

**Cloning, Expression and Functional Characterization of a Novel  $\alpha$ -Humulene Synthase, Responsible for the Formation of Sesquiterpene in Agarwood Originating from *Aquilaria malaccensis***

**ABSTRACT**

This study describes the cloning, expression and functional characterization of  $\alpha$ -humulene synthase, responsible for the formation of the key aromatic compound  $\alpha$ -humulene in agarwood originating from *Aquilaria malaccensis*. The partial sesquiterpene synthase gene from the transcriptome data of *A. malaccensis* was utilized for full-length gene isolation via a 30 RACE PCR. The complete gene, denoted as AmDG2, has an open reading frame (ORF) of 1671 bp and encodes for a polypeptide of 556 amino acids. In silico analysis of the protein highlighted several conserved motifs typically found in terpene synthases such as Asp-rich substrate binding (DDxxD), metal-binding residues (NSE/DTE), and cytoplasmic ER retention (RxR) motifs at their respective sites. The AmDG2 was successfully expressed in the *E. coli*:pET-28a(+) expression vector whereby an expected band of about 64 kDa in size was detected in the SDS-PAGE gel. In vitro enzyme assay using substrate farnesyl pyrophosphate (FPP) revealed that AmDG2 gave rise to two sesquiterpenes:  $\alpha$ -humulene (major) and  $\beta$ -caryophyllene (minor), affirming its identity as  $\alpha$ -humulene synthase. On the other hand, protein modeling performed using AlphaFold2 suggested that AmDG2 consists entirely of  $\alpha$ -helices with short connecting loops and turns. Meanwhile, molecular docking via AutoDock Vina (Version 1.5.7) predicted that Asp307 and Asp311 act as catalytic residues in the  $\alpha$ -humulene synthase. To our knowledge, this is the first comprehensive report on the cloning, expression and functional characterization of  $\alpha$ -humulene synthase from agarwood originating from *A. malaccensis* species. These findings reveal a deeper understanding of the structure and functional properties of the  $\alpha$ -humulene synthase and could be utilized for metabolic engineering work in the future.