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Improving operational stability of thermostable *Pythium myriotylum* secretory serine protease by preparation of cross-linked enzyme aggregates (CLEAs)

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ABSTRACT

Present study was undertaken to develop cross-linked enzyme aggregate (CLEA)of alkaline serine proteases (sp) from *Pythium myriotylum* (*Pm*), a necrotrophic oomycete reported to considerably secrete serine proteases. Among various precipitants screened for *spPm*1-CLEA preparation, ammonium sulfate at 80% saturation (*w/v*) yielded 100% activity recovery and retention of spherical morphology as observed by SEM analysis. Addition of glutaraldehyde as cross-linker at 1% (*v/v*) concentration with optimized ammonium sulfate concentration for 1 hour at 100 rpm yielded 100% activity recovery of *spPm*1-CLEA from 8-day old *P. myriotylum* culture filtrate. Addition of BSA (10 mg/ml) to CLEA cross-linking reaction mix reduced CLEA size from the range of 1.82–1.19 µm to 394–647 nm. *spPm*1-CLEA preparations retained 100% activity at temperature of 80 °C and pH 12.0 signifying their potential commercial applications. In terms of kinetic parameters, present process enhanced kinetic parameters as revealed by 1.67 $U.mg^{-1}$ specific activity, K_m of 0.062 mM and V_{max} of 0.145 $\mu mol.min^{-1}.mg^{-1}$ for the *spPm*1-CLEA compared to 0.288 $U.mg^{-1}$ specific activity, K_m of 0.060 mM and V_{max} of 0.20 $\mu mol.min^{-1}.mg^{-1}$ determined for the free *spPm*1 enzyme. Study has successfully demonstrated the concept of CLEA in enhancing *spPm*1 stability and the results so generated can be translated in future towards development of robust biocatalysts.

KEYWORDS

Cross-linked enzyme aggregate (CLEA); immobilization; *Pythium*; Serine protease

Introduction

Global market for industrial enzymes was estimated at 5.6$billion in 2018 and this demand is expected to increase at 6.8% compound annual growth rate (CAGR) by 2024.[1] Approximately, 75% of the enzymes used in industries are hydrolases like proteases, cellulases, amylases, and lipases.[1] Proteases remain the dominant enzyme type among hydrolases[2,3] due to their extensive use in the detergent and dairy industries.[4] Among proteases, alkaline serine proteases (SPs) are the most important group of commercially exploited enzymes and account for approximately 35% of the total microbial enzyme sales.[5,6] Most of the commercial alkaline proteases viz. Subtilisin Carlsberg, Subtilisin BPN', Alcalase, Esperase and Savinase sourced from *Bacillus* find major applications in detergent industry.[4,7] However these proteases exhibit some drawbacks that include instability in presence of surfactants and oxidizing agents commonly found in detergent formulations besides high production costs.[8]

Identification of diverse proteases from novel microbial paradigm opens up possibilities towards exploiting the broad specificities and stability for various industrial applications.[9,10] SPs from very few wild-type organisms are known to possess appreciable stability to surfactants and oxidants besides exhibiting activity over broad pH and temperature ranges.[7,11] Necrotrophic oomycetes are known to constitutively secrete an array of cell wall degrading enzymes (CWDEs) at the hyphal apex in small apical vesicles to initiate cell necrosis and facilitate nutrient acquisition for survival and colonization.[12] SPs have been identified as the major extracellular proteolytic enzymes secreted by *Pythium myriotylum*, a necrotrophic oomycetous fungus.[13] Further purification and characterization of the secretory protease from *Pythium myriotylum* designated, *spPm*1 revealed retention of proteolytic activity over broad pH (5.0–12.0) and temperature (10–80 °C) ranges with optimal pH and temperature at 8.0 and 60 °C respectively.[14] Acidic or alkaline conditions and elevated temperatures prevailing under industrial conditions limit[15] direct industrial application of *spPm*1. Industrial applicability of *spPm*1 can be enhanced in terms of stability and functionality by immobilization,[15–17] besides reducing autolysis, a major problem limiting direct application of proteases.[18]

Often an empirical approach is followed whereby diverse parameters are considered before arriving at an immobilization protocol yielding optimal results.[19–23] Protein-metal hybrid nanoflower (HNF) system is yet another successful

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