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Ultrasound assisted production of a fibrinolytic enzyme in a bioreactor

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ABSTRACT

The present work illustrates ultrasound assisted production of a fibrinolytic enzyme at 1 L bioreactor scale from *Bacillus sphaericus* MTCC 3672. To alleviate the shortcomings of one factor at a time method of optimization, central composite rotatable design of response surface methodology was employed for optimization of ultrasound assisted production. Different process parameters such as irradiation time, duty cycle and power of ultrasound were varied in 3 different levels in 11 experimental runs. For evaluating mass transfer enhancement effect of ultrasonication on production, control non sonicated fermentation was optimized by varying different agitation speed (300–500 rpm) and aeration rate (8.33–33.33 cc/s). Optimized ultrasonication protocol resulted in 1.48-fold increase in fibrinolytic enzyme yield as compared to non sonicated fermentation, which comprised of ultrasound irradiation at 25 kHz for 10 min with 40% duty cycle and 160 W power on 12 h of growth phase in 1 L bioreactor operated at 400 rpm agitation speed and 16.66 cc/s aeration rate. Declined glucose concentration from 0.1% w/v (non sonicated control run) to 0.05% w/v and breakage of cells cluster emphasized on increased substrate utilization potential and enhanced convection of ultrasound assisted fermentation in a bioreactor. Deliverables of current studies will provide significant insights for enhancement of productivity of various enzymes at a bioreactor level.

1. Introduction

Fibrinolytic enzyme is a promising therapy for treatment of cardiovascular diseases. *Bacillus* being the well-known producer of potent fibrinolytic enzyme was explored at industrial levels. *Bacillus sphaericus*, a mosquito larvicidal toxin producing bacteria has shown potent fibrinolytic activity in extracellular extract [1]. Based on this hypothesis, potent directly acting plasmin type fibrinolytic enzyme was isolated from *B. sphaericus* MTCC 3672 [2]. But, lower productivity at shake flask level is a prime concern and attracts attention of researchers for process intensification [3]. Agitation is the only mass transfer phenomenon used in a shake flask culture and found to be inefficient for higher production yield. Genetic manipulation, *in situ* product removal, introduction of inducer and inhibitors, ultrasound stimulation, and extractive fermentation are noteworthy technologies, recently being explored for maximum production of protease enzyme [4,5].

Ultrasound irradiation is recognized as a most promising tool for process intensification approach benefiting various processes such as adsorption, extraction, hazardous dye degradation, bioconversion, three phase partitioning of enzyme and fermentation [6–8]. Ultrasound irradiation to fermentation broth has significantly improved the production of various therapeutic enzymes such as thrombinase, lovastatin and laccase [9–11]. In ultrasonication, spontaneous bubble generation, bubble growth and collapse of bubbles impart vicious shock wave and mechanical shear to surrounding liquid medium [12]. In fermentation intensification by ultrasonic irradiation, vicious shock of ultrasonication breaks the clusters of cell, increases the porosity of lump and enhances the mass transfer across the bacterial cell with acceleration in substrate uptake by cells. Fermentative production of fibrinolytic enzyme from *B. sphaericus* MTCC 3672 was enhanced by ultrasonic irradiation by 1.82-fold compared to non sonicated shake flask culture [5].

At an industrial scale, enzyme production profile is mainly influenced by agitation speed, aeration rates and viscosity of fermentation medium. Agitation and aeration serves as a source of mass transfer enhancement in aerobic fermentation. Earlier, Chisti et al., had reported 3.5-fold increase in ethanol production from *Kluyveromyces marxianus* (ATCC 46537) by ultrasound stimulation in 7.5 L bioreactor [13]. Along with agitation and aeration, ultrasound has imparted synergistic enhancement in substrate uptake and stimulates metabolic pathway boosting enzyme productivity. Precise mechanism illustrating enhanced productivity of microbial enzyme by ultrasonic stimulus is not well understood; however

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some assumptions have postulated its use for process intensification such as, reduction in mass transfer hindrances, increases in porosity, mechanical shear.

Process optimization by one factor at a time method was found to be inefficient to determine the mutual interaction of process parameters. Hence in recent era, many bioprocesses are optimized by various Design of Experiment (DoE) approaches such as Response Surface Methodology (RSM), Taguchi, Plackett–Burman design and artificial neural network (ANN). Out of above stated DoE approaches, RSM is widely used due to its simplicity and easy interpretation of mutual interaction between the independent variable and responses. RSM comprises of experimental design, model building, and evaluation of different process parameters and its interpretation of mutual interaction with response [14]. To the best of our knowledge, this is the first study on ultrasound assisted production optimization of fibrinolytic enzyme from *B. sphaericus* MTCC 3672 in 1 L bioreactor by response surface methodology.

Hence, necessity of understanding of the production profile at various agitation and aeration combinations and ultrasound stimulated mass transfer enhancement contributes towards setting up objectives for the present study. At various combinations of agitation speed (300–500 rpm) and aeration rate (8.33–33.33 cc/s), production profiles of fibrinolytic enzyme were determined. For further mass transfer enhancement, effect of ultrasound irradiation was evaluated and various process parameters were optimized by central composite rotatable design of response surface methodology. 3 D counter plots were generated to understand mutual interaction between the operating parameters of ultrasonication and response.

2. Materials and methods

2.1. Chemical and reagents

All analytical grade chemicals and media components were purchased from S.D. Fine-Chem Ltd., Mumbai, India and Hi-Media Ltd., Mumbai, India, respectively. Azoalbumin was purchased from Sigma Aldrich, India.

2.2. Microorganism and its maintenance

B. sphaericus MTCC 3672 was obtained from Microbial Type Culture Collection and Gene bank, Chandigarh, India and was maintained on agar slants of medium containing (g/L) peptone, 5; NaCl, 15; beef extract, 1; yeast extract, 2 and agar 20; (pH 7.0) at 37 °C for 1 day and then stored at 4 °C.

2.3. Fermentation medium and inoculum preparation

Optimized fermentation media (% w/v) was composed of glucose, 1.25; NaCl, 0.5; yeast extract, 1.5; $MgCl_2$, 0.0203; $CaCl_2$, 0.01; $MnCl_2$, 0.001; pH, 8.0 [3]. Seed culture was prepared by incubating *B. sphaericus* MTCC 3672 cells in 50 mL of above stated medium in 250 mL Erlenmeyer flask for 6 h and then used as an inoculum for 800 mL of culture media in bioreactor study.

2.4. Bioreactor set up

The bioreactor used was constructed of glass (capacity 1 L) with working capacity of 0.8 L and height to diameter ratio of 1.77 as shown in Fig. 1. The dimensions of bioreactor are as follows: internal diameter 9 cm; height 16 cm; number of baffles 3, baffle height 13 cm; baffle width 0.5 cm; impeller was positioned 3 cm above from the bottom of tank. Six blade pitched turbine downflow impeller was used having 3 cm diameter while maintaining ratio of the diameter of the impeller to the diameter of tank at 0.333. The lid of bioreactor was constructed with five different size ports. The central bigger port was used for impeller attachment; impeller was driven by overhead motor. The other four ports were used for air inlet and outlet, sampling tube and DO probe respectively. Silicon oil was used as an antifoam agent. Air was sparged inside bioreactor with air compressor equipped with air filter (PTFE-0.22 μm) and gas flow rates were measured with the help of precalibrated rotameter. For higher mass transfer of oxygen in bioreactor, sparger tube end was made narrower (diameter 0.05 mm) to generate smaller air bubble. Bioreactor was hermetically sealed and samples were withdrawn after 4 h interval through sampling tube assembly. Dissolved oxygen concentration of fermentation broth was measured with DO probe (galvanic type, Oxygen

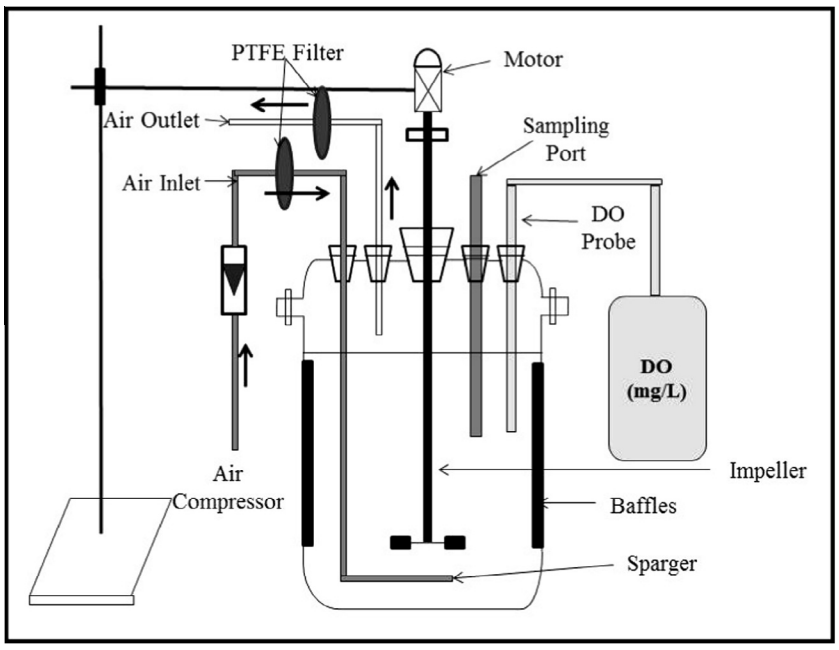

Fig. 1. Schematic of bioreactor used for fermentation of fibrinolytic enzyme from *Bacillus sphaericus* MTCC 3672 (Capacity 1 L).