Chapter

Prospects of Biocatalyst Purification Enroute Fermentation Processes

Michael Bamitale Osho and Sarafadeen Olateju Kareem

Abstract

Biotransformation of broth through fermentation process suffers a major setback when it comes to disintegration of organic substrates by microbial agents for industrial applications. These biocatalysts are in crude/dilute form hence needs to be purified to remove colloidal particles and enzymatic impurities thus enhancing maximum activity. Several contractual procedures of concentrating dilute enzymes and proteins had been reported. Such inorganic materials include ammonium sulphate precipitation; salting, synthetic polyacrylic acid; carboxy-methyl cellulose, tannic acid, edible gum and some organic solvents as precipitants etc. The emergence of organic absorbents such as sodom apple (*Calostropis procera*) extract, activated charcoal and imarsil had resulted in making significant impact in industrial circle. Various concentrations of these organic extracts have been used as purifying agents on different types of enzyme vis: lipase, amylase, protease, cellulase etc. Purification fold and stability of the enzyme crude form attained unprecedented results.

Keywords: Purification, Enzyme, Stability, *Calostropis procera* extract, Activated charcoal, Imarsil

1. Introduction

The fermentation process involves actual growth of the microorganism and product formation under agitation and aeration, optimum environmental conditions to provide uniform and adequate oxygen to the cell for growth and survival. A fermentation process is a biochemical process and, therefore, has requirements of sterility and use of cellular enzymatic reactions instead of chemical reactions aided by inorganic catalysts, sometimes operating at elevated temperature and pressure. Indigenous fermentation process draws the type attention of food scientists for taking the strategies of food security [1]. Microorganisms in fermented foods play major roles in health sector such as production of antimicrobial compounds, antioxidant, and probiotics properties.

The significance of fermentation of different substrates has gained attention in the beginning of the nineteenth century with profound resultant effects. Some products were produced via fermentation viz.; acetone, glycerol, lactic acid, butanol and baker's yeasts. Due to urgent need to treat the World War II army fighters, several metabolites such as amino acids, antibiotics and vitamins were produced

via fermentation. Submerged fermentation with larger volumes under aerobic conditions with moderate process control was established during this period. In subsequent years, the fermentation industry has seen constant improvement with leaps and bounds on the production of high-value metabolites, including various antibiotics and growth hormones, using sophisticated bioreactors [2, 3].

Purification of enzyme is often a multifarious process and a number of procedures are normally employed in succession to obtain adequately high purity state. The idea is to use less expensive but simple methods at nascent stages when the volume is large and more expensive and advanced techniques when the volume is relatively small [4]. The aims are to obtain high final degree of purity; enhanced enzyme activity and reproducibility of the products. It obvious that extraction procedures release a number of other cell components like other enzymes, proteins, polysaccharides and nucleic acids apart from the target enzyme into the medium, this often resulted into increasing the viscosity of the solution depending on their polymeric structure. It is of great significance as to receive knowledge about functional and structural properties of the substance and to predict its applications.

2. Enzyme biosynthesis

Biocatalysis can be defined as the utilization of living materials or molecules to speed up the rate of chemical reactions. The usage of recombinant enzymes, groups of enzymes, naturally occurring enzymes, cell extracts and whole cells, modified or engineered enzymes inclusive. These biological materials have consequential edge over conventional chemical catalysis. Ideally, biochemical reactions occur in aqueous solution, at moderate temperatures and atmospheric pressure which can result in both environmental and economic values as compared with the existing processes at upraised temperatures and pressures, and in organic medium [5]. High cost of enzymes and potential environmental damages resulting from high temperatures and pressures requires substantial energy inputs. Comparatively little known aspect of biocatalysis is the role of protein potency compare to the overall average structure of a protein, mobility/potency is much difficult to estimate experimentally. Like other molecules, proteins are in sustained motion, with stretching, and rotating bonds bending. These motions can bring about to much greater, for example conformational rearrangements following substrate binding or the movement of two domains relative to one another. It is now clear that these motions play important in catalysis and the regulation of enzyme activity [6]. Moreover, recognizing those motions which come up with catalysis will be experimentally challenging. Likewise, modeling them precisely enough to predict their effects in novel arrangement will be tasking.

As important hydrolytic enzymes, amylase and protease represent the two largest groups of industrial enzymes and account for approximately 85% of total enzyme sales all over the globe. At present, more than 3000 different enzymes have been characterized and many of them found their way into biotechnological and industrial applications [7]. One technicality in enzyme technology especially those of starch biosynthetic enzymes is their manipulations to meet the enormous demand of teeming population, safeguard our environment from non-degradable biopolymer and of course satisfy the food industries' need [8]. Some commercially available biocatalysts do not resist industrial processing conditions due to severity of such conditions. Therefore, certain desirable characteristics during isolation and screening of novel enzymes viz.; alkaline stability, halophilicity, and thermostability are foremost to meet the industrial demand. Great deal of attention has been drawn on extremophiles, which are the valuable source of

novel biocatalysts [9]. Extracellular enzymes from these halophiles with polymer-degrading ability at low water activity are of significant in many task processes where concentrated salt solutions hinder enzymatic production. The potency of enzymes to maintain its activeness via organic solvents has attracted considerable interest over the past twenty years.

In contrast to in water, numerous advantages of using enzymes in organic solvents or aqueous solutions containing organic solvents have been observed. Generally, enzymes are easily denatured and their activities disappear in the presence of organic solvents. Therefore, enzymes that remain stable in the presence of organic solvents might be useful for biotechnological applications in which such solvents are used [10]. Because salt reduces water activity, a feature in common with organic solvent systems, halophilic enzymes are thought to be valuable tools as biocatalysts in other low-water-activity environments, such as in aqueous/organic and non-aqueous media.

2.1 Purification protocols and applications

There are considerable ways of maintaining enzyme efficiency. Purification technique is a very powerful device has been arduously used to make some economically attainable and high performance enzymes with improved stability [11]. Enzyme purification is imperative for a full apprehension of the description and established process of enzymes. This is usually a multistep process involving biomass separation, concentration, primary isolation, and purification [12]. The contractual methods for the removal of enzymatic debrises or impurities and colloidal particles from fermentation broth include ammonium sulphate precipitation which encompassing dialysis for almost 16 hours before product could be recovered and also results into protein denaturation due to conformational changes [13].

Large volume of industrial enzymes are usually not purified. Their recovery is often accomplished by an ultrafiltration step. During enzyme production, desired products are synthesis after several concentration and separation techniques known as downstream processing (DSP). Two factors (time and cost) are the major challenges confronting these conventional techniques and their sustainability and efficiency depend on precise choice of purification methods [14, 15]. Here are some examples of strategies undertaken to improve the performance of enzymes with applications in food industry. Wong et al. [16] investigated strategies employed in starch liquefaction with targeted improvement of thermostability using α-amylase, protein engineering through site-directed mutagenesis and mutant displayed increased half-life between 15 min and 70 min at 100°C evolved. Glucoamylase with specific role as starch saccharifier and targeted improvement of substrate specificity, thermostability and pH optimum was characterized with protein engineering through site-directed mutagenesis alone [17]. Xylose (glucose) isomerase displayed isomerization/epimerization of hexoses, pentoses and tetroses as significance role of pH-activity profile with targeted improvement which resulted in protein engineering through directed evolution and the yield number on D-glucose in wild type was sustained between pH 6.0 and 7.5 and improvably at pH 7.3 as compared with mutant strains enhanced by 30–40%.

The application of polyvinyl alcohol or carbowax for protein and enzyme concentration is being restricted by poor water holding capacity. Moreover, gel filtration technique is also considered arduous and costly to the developing nations [18]. Carboxy-methyl cellulose, tannic acid, edible gum and some organic solvents as precipitants also poses the problem of product recovery [13]. In fishing industry, the use of fast, simple and low cost techniques such as using organic solvents vis ethanol and acetone, successive stages of centrifugation and filtration; and

saline solution (ammonium sulfate) were adopted for the separation and partial purification of protein biomolecules obtained from fish by-products beneficiation remains [19–21], aiming to improve the degree of biomolecule purity [21].
The use of ammonium sulphate precipitates during enzyme purification needs protracted separation technique between 12 and 16 h for recovery of product that frequently bring about protein denaturation as gel chromatography is high priced and moderate for developing economies [22]. Application of chromatographic techniques such as gel filtration and ion exchange give rise to purer enzyme fractions, with significant increase in specific activity. These are often used to estimate the molecular mass of the enzyme by comparing protein mixtures of known molecular mass (reference standards) with the unknown.

Pectinase enzyme was precipitated by dissolving it in a 0.1 M, pH 4.2 sodium acetate buffer after mixing with 3 volumes of ice-cold acetone and allowed to stay for 15 min [23]. Chimbekujwo et al. [24] reported the application of SDS-PAGE analysis of purified fungal protease of major protein band with molecular weight of 68 KDa, 13.3 fold and 28% yield. This partially purified enzyme was stable between 30 and 40°C temperature and pH 4–6 which enhanced the activity by Tween-20 and Calcium ions. Moreover, during the production, characterization and anti-cancer application of extracellular L-glutaminase from the marine bacterial isolate, the enzyme was purified through QFF technique by engaging ethanol precipitation and ion-exchange chromatography and resulted into 2-fold purification with molecular weight 54.8 kDa, specific activity 89.78 U/mg, maximum enzyme activity at 40°C and pH 8.2 and ultimately retained 90% activity for an hour [25].

3. Adoption of organic absorbent materials for purification procedures

The emergence of organic materials such as activated charcoal, *Calostropis procera* latex and imarsil has made significant contributions for industrial applications. Some of these organic absorbents are discussed below:

3.1 Activated charcoal and other carbon particles as purifying agents

Activated charcoal is an adsorbent extensively utilized in the treatment of wastewater and industrial contaminants by reason of its high shifting ability and adaptability for a wide range of pollutants. It is produced from any crucially carbonaceous materials. Coal, cotton waste, tree barks, palm kernel shell, and many agricultural by- products can be made to produce activated carbon and their capacity to remove colors has been investigated. Ferreira and coworkers [26] demonstrated the production, characterization of activated charcoal from castor seed cake through activation with phosphoric acid. Treatment of fino sherry wine with activated charcoal, in combination with other clarifying agents, produces a wine with lower polyphenolic content, good organoleptic characteristics, but its receptive to browning is indistinguishable to that recognized in untreated wine, despite starting from lower levels of color potency. Activated charcoal is used to remove compounds that cause objectionable color, odor and taste in water treatment while its industrial applications require elimination of harmful gases and pesticides and including purification of organic compounds [27]. It is established that 80% of activated charcoal globally produced is used in aqueous-phase adsorption of both organic and inorganic compounds [28]. However, the application of activated charcoal in the decolorization of enzyme-converted glucose syrup had been described; though its application for the purification of microbial biocatalysts has been sparse.

One profound advantage of activated charcoal over conventional purification systems is this swift enzyme purification from composite fermentation broth mixture at a very high purification fold. These conventional procedures of purification of enzyme among others include solvent precipitation; gel filtration and salting out technique. From an industrial application stand point of view, they are quite expensive base on the fact that they are associated with some difficulty of scaling up and plugging leading to viscous and particulate materials when treated with crude enzyme extracts. Additionally, it is not economical for developing nations as the materials disposal or enzyme recovery techniques employed in the separation method might escalate the expenses. As a result, the usage of activated charcoal has been considered as preferred option for enzyme purification method.

In addition to the inexpensiveness of activated charcoal, their efficient surface absorption attributes can be exploited for depolarization of fermented medium for efficacious and efficient recovery and purification of industrial enzymes making the downstream processing in large-scale industrial bioprocesses less economical [29]. López et al. [30] in their investigation on the use of activated charcoal in combination with other fining agents as clarifying agents reported that these carbonized materials acted upon the phenolic compounds thus encouraging their precipitation. In the field of enology, many different substances have been employed as fining agents over time such addition of antioxidants (ascorbic acid, sulfur dioxide and bottling under inert atmosphere. The use of bentonite has been well-proven and reported to have a remarkable effect on the protein content of wine and also hastens the precipitation of the thermolabile protein [31] but has also minimized the polyphenolic content of the wine during production [32].

The structure of activated carbon which is based on the graphite lattice corresponds to a non-graphitizable carbon and macromolecular structure of the precursor residues during heat treatment, and losses small molecules by developing and degradation some cross-linking, so that joining cannot occur. Therefore, cross-linking bring about a fixed design with small vigor, thus producing a permeable system and intercepting the ordering expected during graphitization.

3.2 *Calotropis procera* as purifying agent and its industrial applications

Calotropis procera belongs to the family Asclepidaceae being a native of tropical and subtropical region of Africa, the Middle East, and South and South-East Asia [33]. It is a shrub that produces latex with wide pharmacological profile which is a rich source of biologically active compounds [34]. *C. procera* latex contain several chemical compounds which include calotropagenin glycosides/derivatives [35]; saponins, flavonoids and cardenolides [36, 37]; cardioids such as calotoxin, calotropin, uscherin, uscchardin, choline, o-pyrocatechuric acid, glycoside calotropaginin, benzoyllineolone, benzoylisoloneolane, syriogenis and uzariganin etc. [38]. It has been traditionally used for various medicinal purposes such as treatment of animal worms, defense role in plants, acting against herbivorous insects, nematodes and phytopathogenic fungi [39]. Different parts of roots, leaves, flowers and latex from the plant are used in several medicinal preparations [40]. It was also reported to exhibit potent analgesic and weak antipyretic activity in various experimental model, possess antioxidant and anti-hyperglycemic property [41], antihelmintic activity [42, 43]; insecticidal and antifungal proteins and their enzymatic profiles have been characterized [44–46]; and there is an empirical association between antioxidant property and residual peroxidase activity. The milk weed has been established to be efficacious in the chemotherapy of malaria, menorrhagia, fever, leprosy and snake bites. Research works investigated on many biological activities of *C. procera* including osmotin proteins exert antifungal activity [47] and

anti-inflammatory potential in rats. *C. procera* latex dispensed to rats revealed pain-killing effects wound healing and toxic [48].

The leaf of *Calotropis procera* is a natural coagulant used traditionally in waste water treatment and it has also been reported that *Calotropis procera* leaf is effective in removal of environmental pollutant, polyphenolic crystal violet dye from aqueous solution of textile effluent [49] which presumed to be ascribed to the presence of peroxidase in *Calotropis procera* leaf that oxidized phenols to phenoxy radicals. Some studies suggest that the insoluble fraction of *C. procera* latex is associated with the noxious effects of this fluid [50]. Contrariwise, some constituents of this fluid cause toxicity in small ruminants [51].

From the investigation carried out by Mafulul *et al.,* [52] in the extraction, partial purification and characterization of peroxidase from *Calotropis procera* leaves, it was revealed that peroxidise purified from *Calotropis procera* leaves in primary purification procedures resulted in 1.613-fold purification of peroxidase from the crude extract. Subsequently, enzyme precipitation using ammonium sulphate with the dialyzed fraction showed 2.04 purification folds. *Calotropis procera* leaves peroxidase maintained above 50% over a temperature range of 20–70 with optimum temperature 50°C.

Furthermore, considering the availability and abundance of *Calotropis procera* fresh leaves in Nigerian distribution coupled with availability of advance purification method, this plant tends to provide a very cheap source of peroxidase for phenolic pollutants' bioremediation for waste treatment especially in oil spill region of Niger Delta. It provides potential alternative peroxidase that can compete with commercially available peroxidases for biotechnological applications.

3.3 Imarsil - an inexpensive synthetic chromatographic absorbent

Imarsil is a novel, inexpensive synthetic chromatographic absorbent and oxidized natural polymer of *Brachystegia nigerica. B. nigerica* is a legume used especially in the eastern states of Nigeria as condiment to thicken soup. Its thickening characteristics have been attributed to the presence of hydrocolloid property or gelling property [53]. Imarsil possesses quick and simple recovery approach more importantly in the clarification of microbial biocatalyst from fermentation broth [11]. Cherry and Fidants, [54] demonstrated the use of carboxy-methyl cellulose, edible gum and tannic acid as precipitants and as well as organic solvents also poses the problem of product recovery. Gel filtration technique is also considered assiduous and expensive in the developing countries.

Several procedures of concentrating protein and dilute enzyme from fermentation cell extracts and media using agricultural residues as coagulants. Furthermore, Kareem *et al.* [55] investigated the use of Imarsil and activated charcoal to purify crude lipase in a two-step purification fold which brought about an increase in specific activity from 5.29 to 20.8 Umg^{-1} with protein reduction of 18.24% in the supernatant and ultimate 3.93-fold purification. The study on crude amylase purification showed that a 40-fold purification was attained with 50% final yield of the total fungi amylase in a 3-step purification technique. The elution pattern of *Rhizopus oligosporus* SK5 amylase on Sephadex G-100 column had peaks at fractions (19–22) and (34–38). This purification fold value is conceived greater than values obtained in previous work [56].

Osho et al. [57] studied on production and optimization of bacterial cellulase using agricultural cellulosic biomass by solid state cultivation where the enzyme was clarified with Whatman No 1 filter paper, partially purified with Imarsil (1% w/v) and incubated at 4°C for 3 h. It was reported that at temperature ranges of 40–90°C, enzyme activity increases in crude and partially clarified states as the relative

activity also increased to 50 and 60°C for both forms of cellulase respectively. A decline in activity was noticed as temperature increases for both solutions. However, 90% activity of the partially purified enzyme was retained between 50 and 55°C and activity peaked at 60°C. Partial clarification of enzyme is therefore needed to enhance their stability even at much temperature. Kareem and other coworkers [58] outlined that partial clarification of enzyme using activated charcoal preceding gel filtration will established a high purification fold thus preventing some awkwardness of plugging and scaling up when treating crude extracts that sometimes contain particulate and viscous materials. These studies have further substantiated the use of Imarsil as a coagulating-flocculating agent in purification of crude enzyme extracts.

4. Conclusion

It has been proven that enzymes could be recovered from the fermentation broth by these organic absorbents and flocculating materials, making fermentation procedure less laborious. Following elution process, a highly concentrated and purified enzyme would be obtained at reasonable time. This technique seems to be rapid, cheap and promising in downstream processing of industrial enzymes which leads to an aqueous enzyme concentration. They are also established to be faster and easier to implement than the two or three-step processes of conventional precipitation, dialysis and subsequent chromatography. Thus, these natural coagulating-flocculating materials are of great importance in that they are effective in removal of pollutants and debris from fermented broths without necessarily affects the functional and structural formation of industrial enzymes.

Conflict of interest

The authors declare no conflict of interest.

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