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Immobilization of *Streptomyces clavuligerus* on loofah sponge for the production of clavulanic acid

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Abstract

Clavulanic acid, a naturally occurring powerful inhibitor of bacterial β-lactamases, is produced by *Streptomyces clavuligerus*. The high void volume, permeability, and low cost of fibrous matrices prompted the use of *Luffa cylindrica* as a matrix for the immobilization of *S. clavuligerus* for the production of clavulanic acid. Immobilization of *S. clavuligerus* onto loofah sponge discs was studied with respect to the optimization of the inoculum size (number of discs) and its reusability for clavulanic acid production. Best yield of 1125 μg ml^{-1} clavulanic acid was reached with two discs of loofah sponge (each approximately 0.136 g dry weight) and 120 h duration in the first cycle. Data obtained during four reusable cycles showed reduction in the initiation time of clavulanic acid production, resulting in higher levels of clavulanic acid in shorter time duration. Immobilization of *S. clavuligerus* on to loofah sponge discs, therefore, permit repeated reuse under the specified fermentation conditions for clavulanic acid production.

Keywords: Clavulanic acid; *S. clavuligerus*; Immobilization; Loofah

1. Introduction

All natural antibiotics are almost universal secondary metabolites, generally produced at low growth rates or after growth has ceased. These are non-essential for growth of the producing organisms in pure culture, and typically possess unusual structures not found in primary metabolites of central metabolism. It is difficult to produce antibiotics continuously during prolonged cultivation of microbial cells. Antibiotic cultivation has been performed in batch system, but immobilized cells are useful for continuous/semi-continuous production of antibiotics. The production of antibiotics such as penicillin, chlortetracycline (Mahmoud and Rehm, 1987), theinamycin (Arcuri et al., 1986), nikkomycin and tylosin (Veelken and Pape, 1982), and actinomycin D (Dalili, 1988), cephamycin C (Devi and Sridhar, 2000) has been studied with immobilized cells.

Immobilization of the microbial biomass in the polymeric gel matrices is most extensively studied (Leenen et al., 1996; Arica et al., 2001). However, production of large amount of gel beads needed for its commercial application is expensive and also requires specialized equipment. Furthermore, the use of such polymeric matrices results in a closed structure with restrictive diffusion and low mechanical strength (Hu and Reeves, 1997).

In the practical utilization of living cells entrapped in alginate gel, diffusion of essential nutrients, oxygen transfer, physical and chemical properties of the gel and immobilization procedure are the important factors affecting microbial metabolism and the efficiency of the system. Although immobilized cells have received a lot of attention, it is not possible to make a general statement about the behavior of microorganisms in alginate. Literature results are not uniform, but vary according to the type of microorganism, of immobilizing matrix and of productive system.

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Calcium alginate gels are apt to disrupt by mechanical stress and are gradually solubilized in the presence of calcium-ion-trapping reagents, such as phosphate ion and citrate.

Devi and Sridhar (2000) reported on the production of cephamycin C in repeated batch operations from immobilized *Streptomyces clavuligerus* with two different supports – via sponge and 4% alginate; and cephamycin C production in repeated batch operations were estimated. A gradual increase was observed in cephamycin C production from 48–96 h during the fermentation. Sponge immobilized cells supported higher production than the alginate immobilized cells in repeated batch operations.

Freeman and Aharonowitz (2004) reported immobilization of *S. clavuligerus* cells in crosslinked, prepolymerized, linear polyacrylamide gels for antibiotic production. Cells were suspended in a solution of preformed, linear, water-soluble polyacrylamide chains, partially substituted with acylhydrazide groups. The prepolymerized backbone polymer was crosslinked, in the presence of viable cells, by stoichiometric amounts of dialdehydes such as glyoxal, glutardialdehyde, and periodate-oxidized polyvinyl alcohol. The crosslinking reaction, carried out in cold, neutral physiological conditions resulted in cells entrapped in gels with physical properties similar to those of the common polyacrylamide gels. However, cell damage generally caused by the acrylamide monomer was avoided. Resting *S. clavuligerus* cells possessing a high capacity for antibiotic production, entrapped as per this procedure produced cephalosporins continuously for 96 h with yields similar to those of free resting cells.

A matrix for immobilization should ideally be strong, resistant to operating conditions, and preferably have an open structure. The plant-derived loofah (*Luffa cylindrica* L.) sponge is an inexpensive and easily available biological, and therefore, renewable, matrix produced in most of the tropical and subtropical countries. The sponge is made up of interconnecting voids with an open network of fibrous support giving the potential for rapid contact of immobilized cells with the surrounding aqueous medium. Merits of the loofah biomatrix include freedom from materials that might be toxic to microbial cells, simple application and operation technique, and a high stability during long-term repeated use. The high void volume, permeability, and low cost of fibrous matrices make it particularly attractive. The simplicity of the immobilization technique, the strong binding and the low cost of the loofah sponge can help to find future applications for whole cells immobilization for various applications. Recently, loofah sponge has been evaluated for the production of fungal biomass immobilized loofah sponge discs (FIBLS) for removal of heavy metal ions in chlorinated compounds from aqueous solutions (Iqbal et al., 2005). Saeed and Iqbal (2006) have reported the use of loofah sponge for immobilization of *Synechococcus* sp. isolated from wastewater as a biosorbent of cadmium from the aqueous solutions. Akhtar et al. (2003) demonstrated the use of microalgal-loofah sponge immobilized disc as a new efficient biosorbent for the removal of Ni(II) from aqueous solution. Bazaraa et al. (1998) reported on bioreactor for continuous synthesis of compactin by *Penicillium cyclopium* fungal spores, immobilized onto-into loofah sponge.

To the best of our knowledge there are no reports on immobilization of *S. clavuligerus* on loofah sponge for clavulanic acid production. The objective of the present work was to produce fungal biomass immobilized loofah sponge discs to ascertain semi-continuous method for production of clavulanic acid by *S. clavuligerus*.

2. Methods

2.1. Media components

Glucose, sucrose, glycerol, yeast extract, peptone, malt extract, agar, calcium chloride, magnesium sulphate, sodium chloride, zinc chloride, manganese chloride, glutamic acid, ferric chloride, potassium dihydrogen phosphate, L-arginine, were purchased from Hi-Media Limited, Mumbai. HPLC solvents were purchased from SD Fine Chemicals Ltd., Mumbai.

2.2. Maintenance of the microbial culture and fermentative production of clavulanic acid

Strain of *Streptomyces clavuligerus* MTCC 1142 was procured from MTCC, Chandigarh, India. Clavulanic acid production was carried out in two stages. In the first stage, cells were grown in the seed culture medium; and in the second stage seed culture was inoculated into the fermentation medium for clavulanic acid production. *S. clavuligerus* MTCC 1142 was maintained on slants of a defined medium containing 0.4% yeast extract, 1% malt extract, 0.4% glucose and 2% agar with a pH adjusted to 7.2 $\pm$ 0.2. The slants grown at 25 °C for four days were used for inoculation into a seed culture medium (2% glycerol, 1% bacteriological peptone, and 1% malt extract with pH adjusted to 7.0 $\pm$ 0.2). For the preliminary studies, 2% of seed culture grown for 96 h in an incubator shaker at 25 °C and at 200 rpm was used for inoculation into the production medium.

2.3. Fermentative production of clavulanic acid

Composition of the production medium used in the present study was optimized in our laboratory (Saudagar and Singhal, 2006; Saudagar and Singhal, 2007) and is summarized as follows, 15.0 g l^{-1} glycerol, 20.0 g l^{-1} sucrose, 1.7 g l^{-1} arginine, 16.8 g l^{-1} glutamic acid, 0.4 g l^{-1} calcium chloride, 0.1 g l^{-1} ferric chloride, 2.0 g l^{-1} potassium dihydrogen phosphate, 5.0 g l^{-1} sodium chloride, 0.1 g l^{-1} manganese chloride, 0.05 g l^{-1} zinc chloride and 1.0 g l^{-1} magnesium sulphate with a pH adjusted to 7.0 $\pm$ 0.2. The pH was maintained every 6 h by addition of 1 M HCl/1 M NaOH under sterile condition. Fermentation runs