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Process development of eicosapentaenoic acid production

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ABSTRACT

Eicosapentaenoic acid (EPA), a well-known member of omega-3 fatty acids, is considered to have a significant health promoting role in the human body. It is an essential fatty acid as the human body lacks the ability to produce it in vivo and must be supplemented through diet. Microbial EPA represents a potential commercial source. GC/MS analyses confirmed that bacterial isolate 717, similar to *Shewanella pacifica* on the basis of 16S rRNA sequencing, is a potential high EPA producer. Two types of bioreactors, a Stirred Tank Reactor (STR) and an Oscillatory Baffled Reactor (OBR), were investigated in order to choose the optimum system for EPA production. The EPA production media was optimised through the selection of media components in a Plackett–Burman (PB) design of experiment followed by a Central Composite Design (CCD) to optimise the concentration of medium components identified as significant in the Plackett–Burman experiment. The growth conditions for the bioreactor, using artificial sea water (ASW) medium, were optimised by applying Response Surface Methodology (RSM). This optimisation strategy resulted in an increase in EPA from 33 mg/l (10 mg/g biomass), representing 8% of the total fatty acids at shake flask level, to 350 mg/l (46 mg/g biomass) representing 25% of the total fatty acids at bioreactor level. During this study the main effects and the interactions between the bioreactor growth conditions were revealed and a polynomial model of EPA production was generated. Chemostat experiments were performed to test the effect of growth rate and temperature on EPA production.

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1. Introduction

Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid (PUFA) with 20 carbon atoms and five double bonds. As the first double bond appears on the third carbon atom from the methyl end it is an omega-3 fatty acid. Industrial production of EPA has gained more attention recently due to the proven clinical importance of EPA in reducing the risk of cardiovascular diseases, lowering of plasma cholesterol and decreasing the incidence of breast, colon and pancreatic cancers, in addition it plays an important role in controlling various biological processes as it is a precursor for a number of vital eicosanoid signalling compounds [1,2].

As humans lack the ability to produce EPA in vivo, the main EPA source is through dietary supplements [3]. Although fish oil is the main commercial source of EPA as a dietary supplement, there are many limitations on its wider usage such as high purification cost, complex composition, potential heavy metal contamination and unacceptable odour. Recently, fish oil was found to interfere with chemotherapy causing cancer cells to become less sensitive to such treatments [4], due to the presence of 12-oxo-5, 8, 10-heptadecatrienoic acid and hexadeca-4, 7, 10, 13-tetraenoic acid which, even in minute quantities, induces resistance to a wide spectrum of chemotherapeutic agents. As a result, microbial EPA may be a promising alternative [5,6].

EPA plays a critical role in the bacterial membrane, especially at low temperatures, as it maintains fluidity of the membrane in extreme cold environments [7], and is essential for cell division and membrane organisation [8]. In addition to its role at low temperature, EPA also plays a role as an antioxidant by protecting the cell from oxygen free radicals [9] and in facilitating the transport of hydrophilic and hydrophobic compounds across the bacterial membrane [10].

PUFA production was studied, one factor at a time (OFAT), in *Shewanella* sp. GA-22 by [11] demonstrating that it is carbon-temperature dependant. Different carbon sources (crude oil, gasoline, glucose, glycerol pyruvate n-tetradecane and Tween) were used as sole carbon source in the media and showed marked influences on PUFA production. In addition, temperature also showed a significant effect on PUFA production with at least a two-fold increase from 2% (w/v) at 20 °C to 5% of the total fatty acids at 4 °C. Corn steep liquor and marine industrial waste liquid were

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used as a carbon source for a marine bacteria identified as *Shewanella putrefaciens*, to reduce the overall fermentation costs [12], achieving 200 mg of EPA per litre of broth.

The key controlling factor in PUFA synthesis is the temperature. Temperature, as the sole tested variable, significantly affected PUFA production in bacteria with maximum productivity achieved between 5 and 25 °C with no production reported at temperatures higher than 25 °C [13,14]. The same effect of the temperature was observed, in an OFAT approach, in *Shewanella olleyana*, decreasing the temperature from 24 °C to 4 °C doubled the production of EPA from 10.2% to 23.6% of the total fatty acids [15]).

The main aim of this work was to develop a bioprocess for industrial production of EPA through two approaches: a) defining the composition of an optimal production medium, and b) optimising the growth conditions at a bioreactor scale. A statistical design of experiments was undertaken to achieve this target using a Plackett–Burman (PB) design as an initial screening tool followed by a Central Composite Design (CCD) for optimisation. The statistical designs enabled the assessment of the statistical significance of the main effect of each factor and the interactions among them as well as the development of a predictive model for numerical optimisation of the process conditions.

Given the reported antioxidant properties of EPA, the effective dissolved oxygen distribution throughout the bioreactor is potentially an important factor in large scale EPA production. Two bioreactor types with different mass transfer abilities were investigated: the stirred tank reactor (STR) is traditionally used in bioprocessing, whilst the oscillatory baffled reactor (OBR) was reported to provide a higher oxygen transfer rate with k_La values reaching an average of 75% above those achieved in STR for yeast fermentation processes [16].

2. Materials and methods

2.1. Cultivation conditions and strain identification

Five different deep sea core sediment and fluff samples were collected from the Mid Atlantic ridge by research personnel at the Dove Marine laboratory, Newcastle University and kindly provided for this research. After initial screening for EPA production, isolate 717 was identified as the highest EPA producer and was selected for optimisation. A loopful of cell biomass of this isolate, incubated on Bacto Marine Agar (DIFCO 2216) at 20 °C for 48 h, was transferred to a 250 ml sterile flask containing 50 ml of marine broth and incubated at 20 °C in an orbital shaking incubator at 160 rpm for two days. The culture was collected into a 50 ml sterile Falcon tube and centrifuged at 6000 rpm at 4 °C for 15 min. The cell pellets were then transferred into 2 ml sterile centrifuge tubes with 30% glycerol and stored at −20 °C for subsequent use.

Genomic DNA was extracted and 16S rRNA was amplified using primers 27f and 1592r [17] by PCR, purified and sequenced using standard methods [18]. The resultant almost complete sequence was blasted against the Genbank database to identify the closest type strains and aligned against sequences in the genus *Shewanella* retrieved from the GenBank and RDP databases using Clustal W in MEGA 3.1 [19]. The aligned sequences were used to construct a phylogenetic tree based upon Jukes and Cantor distances and the neighbour joining algorithm in MEGA 3.1 [19].

2.2. Seed culture in artificial sea water

A loopful of biomass from culture plates was transferred into a 250 ml flask containing 50 ml of artificial sea water (peptone 3.5 g/l; yeast extract 3.5 g/l; NaCl 23 g/l; $MgCl_2$ 5.08 g/l; $MgSO_4$ 6.16 g/l; $Fe_2(SO_4)_3$ 0.03 g/l; $CaCl_2$ 1.47 g/l; KCl 0.75 g/l; Na_2HPO_4 0.89 g/l; NH_4Cl 5.0 g/l) [20] and grown at 20 °C in an orbital shaker incubator at 160 rpm for 24 h.

2.3. Growth in production media

Growth was performed in 250 ml sterile flasks with 50 ml of medium at the given temperature for two days in an orbital shaker incubator at 160 rpm. The basal medium used was 1 g/l yeast extract, 10 g/l NaCl, 6 g/l $MgSO_4$ and 0.75 g/l KCl, while for the CCD experiments the same basal media excluding yeast extract was used. Final biomass from each flask was collected into a 50 ml Falcon tube and centrifuged at 6000 rpm for 15 min at 4 °C. The cell pellets were transferred into a 1.5 ml screw tube and freeze-dried overnight.

2.4. Fatty acid methyl ester (FAME) preparation

20 mg of freeze dried cells were suspended in 2 ml of 5% methanolic HCl and heated at 70 °C for 2 h in sealed tubes. Fatty acid methyl esters were extracted from the cells with 0.6 ml hexane and then dried under nitrogen gas [21].

2.5. FAME profiling

The single point internal standard method was used for the determination of EPA concentration. Methyl nonadecanoate (≥99.5% GC capillary purity, Sigma–Fluka) was used as an internal standard.

Gas chromatography (GC) with flame ionisation detector (FID) on a Hewlett-Packard 5890 series 2 chromatograph, with a SGE forte-BPX70 column; 30 m × 0.25 μm film thickness from SGE Analytical Science LTD with helium as a carrier gas was used for FAME profiling .The GC temperature was held at 210 °C for 30 min.

GC–MS analysis was performed on an Agilent 7890A GC in split mode, injector at 280 °C linked to an Agilent 5975C MSD with electron voltage 70 eV, source temperature 230 °C, quad temperature 150 °C, and multiplier voltage 1800 V, interface temperature 310 °C, controlled by HP Compaq computer using Chemstation software. The sample (1 μl) in hexane was injected using HP7683B auto sampler with the split open. Separation was performed on an Agilent fused silica capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm dimethyl poly-siloxane (HP-5) phase. The GC was temperature programmed from 30 to 130 °C at 5 °C/min then to 300 °C at 20 °C/min and held at the final temperature for 5 min with Helium as the carrier gas (flow rate of 1 ml/min, initial pressure of 50 kPa, split at 10 ml/min).

2.6. Bioreactor cultivations

Two bioreactor types were investigated in order to determine the optimum for EPA production. The Stirred Tank Reactor (STR) was an Applikon Biotechnology autoclavable 2 L Rushton turbine bioreactor with power number P_o 6, impeller diameter D_i 0.045 m and reactor vessel diameter D_R 0.105 m. The Oscillatory Baffled Reactor (OBR) was a custom made tall cylindrical glass column, 0.024 m in diameter and 1 m in length with a total volume of 1 L. Orifice plate baffles, 0.001 m thick each, connected by stainless steel rods and arranged periodically 0.036 m apart were inserted into the entire length of the column. The incubation temperature was 20 °C for both types of cultivation.

The comparison between the two bioreactor types was carried out by introducing the same power densities (250 W/m^3) in both reactors for the same process and comparing their performance in terms of biomass and product concentrations. The power densities were calculated as follows:

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