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Enzymatic Agarooligosaccharides Development
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Comprehensive *In-Silico* Study Combined with Experimental Validation to Reconstruct α-Agarase for Enzymatic Agarooligosaccharides Development

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ABSTRACT

Enzymatic synthesis has gained attention from the industry and researchers due to its sustainable process and the fact that no byproducts have been yielded. However, most agarooligosaccharides (AOS) production utilized chemical synthesis as the study of α-agarase remained scarce. While it was possible to discover desired α-agarase from natural sources, it tended to be time- and cost-consuming with unpredictable outcomes. This study aimed to explore effective α-agarase using state-of-the-art *in-silico* approaches to shortlist and reconstruct the potential candidate and wet-lab experiment to validate the outcomes. Biological databases from CAZy, UniProtKB, PDB, and cross-references from journals were involved to gather all information on α-agarases for enzyme curation. The selected candidate was analyzed whether the removal of non-catalytic regions was required to reduce cost production. Then, structure modeling using SWISS-MODEL was performed for further assessment of docking using CB-Dock2 and physicochemical properties incorporating OphPred, Protein-Sol, and SCooP. Experimental validation was performed by conducting screening, expression by involving SDS-PAGE and Bradford assay, and activity assays interpreted using TLC and DNS assay. It revealed that α-agarase AgaA33 from *Thalassomonas agarivorans* JAMB-A33 was selected for its overall score and optimal temperature, non-catalytic regions were removed to reduce synthesis costs, preserving only GH96 attached to His-tag. Structural models and cavity analyses confirmed the preservation of functional domains. pH and thermal stability prediction assessments possessed desired output with the pH value of 0.46 and T_m of 24.3 °C higher than native. However, the solubility prediction was 0.012 lower than PopAvrSol. Recombinant AgaA33 was expressed in *E. coli* BL21 (DE3), but experimental activity assays revealed low catalytic performance, with the higher protein concentration on pellet fraction, likely due to inclusion body formation and impaired protein folding. Despite these challenges, this study underscores the utility of computational tools in enzyme reconstruction and highlights areas for further optimization. Future work should focus on improving solubility, folding efficiency, and activity to harness the potential of α-agarase for AOS production.

Keywords: α-agarase, AOS, activity assay, enzyme engineering, *in-silico*