ABSTRACT

Owing to vast therapeutic, commercial, and industrial applications of microbial proteases microorganisms from different sources are being explored. Proteases have vast biotechnological applications in various industries like detergent, leather, agriculture, poultry, pharmaceutical, food, etc. The water-living *Monopterus cuchia* (Freshwater Asiatic mud eel) is an exclusively insect-feeding fish, so the animal is presumed to harbor proteolytic enzyme producing microorganisms (bacteria) in the gut. In this regard, the gut microbiota of *M. cuchia* was isolated and examined to assess the production of proteases by them. All the isolates were screened on skimmed milk and gelatin agar plates. The protease-positive isolates were characterized morphologically, biochemically, and at the molecular level. Out of 20 bacterial isolates, 6 belonging to five different genera viz. *Bacillus, Priestia, Aeromonas, Staphylococcus,* and *Serratia* demonstrated proteolytic activity. *Bacillus safensis* strain PRN1 demonstrated the highest protease production as confirmed by the largest hydrolytic clear zones in both skimmed milk agar (15±1 mm) and gelatin agar (16±1 mm) plates. The optimized parameters like time, pH, temperature, carbon, and nitrogen for the highest activity of protease and growth of *B. safensis* PRN1 include 72 h (OD_{600}=0.47,1213 U/mL), pH 8 (OD_{600}=0.83, 403.29 U/mL), temperature 40°C (OD_{600}=1.23, 1021.91 U/mL), fructose (OD_{600}=1.14, 722 U/mL), and gelatin (OD_{600}=1.74, 876.83 U/mL). Furthermore, the crude enzyme showed antibacterial and blood-stain removal properties. The Ammonium sulfate $(NH_4)_2SO_4$)-precipitation and gel filtration chromatography were used for the purification of the protease. The SDS-PAGE demonstrated the purified enzyme to be a monomer of molecular weight 33 kDa. The enzyme demonstrated the maximum activity at pH 8 and temperature 60°C. The activity was strongly inhibited by PMSF confirming that it belongs to the family of serine-proteases. The compatibility of the enzyme with surfactants and commercial detergents demonstrates its potential use in the detergent industry. The study also refers to an eco-friendly enzyme-mediated approach as opposed to the conventional chemical approaches in the detergent and laundry industries that are hazardous to human health and the environment. *B. safensis* PRN1 harboring the protease enzyme has not only to be isolated from the gut of the animal but also to culture in a growth medium. It was found to be difficult to culture and extract the bacteria and its protease enzyme respectively. Therefore, it was desirable to clone the protease gene and transfer the plasmid into competent cells of *E. coli* BL21 (DE3) for the production of the enzyme. Accordingly, the *knbs*^{SP1}gene encoding the trypsin-like serine protease KNBS^{SP1} was isolated from *B. safensis* PRN1,

and cloned into an expression vector (PET-28a). The gene was expressed in the *E. coli* cells BL21 (DE3) and Ni-NTA affinity chromatography single step was used to purify the recombinant protease (rKNBS^{SP1}) enzyme. The molecular weight of the rKNBS^{SP1} was 33 kDa as determined in SDS-PAGE. The rKNBS^{SP1} showed optimal activity at a temperature 60°C and pH 8. It retained above 70% of its enzyme activity at pH 10 and 70°C respectively. The recombinant protease enzyme was inhibited by PMSF and diisopropyl fluorophosphates suggesting its inclusion into the serine family of protease. The rKNBS^{SP1} enzyme demonstrated outstanding compatibility and stability with metal ions, commercial detergents, and surfactants, exhibiting 97.8 ± 2.5% stability with Surf Excel, a commercial laundry detergent, and 96.9 ± 2.0% stability with SDS. The kinetic constant values of the rKNBS^{SP1} enzyme were 1.03 ± 0.14 mg mL^{-1} and 359.7 ± 8.15 U mg^{-1} with the substrate azocasein. The turnover number value was 65.25 min^{-1} with a catalytic efficiency of 63.34 min^{-1} mg mL^{-1}. The primary, secondary, tertiary structures and the protein-protein interaction of the protease enzyme were elucidated. The multiple sequence alignment confirmed KNBS^{SP1} to be a trypsin-like serine protease enzyme. The assessment of the secondary structure demonstrated the predominance of coils with the increase in KNBS^{SP1} stability and activity under high temperature and alkaline conditions. Furthermore, docking of the modeled trypsin-like serine protease (KNBS^{SP1}) showed good affinity towards the casein substrate. The recombinant protease demonstrated blood-stain removal and goat skin dehairing properties. All these remarkable characteristics make the protease a potential eco-friendly candidate in the detergent formulation and leather processing industries. Additionally, a comparative analysis of the blood parameters of *Monopterus cuchia* with human blood was done. The haemoglobin value as well as other blood parameters of *M. cuchia* are comparatively higher than the human blood. Therefore, the prescription of cuchia raw blood to severely anaemic patients by traditional medicine men has been scientifically validated.