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Development of an immobilized biocatalyst with lipase and protease activities as a multipurpose cross-linked enzyme aggregate (multi-CLEA)

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ABSTRACT

This study focused on the production of a novel multi-CLEA comprising the enzyme activities of lipase and protease from fish viscera. A multi-CLEA is a single biocatalyst that can catalyze separate unrelated reactions, but these reactions can be conducted in one application. Tests pertaining to the effect of various additives on the multi-CLEA's activity were performed. Response Surface Methodology's Face Centered Central Composite Design (FCCCD) was employed to optimize the preparation parameters of the multi-CLEA in an aqueous medium. It was found that 55% (w/v) of ammonium sulfate, 65 mM of glutaraldehyde, and 0.113 mM of bovine serum albumin were the optimum levels of additives to prepare the multi-CLEA with the protease and lipase recovery activity of 43.82% and 99.91%, respectively. Multi-CLEAs were found to retain an average of more than 34% of the initial activity after five consecutive batches for both enzymes. Finally, the multi-CLEA was utilized to catalyze two reactions: improved washing process and biodiesel production. The stain removal percentage of a commercial detergent was improved by 67.78% after adding multi-CLEA. In addition, the multi-CLEA catalyzed biodiesel production from vegetable oil with a percentage conversion of 51.7%. Such results demonstrated that the multi-CLEA is a promising catalyst for biotechnological applications.

1. Introduction

The use of biotechnological immobilized enzymes has been established in many industrial processes [1]. Enzyme immobilization aims to enhance the economics of biocatalytic processes since the technique enables re-use of enzymes for an extended period of time and enables simpler catalyst separation from the product mixture at the end of the reaction [2]. If immobilization is properly carried out, it may improve various enzyme features (mainly stability, but also activity, selectivity, specificity, or inhibitions problems) [3,4], CLEA technology produces catalysts with high activity volumes, since the activity dilution caused by inert carriers was avoided [5]. Sheldon [6] introduced the immobilization method whereby the crude enzyme extract can be immobilized directly at the production site, without the need for purification, after performing protein precipitation. The physical aggregates of the resultant enzyme molecules are joined together by non-covalent bonds and can be easily re-dissolved in water [7]. Enzyme aggregates are then cross-linked using a cross-linking agent, such as glutaraldehyde, which is commonly used in the design of immobilized biocatalysts. Glutaraldehyde molecules are small molecules that can penetrate an aggregate's internal structure, and hence react with the amino residues on the enzyme surface [6]. Despite the enormous advantages of the CLEA method, Garcia-Galan [8] reported several disadvantages of CLEA technology. Among these problems, CLEAs are not mechanically resistant and that they are too soft for many industrial applications. Since CLEA does not require a purification step, other contaminant proteins in the solution may generate other problems, and recovery of CLEAs is a difficult process. Additionally, CLEAs have small pore sizes that can somehow reduce the diffusion rate of the substrate thereby affecting the enzyme activity. However, there are several procedures that might be considered in order to reduce CLEA' drawbacks; CLEAs may be hardened by dehydration and an intense cross-linking to increase their mechanical resistance [8], or they may be further

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immobilized on sol–gel [9], or one inert protein or polymer may be added [6–10]. Precipitation of enzymes by ammonium sulfate may also achieve the required purified enzymes [11].

A new concept in CLEA technology was introduced after the discovery of the possibility of forming various catalyzing reactions with a single CLEA particle. The term combi-CLEA was introduced by Sheldon et al. [11] to refer to CLEAs that catalyze a sequence of reactions. Subsequently, it is possible that combi-CLEA can catalyze non-cascade reactions. Dalal et al. [12] defined such a multipurpose CLEA (multi-CLEA) as "biocatalyst for many unrelated biological activities". Dalal et al. [12] provided an example of a multi-CLEA that is prepared from porcine pancreatic acetone powder that had lipase, α-amylase and phospholipase A2 activities to perform three different reactions. Banerjee et al. [13] prepared a novel biocatalyst consisting of two different lipases CAL B (a non-specific lipase) and Palatase (1, 3-specific enzyme) for synthesis of biodiesel from oil. Another example of the applications of co-immobilized enzymes was carried out by Minakshi [14] to determine the level of triglycerides in serum by immobilizing lipase, glycerol kinase, glycerol-3-phosphate oxidase and peroxidase. This study focused on the combined activities of lipase and protease in a single biocatalyst since it is in demand. This single biocatalyst will be able to perform several biotransformation reactions at once and hence, can save time, effort, and cost.

Over the years, a variety of hydrolases have been isolated from the internal organs of fish. Recently, recovery and characterization of various hydrolases from fish viscera have been carried out, for example alkaline phosphatase, lipase, acetylglucosaminidase, protease, α-amylase, cellulase, etc., which led the way to the development of some new applications of these enzymes in biotechnological processes [15,16].

Proteases are one of the most important hydrolases obtainable from fish viscera [17]. It was reported that proteases obtained from different origins are very promising for hydrolysis of proteins for commercial uses due to their biological origin [18]. Meanwhile, most of the available reports regarding protease production are those on proteases from microbial origin [19–21]. The demand for lipases is rapidly growing as well, due to their uses in many industries and industrial processes, such as organic chemical processing, detergent formulations, synthesis of bio surfactants, the oleo chemical industry, the dairy industry, the agrochemical industry, paper manufacture, nutrition, cosmetics, and pharmaceutical processing [22].

This paper reports the preparation steps of a single biocatalyst from channel catfish viscera *Ictalurus punctatus* which can catalyze several reactions of industrial relevance. *Ictalurus punctatus* is a common species of channel catfish that is generally found at the bottom of rivers, ponds, and reservoirs [15]. The multi-purpose cross-linked enzyme aggregate (multi-CLEA) described here possesses both lipase and protease activities. Thus, in this work, a study of different additives was carried out in order to improve the activity of this biocatalyst i.e., BSA, SDS, heptane and oleic acid; as interfacial activation, inner co-feeder and bioimprinting, and optimization of the preparation parameters were carried out. Furthermore, the multi-CLEA produced was then used was tested for its effect on the performance of the commercial washing detergent in removing food stains was investigated, and was used to catalyze esterification and transesterification reactions on vegetable oil.

2. Materials and method

2.1. Reagents

All chemicals and reagents were obtained from Essen Haus Sdn. Bhd and Merck Sdn. Bhd. They were of analytical grade and were not further purified. A Tecan Microplate Reader (Switzerland) was used to measure the absorbance, and a Sartorius Shaker (Germany) was used for preparation of the multi-CLEA. The channel catfish viscera were collected from a local market from Selangor, Malaysia.

2.2. Preparation of crude enzymes sample

Visceral parts of channel catfish *I. punctatus* were washed and blended with 1 M phosphate buffer (pH 7.3), at a ratio of 2:1 buffer-to-viscera and the resulting mixture was sieved through a muslin cloth. The filtrate was centrifuged at 4 °C, at a speed of 12,000 rpm, for one hour. This was followed by precipitating the supernatant with a 4 M ammonium sulfate solution, at 4 °C, with continuous stirring for 24 h. The protein concentration of the crude sample was measured using a Bradford assay [23]. The sample was dissolved in Phosphate Buffer Saline (PBS), and then followed by dialysis against minimal PBS (pH 7), in centrifugal concentration, (10000 MWCO), for four hours with continuous mixing, at 4 °C [15].

2.3. Lipase activity assay

A method described by Zhu et al. [24] was used to measure the activities of free-lipase, and lipase of multi-CLEA obtained from the channel catfish viscera. The method uses *p*-nitrophenyl palmitate as substrate, and *p*-nitophenol was measured as a product of the hydrolysis of *p*-NPP. The reaction mixture contained 1 mL of enzyme solution and 0.4% and 0.1% (w/v) of Triton X-100 and gum arabic, respectively. After incubating the reaction mixture at 37 °C for 15 min, the optical density was measured at a wavelength of 410 nm. One unit of lipase activity is defined as the amount of enzyme required to produce 1 μM of *p*-nitophenol per minute, under experimental conditions.

2.4. Protease activity assay

The enzyme activity of protease of multi-CLEA and free-protease was determined by a method mentioned by Mahmod et al. [15] with casein as a substrate. To terminate the reaction, 10% trichloroacetic acid (TCA) was used. Produced tyrosine was measured at a wavelength of 660 nm, and a tyrosine standard curve was plotted. One unit of protease activity is defined as the amount of casein that is hydrolyzed in 1 min of incubation at 35 °C for 1 mL of enzyme solution.

Immobilization yield in terms of enzyme recovery was determined as the relative activity of the CLEAs with respect to the same amount of native soluble enzyme that was used to prepare CLEAs [25]. However, the recovered activity for both lipase and protease of multi-CLEA were calculated using the following equation [26]:

$$\text{Recoveredactivity (\%)} = \frac{\text{Activity of CLEA}}{\text{Activity of free enzyme used for CLEA preparation}} \times 100\% \quad (1)$$

2.5. Preparation of multi-CLEAs

2.5.1. Selection of additives

To analyze the efficacy of additives on the activity of multi-CLEA, four different additives were tested. The four additives were; Bovine Serum Albumin (BSA) (50 mg) as a co-feeder, 20 mg of Sodium Dodecyl Sulfate (SDS), *n*-Heptane (50% v/v) for interfacial activation and oleic acid (150 mg) for bioimprinting. Wherein, the precipitant and the cross-linker values were fixed at ammonium sulfate = 50% (w/v) and glutaraldehyde = 60 mM. To prepare multi CLEA, 1 mL of crude enzyme was used and the reaction was carried out for 17 h at room temperature, with continuous stirring