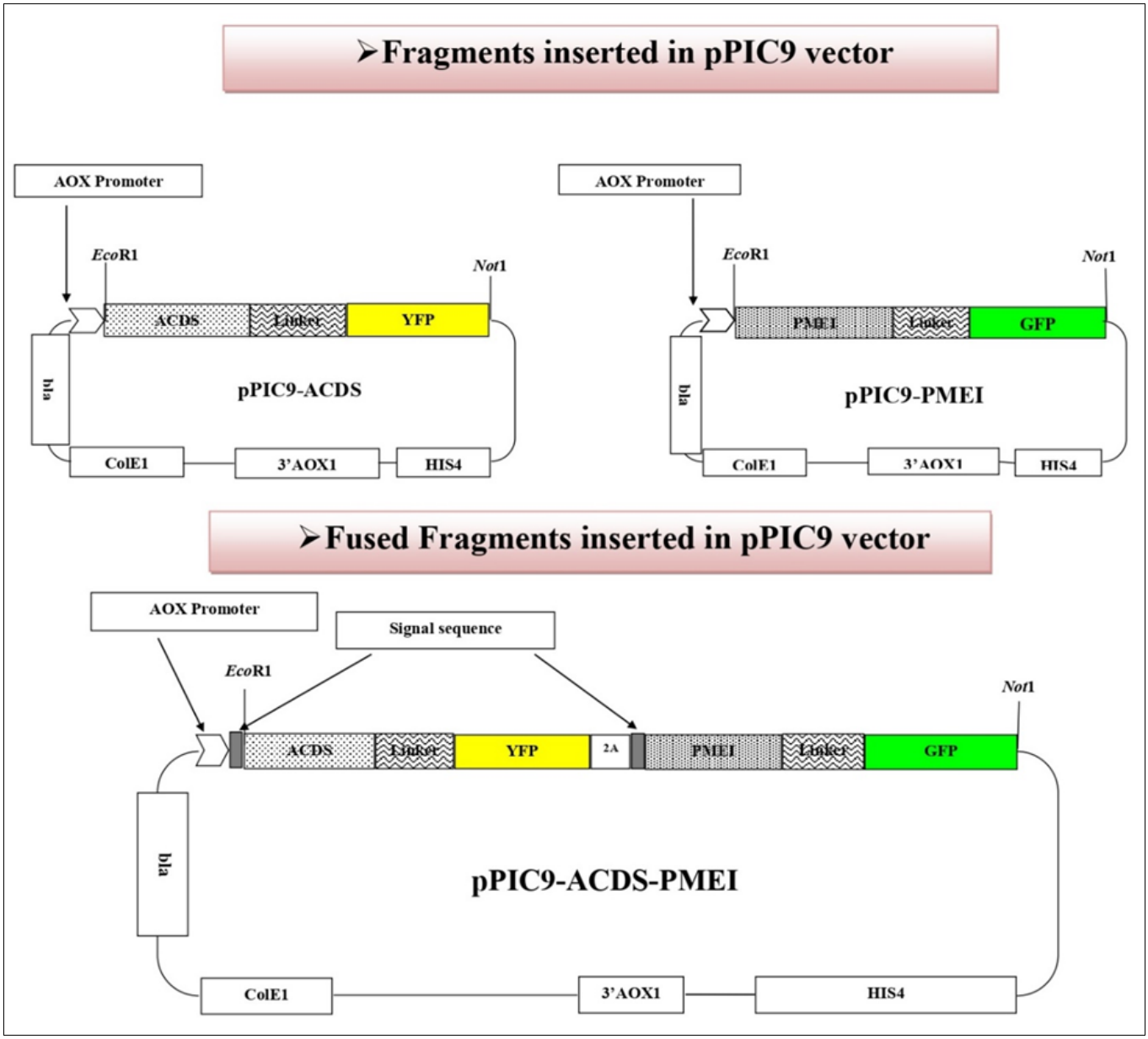

Fig 1: Fusion Gene cloning in pPIC9 Vector. The figure showing the individual construct cloning and Final fusion construct cloning in pPIC9 vector

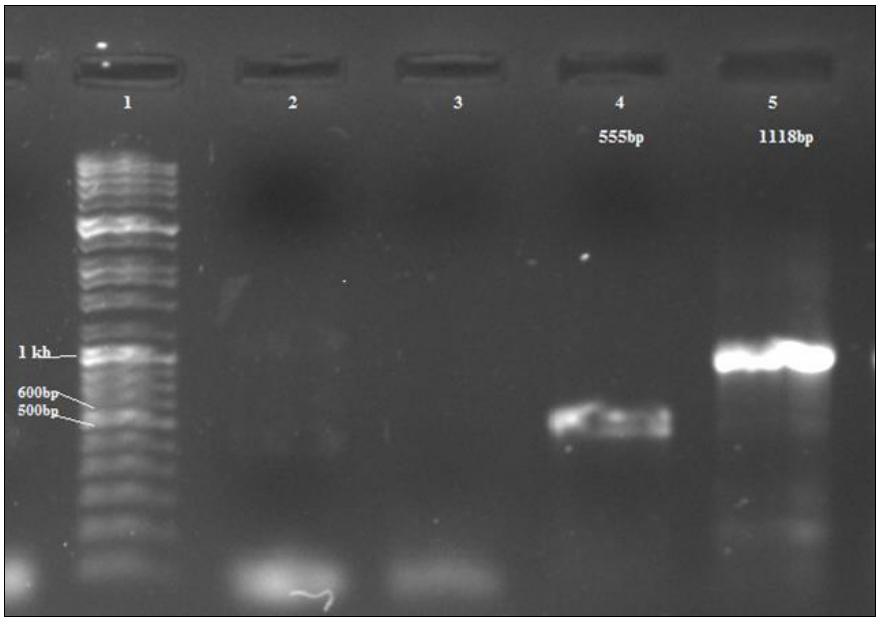

Fig 2: Gene Amplification From cDNA

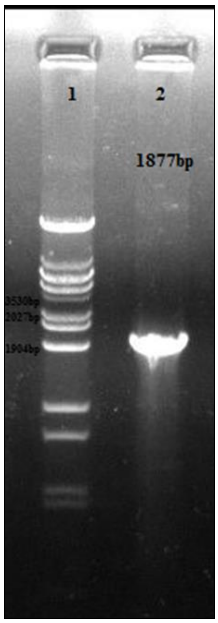

Fig 3: Amplified Construct 1

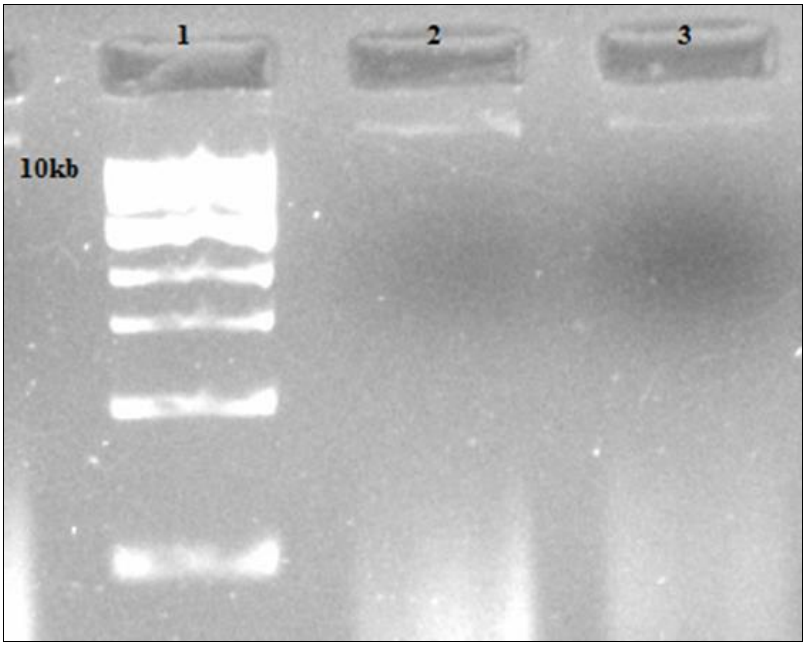

Fig 4: Gel image of Plasmid Isolation

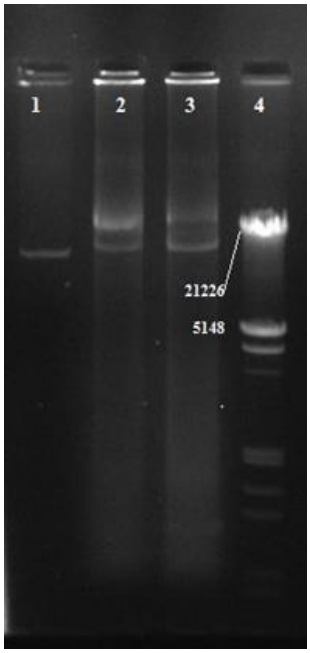

Fig 5: Plasmid Digestion

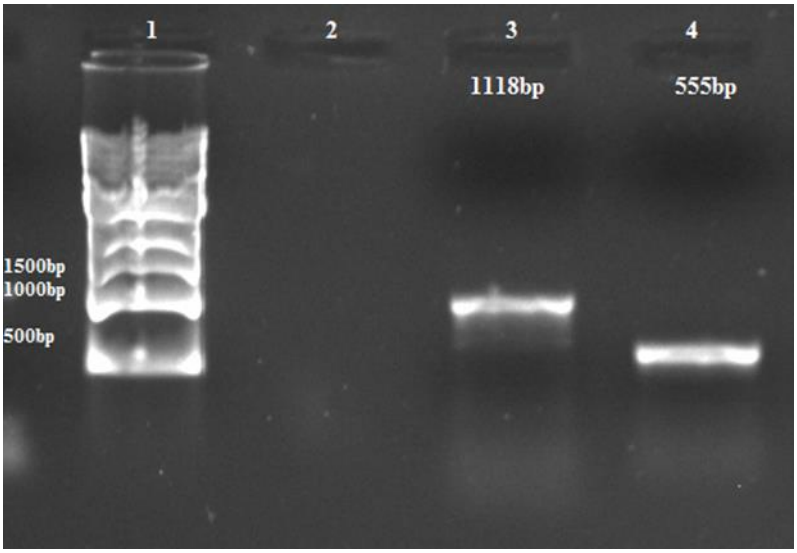

Fig 6: Gene Amplification from transformed colony

Results

The present investigation focused on the coexpression of ACC deaminase (*acds*) and PMEI as a self-processing fusion protein in *Pichia pastoris*. This research employed a variety of molecular biology techniques, including RNA isolation, gene amplification, cloning, and transformation. Fresh tomato fruits were selected as the source for RNA extraction to amplify the PMEI gene, while *Trichoderma asperellum* culture served as the source for the isolation of the ACC deaminase gene. RNA was extracted from the tomato fruits, converted to cDNA, and used as a template for amplifying the PMEI gene. Similarly, cDNA synthesized from *Trichoderma asperellum* RNA was used for amplifying the *acds* gene. Both genes were successfully amplified, with the PMEI gene producing a 555 bp product and the *acds* gene yielding a product of 1118 bp. The amplification results were visualized on a 1X EtBr agarose gel, as shown in Figure 2, confirming the successful amplification of the target genes. The construction of fusion proteins required specific modifications to the amplified genes. For the synthesis of Construct 1, the *acds* gene was amplified with a $(G4S)_3$ linker at its 3' end. Concurrently, the YFP gene was amplified with a $(G4S)_3$ linker at the 5' end and a 2A peptide at the 3' end. Gel electrophoresis confirmed the successful amplification of both *acds* and YFP genes. The two genes were fused using overlap PCR, resulting in a final construct of approximately 1877 bp, as depicted in Figure 3. Similarly, for the preparation of Construct 2, the PMEI and GFP genes were amplified with overhang sequences designed to facilitate their fusion. The PMEI gene, amplified as a 620 bp product, and the GFP gene, amplified as a 762 bp product, were fused via overlap PCR to generate the final fusion construct.

The fusion construct, measuring approximately 3.5 kb, was subsequently cloned into the pPIC9 expression vector. This vector, containing the construct, was introduced into *E. coli* DH10α cells for plasmid amplification. The plasmid was then isolated from the transformed *E. coli* cells, with the plasmid size determined to be approximately 11.5 kb, as illustrated in Figure 4. To prepare the plasmid for transformation into *Pichia pastoris*, it was linearized using SacI digestion. The digestion results confirmed the linearization of the plasmid, as shown in Figure 5.

Following transformation of *Pichia pastoris* with the linearized plasmid, colony PCR was performed to confirm the integration of the *acds* and PMEI genes into the host genome. Specific primers were used to amplify the *acds* and PMEI genes, producing fragments of 1120 bp and 555 bp, respectively, which were visualized on a 1X EtBr agarose gel. These results, displayed in Figure 6, confirmed the successful transformation of the target genes into *Pichia pastoris*. This study successfully demonstrated the synthesis, cloning, and expression of a self-processing fusion protein in *Pichia pastoris*, setting the stage for further functional studies.

Discussion

The successful amplification and cloning of the *ACCD* and *PMEI* genes into a self-processing fusion construct is a significant achievement. The results align with previous studies that have successfully cloned and expressed similar genes in various organisms. Shah *et al.* (1998) [10] isolated and characterized the ACC deaminase gene from *Enterobacter cloacae*, where the gene amplified to 1 Kb, showing homology with ACC deaminase genes from *Pseudomonas*. Similarly, Campbell and Thomson (1996) [1] isolated an ACC deaminase gene from *Pseudomonas*, which yielded an 817 bp amplified fragment. These studies corroborate the findings in this study, where the *ACCD* gene was successfully amplified at a length of 1118 bp.

For the PMEI gene, Hong *et al.* (2010) [7] isolated a functional PMEI gene from wheat, which was approximately 579 bp in size. The PMEI gene isolated in this study was 555 bp, a size similar to the findings of Srivastava *et al.* (2012) [11], who isolated a 567 bp PMEI gene from banana. These comparisons validate the methodology used for the amplification of the PMEI gene in this study.

The fusion of genes using the 2A peptide sequence, as done in this study, has been widely used in previous research. Subramanian *et al.* (2017) [12] successfully fused GFP with a target gene using the 2A peptide, leading to the expression of two distinct proteins. Tang *et al.* (2016) [13] similarly fused YFP and RFP genes using the 2A peptide, observing the separation of the two proteins, despite their translation from the same mRNA. These results are consistent with the findings in this study, where a YFP gene and an *ACCD* gene were successfully amplified with overhangs and fused through a $(G4S)_3$ linker and 2A peptide sequence.

The plasmid isolation and digestion results indicate successful cloning and preparation of the vector for transformation into *Pichia pastoris*. The plasmid digestion with SacI produced the desired linearized product, which is essential for successful transformation into the host organism. The colony PCR results from transformed *Pichia pastoris* colonies confirmed the successful integration of both *ACCD* and *PMEI* genes into the host genome, which is a crucial step for subsequent protein expression.

Conclusion

This study successfully co-expressed *ACCD* and *PMEI* as a self-processing fusion protein in *Pichia pastoris*, demonstrating efficient production of both functional enzymes. The use of the 2A peptide linker enabled balanced expression and post-translational cleavage, preserving protein activities. The results validate *Pichia pastoris* as an effective system for dual protein expression with potential applications in agricultural biotechnology to mitigate stress and enhance crop resilience. Future work may focus on optimizing expression conditions and extending this system to other stress-related proteins.

Conflict of Interest

Authors declare that they have no conflict of interest.

Financial Support

Not available

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