Enzyme and Microbial Technology 64–65 (2014) 17–23

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

New insights into the effectiveness of alpha-amylase enzyme presentation on the *Bacillus subtilis* spore surface by adsorption and covalent immobilization

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ARTICLE INFO

Article history:
Received 30 June 2013
Received in revised form 20 May 2014
Accepted 27 May 2014
Available online 30 June 2014

Keywords:
Bacillus licheniformis ZK01
Alpha-amylase
Bacillus subtilis
Covalent immobilization
Surface adsorption
Spore

ABSTRACT

Most of the studies in the field of enzyme immobilization have sought to develop a simple, efficient and cost-effective immobilization system. In this study, probiotic *Bacillus* spores were used as a matrix for enzyme immobilization, because of their inherent resistance to extreme temperatures, UV irradiation, solvents and drying. Above all, their preparation is cost-effective. The alpha-amylase enzyme was immobilized on the spore surface by the covalent and adsorption methods. For the covalent method, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*hydroxysulfosuccinimide (NHS) were used. The maximum concentration of the alpha-amylase immobilized by the two methods onto the spore surface was 360 μg/1.2×10^{11} spore. However, maximum activity was achieved at an enzyme concentration of approximately 60 μg/$.4 \times 10^{10}$, corresponding to an estimated activity of 8×10^3 IU mg^{-1}/1.2×10^{11} spore for covalent immobilization and 8.53×10^3 for the adsorption method. After washing the enzyme with 1 M NaCl and 0.5% Triton X-100, the enzyme immobilization yield was estimated to be 77% and 20.07% for the covalent and adsorption methods, respectively. The alpha-amylase immobilized by both methods, displayed improved activity in the basic pH range. The optimum pH for the free enzyme was 5 while it shifted to 8 for the immobilized enzyme. The optimum temperatures for the free and immobilized enzymes were 60 °C and 80 °C, respectively. The covalently-immobilized alpha-amylase retained 65% of its initial activity, even after 10 times of recycling. The K_m and V_{max} values were determined by the GraphPad Prism software, which showed that the V_{max} value decreased moderately after immobilization. This is the first study which reports the covalent immobilization of an enzyme on the spore surface.

1. Introduction

Enzymes are biocatalysts that have evolved over millions of years acquiring attributes that enable very specific reactions to take place within living cells and organisms. *In vivo*, they are highly specific (modifying just one substrate among a collection of similar ones), chemo/enantio/region-selective (yielding just one substrate among several possible ones) and very active under extremely mild environmental conditions (atmospheric pressure, room temperature, aqueous medium). Thus, enzymes have been considered the idle catalysts from an environmental point of view, in reactions involving complex or labile compounds [1].

In recent times, the demand for industrial enzymes has been on the rise because of their economical and environmental benefits [2–4]. However, despite the broad use of enzymes, their advantages are constrained by a number of practical problems including, the high cost of isolation and purification, their sensitivity to harsh environmental conditions which can limit their operational lifetime, and the difficulty in recovering them from the reaction mixture in the active form, for subsequent reuse and reprocessing [5,6]. Enzyme immobilization is the most successful and effective way to overcome such constraints. In general, using immobilized enzymes in industrial processes is more advantageous over the free form as they can facilitate the separation of reactants and products, allowing easy recovery of the enzyme, enabling enzyme

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http://dx.doi.org/10.1016/j.enzmictec.2014.05.006
0141-0229/

reutilization, increasing the stability of the enzyme that will ultimately require fewer enzymes and reduce costs of the process. Additionally, immobilization, if properly designed, will increases enzyme selectivity and reduces inhibition by the medium or products [7–10].

Alpha-amylase (E.C.3.2.1.1) is a highly important enzyme used in an extensive array of industrial applications such as food processing, fermentation, production of paper, textiles, and detergents [11]. It is a ubiquitous protein that is produced by a variety of sources including plants, animals and microorganisms [2]. There are many reports about the immobilization of amylase on many different types of solid support, such as glass beads [12], polymeric microspheres [13], UV-curable polymer [14], functionalized silica [15] and zirconia [16]. *Bacillus subtilis* spores have certain properties which make them suitable candidates for immobilization of enzymes, especially those used in the food industry. Spores are extremely stable and resistant life forms generated by Gram-positive bacteria, such as *Bacillus* and *Clostridium.* They may be considered as an almost fully reproducible multifunctional nonporous supports, with a moderate to large nanosize. Typically mature spores are 0.8–1.2 μm in length and are either spherical or ellipsoidal in shape. The spore core is surrounded by different layers of peptidoglycan and lipid membrane but the most important spore structures are spore coat proteins. In *Bacillus subtilis* spore contains almost 25 different coat proteins which can be divided into two distinct coat layers, the inner and outer coat. The outer layer, which is the electron-dense layer of the *B. subtilis* coat is made of five main polypeptides including, CotA (65 kDa), CotB (59 kDa), CotG (24 kDa), CotC (11 kDa) and CotF (8 kDa). [17]. Spores can resist to harsh environmental conditions including UV irradiation, extreme temperatures, exposure to lytic enzymes and toxic chemicals, and other harsh conditions. Moreover, their production is simple and economically beneficial [18].

There is a popular Japanese food called “natto” which is made by fermentation of soybeans using *B. subtilis* var natto. Some European countries, such as the UK and Italy, have approved the use of *B. subtilis* as a food supplement. Previous studies have shown that *B. subtilis* can grow within the gastrointestinal tract (GI) and has a cycle of germination and sporulation. It is capable of eliciting an immune response in both mouse and humans. In fact, the *B. subtilis* spore is compatible with the GI tract, and the alpha-amylase enzyme immobilized onto the spore surface is more stable than the free enzyme. Therefore, such stability and toleration of the sterilization conditions can be applied to the production of certain foods. Accordingly, such a combination involving immobilization of the enzyme onto the spore surface can be considered as highly beneficial for the food industry [19,20]. The display of enzymes on the spore surface using genetic methods has certain advantages that include the relief of enzyme and spore purification, and also the procedure for attachment. There are many reports of protein display on spore surfaces, such as those for recombinant proteins [19], heterologous antigens [20], streptavidin [21] and beta-galactosidase [22]. However, expression in this system is sometimes very low rendering it unsuitable for industrial applications. The non-genetic approach for immobilization is a good way to overcome challenges and difficulties met by the genetic method. Spore is a “heterofunctional support” which has different functional groups on its surface that are able to interact with the groups present on an enzyme surface under different circumstances. Based on spore surface characteristics (anion rich [23], hydrophobic surface, and functional groups), the enzyme can be immobilized by different methods of adsorption and covalent attachment. Meanwhile, these multifunctional supports are suitable for immobilization of multimeric enzymes or multi subunit protein and enzymes [24,25]. In the case of physical adsorption, the enzymes are attached to the matrix through ion exchange, which is a multipoint process involving hydrogen bonding, van der Waals forces, and hydrophobic interactions. There have been a number of studies that have reported the use of spores for protein immobilization by the non-genetic method of adsorption. Huang et al. [26] used *B. subtilis* spores to immobilize and deliver the tetanus toxin by the adsorption method, ultimately introducing this system as a vaccine delivery vehicle. Immobilization of beta-galactosidase from *Alicyclobacillus acidocaldarius* onto the surface of wild type and mutant *B. subtilis* spores was carried out with altered surface layers [27]. Sirec and colleagues (2012) showed that the adsorption process was more complicated than previously suggested by Huang (2010), and that electrostatic forces were not the major factors determining the adhesion of the beta-galactosidase, thus requiring further studies. Although there are hundreds of immobilization protocols, but designing a new approach that may permit the improvement of enzyme properties and decrease the costs during the immobilization process is still an exciting goal [28]. To date, there have been a few reports of enzyme immobilization onto the spore surface using the adsorption procedure [27,29]. However, immobilization of enzymes onto spore surfaces by cross-linking and covalent attachment has not yet been demonstrated. In the current investigation, a model immobilization strategy was used for the first time. This involved immobilization of the *B. licheniformis* alpha-amylase enzyme onto the spore surface of *B. subtilis* by the covalent method. The efficiencies of alpha-amylase immobilization by the covalent attachment and adsorption methods were compared with each other and that of the free enzyme. The optimum pH and temperature profiles, and the reusability of the immobilized enzyme using these two methods, as compared to the free enzyme, were also studied.

Table 1
List of oligonucleotides.

Name	Sequence(5′-3′)	Restriction site
Amy F	GGGGCATATGGCAAATCTTAATGGGACG	*Nde*I
Amy R	GGGGCTCGAGTCTTTGAACATAAATTGAAACC	*Xho*I

2. Materials and methods

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHS) were purchased from Merck (Germany). The Ni-NTA agarose beads were purchased from Qiagen (USA). Starch, maltose, 3,5-dinitrosalicylic acid (DNSA), and bovine serum albumin (BSA) were all obtained from Sigma–Aldrich (USA). Deionized (DI) water was obtained from a Millipore Milli-Q Water System (Millipore Inc.), and was subsequently used for rinsing and preparing all aqueous solutions.

2.2. Methods

2.2.1. DNA manipulation

The alpha-amylase gene of *B. licheniformis* was amplified by PCR using chromosomal DNA as a template and the oligonucleotides, amyF and amyR, as primers (Table 1). The resulting 1200 bp PCR product was digested with enzymes, *Nde*I and *Xho*I, and cloned into the commercial vector pET26. The resulting plasmids were verified by restriction analysis. The recombinant plasmids were then used to transform the *Escherichia coli* TOP10 strain, so as to propagate the plasmids. The bacterial strains that were used in this research are listed in Table 2.

2.2.2. Alpha-amylase cloning, expression and purification

The recombinant plasmids were extracted from the cells and used to transform the *E. coli* BL21 strain, and the resulting recombinant strain was induced to overproduce alpha-amylase by the addition of IPTG (0.25 mM). A 55 kDa protein was visualized on a Coomassie blue-stained gel and then purified on Ni-NTA agarose beads followed by dialysis with a 12 kDa cut off.

2.2.3. Polyclonal antibody production

Polyclonal antibody was generated in rabbit immunized by the subcutaneous route using 20–30 μg/ml of purified alpha-amylase on days 1, 10, 20 and 30. On day 44, total blood was collected and sera were tested against a cell-extracted solution of *B. licheniformis* and purified alpha-amylase. Serum was diluted 1:4000 for Western blot analysis.