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Optimization of the aeration and agitation speed of *Aeribacillus palidus* 418 exopolysaccharide production and the emulsifying properties of the product

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ABSTRACT

The specific properties of exopolysaccharides (EPS) from thermophilic microorganisms have attracted interest in their optimized production. In this study, the ability of *Aeribacillus pallidus* 418 to grow and produce polysaccharide in a 5-l stirred tank bioreactor was investigated. Agitation rates of 100, 200, 600, 900, and 1100 revolutions per minute (rpm), at an air flow rate of 0.5 gas volumes per unit medium volume per minute (vvm), and aeration rates of 0.25, 0.5, 1.0, and 1.5 vvm, at an agitation rate of 900 rpm, were examined. A maximum EPS yield of 170 μg/ml has been registered in a single impeller bioreactor equipped with an original Narcissus impeller at agitation speed of 900 rpm, with an aeration rate of 0.5 vvm. The bioprocess oxygen uptake rate (OUR) and oxygen mass transfer coefficient (K_La) were evaluated. The emulsifying properties of the specific EPS produced by *A. pallidus* 418 were determined. Stable oil-in-water emulsions, a low level of separated water phase and high dispersion stability were found, which together demonstrate the prospects for the industrial exploration of EPS production. Enhanced synergism between the *A. pallidus* 418 synthesized EPS and various commercially used hydrocolloids was observed; superior synergy was achieved in combination with xanthan gum.

1. Introduction

The unusual conditions in thermophilic niches result in the synthesis of compounds with unusual properties by the resident thermophilic organisms. Whereas the enhanced resistance of enzymes to high temperature, detergents and solvents is well studied, the unique properties of exopolysaccharides (EPSs) synthesized by thermophiles are not yet well known due to the comparatively low number of isolated thermophilic producers [1]. In general, these thermophilic processes show comparatively low EPS yields and correspondingly high production costs that could limit their application in value-added products. Due to the sometimes-limited cell growth in bioreactors and failure to release metabolites, the capacity for growth and production in such conditions should be examined [2].

In aerobic bioprocesses, oxygen is a key substrate that needs to be continuously supplied [3]. Oxygen affects the life cycle of aerobic microorganisms by inducing or repressing several enzyme systems of primary or secondary metabolism, thus activating the oxidative reactions for nutrient utilization and energy generation [4]. Product formation could be increased by optimizing the agitation and aeration rates. Both variables are crucial in influencing the availability of nutrient and dissolved oxygen and controlling the rate of metabolite release from the cells [5]. Due to the elevated temperatures of thermophilic fermentations reducing the solubility of gas in the medium, oxygen transfer is often the rate-limiting step for aerobic bioprocesses. The size of gas bubbles and their dispersion throughout the reactor volume are critical for culture performance. The smaller the bubble size, the larger the surface area for gas contact, which can improve the oxygen transfer rate [6]. Extensive literature on these issues is available, and a considerable portion has been published in recent years [7]. However, the optimization of agitation and aeration conditions in thermophilic processes for EPS production has not been investigated.

Despite the relatively high cost of thermophilic EPS production, there are several advantages: (1) the high growth rates at elevated temperature allow for short fermentation processes; (2) the

Abbreviations: EPS, exopolysaccharide; rpm, revolutions per minute; vvm, gas volume flow per unit volume medium per minute; OUR, oxygen uptake rate; K_La, oxygen mass transfer coefficient; SE, standard error; T, translucency index.

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viscosity of culture liquids containing synthesized EPSs is lower, which suggests lower energy consumption and better mass transfer; (3) the synthesized products are non-pathogenic as a rule; (4) the EPSs synthesized by thermophilic bacteria are expected to maintain their properties at high temperatures, which is desirable for any polymer solution; (5) thermophilic EPSs form stable oil/water emulsions; and (6) their high molecular weight and unusual structures enhance the industrial interest in their production.

The exploration of EPSs as emulsifying and stabilizing agents has been justified by their prospective applications as replacements for synthetic emulsifiers in the food and cosmetic industries. There are few reports on the emulsifying properties of some high molecular weight bioemulsifiers produced by bacteria [8,9]. Among the thermophilic producers, a highly stable bioemulsifier isolated from *Aeribacillus pallidus* YM-1 with emulsifying properties promising for biotechnology applications was recently observed by Zheng et al. [10]. The monosaccharide composition, chain structural conformation and molecular weights of EPSs could influence their interaction at the oil/water boundary of emulsions in different ways. Due to the variety of polysaccharide properties and their ability to show synergistic effects, a combination of two or more polysaccharides is often used in commercial products. Several authors have reported effective synergism between different EPSs [11–13].

The medium, temperature, and pH have been optimized in flask cultures for EPS production by *A. pallidus* 418. The presence of two different EPSs, electroneutral EPS 1 and negatively charged EPS 2, both containing mannose as the main sugar, was identified in the EPS fraction [14]. Their composition did not change following cultivation in different media or with various shaker conditions. Both EPSs showed high molecular weight and thermostability [14]. The present study objectives were to analyze the effects of agitation and aeration on the production of EPS by *A. pallidus* 418 in a bench-top reactor and to analyze the specific EPS emulsifying and stabilizing properties.

2. Materials and methods

2.1. Microorganism

A strain 418, producing an exopolysaccharide was isolated from one of Rupi Basin hot springs located in South-West Bulgaria (latitude: 41.4 N, longitude: 23.25 E). The temperature of water was 55 °C and pH of 7.8. Based on its phylogenetic and phenotypic characteristics the strain was identified as belonging to the species *A. pallidus* [14].

2.2. Experimental setup and operation mode

A 5-l single impeller jacketed glass reactor (LF-2, ČSAV, Vývojove dilny, Prague, Czech Republic) of internal diameter 0.15 m was employed. A hydrofoil impeller Narcissus of diameter 0.05 m was used. The temperature was maintained at 55 °C by an internal cartridge heater. Precise temperature control was achieved with a solid-state thermistor regulator providing a control accuracy of ±0.2 °C. Foaming was regulated by the addition of Antifoam 204 (Sigma–Aldrich). Dissolved oxygen concentration (Ingold) and pH probes were used. The reactor was filled with 2.4 l of culture medium and then sterilized by autoclaving at 121 °C for 20 min. The medium was inoculated with 3.5% (v/v) of an 18-h culture under aseptic conditions. The inoculum was prepared in 100-ml Erlenmeyer flasks in a similar medium. The bioreactor was operated in batch mode for 24 h with constant temperature and pH for the different experiments performed. The pH value was maintained at 7.0 due to the buffer properties of the medium. The effect of agitation on growth and EPS production was studied at 100, 200, 600, 900, and 1100 rpm. The effect of aeration rate was studied at 0.25, 0.5, 1.0, and 1.5 vvm, with 900 rpm agitation providing the highest EPS yield. The dissolved oxygen level (%) was measured in all experiments. Samples were taken at 2-h time intervals. Following centrifugation, the EPS in the supernatant solutions was analyzed.

2.3. Measurement of growth and EPS production

Culture growth was determined by measuring samples' turbidity at 660 nm. One unit OD was established to correspond to 1.05 mg/ml dry *A. pallidus* 418 cells.

The EPS in the supernatant was recovered by centrifuging the cells in a stationary phase of growth (at 4000 × *g* for 10 min). The supernatant was treated with an equal volume of cold absolute ethanol added dropwise under stirring in an ice bath, which was held at −18 °C overnight and then centrifuged at 13,000 × *g* for 30 min. The pellets were washed twice in ethanol, dissolved in hot water, dialyzed against distilled water and lyophilized. The samples were tested for carbohydrate, protein, nucleic acid and ash contents. The carbohydrate content was determined using the phenol/sulfuric acid method with glucose as standard [15]. Protein concentration was determined by the Bradford test using bovine serum albumin (BSA) as standard. The nucleic acid content of the samples was determined by the absorbance of 260-nm UV light. All analyses were performed in triplicate, and an average value is presented as the result.

2.4. Measurement of oxygen uptake rate (OUR) and mass transfer coefficient (K_La)

The OUR and K_La were determined by the unsteady stop-gassing measurement technique [7]. An Ingold oxygen probe was used, and the dynamic measurement was conducted in line with the probe dynamics [16]. The OUR was determined from the depletion of the dissolved oxygen concentration ($(dC_0/dt)_r$) following the interruption of the air flow and the occurrence of net respiration (r). The OUR was calculated from the equation:

$$\left(\frac{dC_0}{dt}\right)_r = \text{OUR} \quad (1)$$

At air flow restart with known OUR, K_La was determined from the overall oxygen balance of the absorption stage (a) following the integration of the equation:

$$\left(\frac{dC_0}{dt}\right)_a = K_La(C_0^* - C_0) - \text{OUR} \quad (2)$$

where $(dC_0/dt)_a$ is the accumulation of oxygen in the liquid phase and C_0^* is its equilibrium value.

2.5. Investigation of emulsifying properties

The emulsifying properties of EPSs from *A. pallidus* 418 were investigated according to the method described by Kuncheva et al. [17]. Commercially available sunflower oil was the preferred oil. First, the EPS was dissolved in the water phase at 45–50 °C in a vessel equipped with a magnetic stirrer. Then, the oil was added (1:1), and homogenization and emulsification were performed by using an IKA ULTRA-TURRAX T 1 (IKA, Germany) disperser at 3600 rpm. The emulsifying capacity of the synthesized biopolymer fraction was studied at concentration steps of 0.5% EPS in water starting from 0.5% to 2.5% (w/v). The quality of the emulsions was determined by measuring the light penetration, which was recorded as the percent translucency index (T, %) by using a Camspec M 107 (Camspec Analytical Instruments, Camspec, UK) spectrophotometer at wavelength 540 nm. The emulsion stability was evaluated by measuring the amount of residual oil and water phases separated from the emulsion following centrifugation at 3000 × *g* (Hettich EBA 20, Germany) for 20 min.

The synergistic effect was investigated by adding xanthan gum, guar gum, cellulose gum, or Na-alginate (FLUKA, Switzerland) at concentration 0.5% (w/v). Concentration (in %) refers to the hydrocolloid content in the water phase, further mixed with the oil phase (1:1).

2.6. Statistical analysis

All analyses were performed in triplicate, and the average values are presented. The standard error (SE) calculated from EPS and biomass data deviation were 8% and 5%, respectively. The curves reflecting the changes of dissolved oxygen concentration, as a result of the experimental stopping of the gas fluid were integrated by parallel approximations at various time intervals along the two sections of the curves, i.e., the respiration and the absorption one. As a result, the deviations' SE was found to be 15% for OUR and 5% for K_La.

3. Results and discussion

3.1. The effect of agitation on growth and EPS production

In stirred bioreactors, several variables affect mixing and mass transfer; these variables include the type and number of stirrers, the stirrer speed, and the gas flow rate used [7]. A number of impellers have been reported to ensure the uniform distribution of substrates and high heat and mass transfer rates in polysaccharide-producing broths [18]. A moderate-shear single impeller Narcissus was used in this study. In fermentation media with large biopolymer molecules, the impeller is comparable to the conventional six flat-blade