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Response surface methodology for optimising the culture conditions for eicosapentaenoic acid production by marine bacteria

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Abstract Polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA), are increasingly attracting scientific attention owing to their significant health-promoting role in the human body. However, the human body lacks the ability to produce them in vivo. The limitations associated with the current sources of ω-3 fatty acids from animal and plant sources have led to increased interest in microbial production. Bacterial isolate 717 was identified as a potential high EPA producer. As an important step in the process development of the microbial PUFA production, the culture conditions at the bioreactor scale were optimised for the isolate 717 using a response surface methodology exploring the significant effect of temperature, pH and dissolved oxygen and the interaction between them on the EPA production. This optimisation strategy led to a significant increase in the amount of EPA produced by the isolate under investigation, where the amount of EPA increased from 9 mg/g biomass (33 mg/l representing 7.6 % of the total fatty acids) to 45 mg/g (350 mg/l representing 25 % of the total fatty acids). To avoid additional costs associated with extreme cooling at large scale, a temperature shock experiment was carried out reducing the overall cooling time from the whole cultivation process to 4 h only prior to harvest. The ability of the organism to produce EPA under the complete absence of oxygen was tested revealing that oxygen is not critically required for the biosynthesis of EPA but the production improved in the presence of oxygen. The stability of the produced oil and the complete absence of heavy metals in the bacterial biomass are considered as an additional benefit of bacterial EPA compared to other sources of PUFA. To our knowledge this is the first report of a bacterial isolate producing EPA with such high yields making the large-scale manufacture much more economically viable.

Keywords Polyunsaturated fatty acids (PUFAs) · Eicosapentaenoic acid (EPA) · Response surface methodology · Bioreactor

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Introduction

Eicosapentaenoic acid (EPA) accumulates within the bacterial membrane and plays a critical role in the adaptation of psychrophilic and piezophilic bacteria to the extreme low temperatures and high pressures in their environment. A high percentage of EPA in the total fatty acids is required to sustain the fluidity of their membrane under such conditions [1, 2]. Disruption of the EPA gene cluster led to the conversion of *Shewanella livingstonensis* from a cold-resistant organism to a cold-sensitive mutant [3].

EPA protects bacterial cells not only against extreme cold environments but also against high pressure stress, explaining the abundance of EPA among psychrophilic and piezophilic bacteria. *Shewanella violacea* DSS12 is an EPA producer that can grow at low temperature and relatively high optimum pressure (30 MPa). However, its

EPA-less mutant has an unstable and disordered membrane compared to the wild type, over a wide range of pressures. These results suggest that EPA preserves the bacterial membrane stability when exposed to significant changes in pressure [4]. EPA was also found to protect the microbial membrane against oxidative damage [5, 6]. In addition to its protective functions, EPA was found to be critical in controlling various physiological processes including regulating the movement of hydrophobic and hydrophilic compounds across the membrane, cell division and membrane organisation [7, 8].

Recently EPA has attracted extensive attention because of its benefits to human health. The health benefits of EPA include reducing the incidence of breast, colon and pancreatic cancers [9]. In addition, by reducing plasma triacylglycerols, blood pressure, platelet aggregation, inflammation and improving vascular reactivity, EPA was found to decrease the possibility of death from cardiovascular events [10]. EPA has a protective function against atherosclerosis and this contributes to the low percentage of cardiovascular diseases in human populations with high fish utilisation [11, 12]. EPA is an essential fatty acid because of the inability of the human body to synthesise it either de novo or to the required levels from its precursor fatty acids (linoleic and α-linolenic acids) by de-saturation [13].

EPA is classed as a natural product because of the difficulty of producing stereospecific double bonds synthetically. Fish, such as mackerel, sardines, and herring, are basic sources for the commercial production of omega-3 fatty acids including EPA [14, 15]. However, the introduction of fish oil EPA in food for dietary and health purposes is complicated by the unpleasant smell and taste, in addition to the expensive purification steps required to separate EPA from the complex mixture of fatty acids and potential heavy metal contamination present in the fish oil. As a result, scientists are trying to find alternative ways of obtaining EPA to satisfy the increasing demand for omega-3 products, and microbial sources were identified as the most suitable potential sources [16].

The aim of this work is to develop a potential economically viable EPA production bioprocess using a marine bacterium by optimising growth conditions including pH, temperature and dissolved oxygen (DO) within the bioreactor.

As one-factor-at-a-time studies, the previous investigations on the effect of different culture conditions on EPA production explored only the main effect of such factors and ignored any interactions. In addition, previous work was performed at shake flask level without accurate monitoring or controlling of these factors over the production period.

Response surface methodology (RSM) was found to be effective in processes optimisation for many industrial products including paints and coatings, foods and beverages, and pharmaceuticals [17]. RSM is able to capture the main effect of each factor and the interactions between them. For example, RSM was applied to optimise the significant variables, including glucose, yeast extract, NaCl, pH and incubation time, affecting the docosahexaenoic acid (DHA) production by *Schizochytrium* sp. S31. The optimum combination of these factors led to a maximum DHA concentration of 516 mg/l [18]. Another *Schizochytrium* sp. was found to produce 13.8 g/l of DHA after performing RSM to optimise and test the effects of glucose, yeast extract, corn steep liquor and soy peptone [19].

Materials and methods

Strain and cultivation conditions

Bacterial isolate named 717, isolated from deep sea core sediment collected from the Mid-Atlantic Ridge by research personnel at the Dove Marine Laboratory, Newcastle University, was kindly provided for this research. This isolate was identified by the authors previously as *Shewanella* sp. 717 (Genbank accession no. JX203388). A loopful of 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 2 days. The culture was collected in a 50-ml sterile Falcon centrifuge tube and centrifuged at 6,000 rpm at 4 °C for 15 min. The cell pellets obtained were then transferred into 2-ml sterile centrifuge tubes with 30 % glycerol and stored at −20 °C for subsequent use.

Growth in production medium

Initial experiments were carried out in artificial sea water (ASW) typically used for the growth of marine bacteria [20] (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). Subsequently, the growth was performed in 250-ml sterile flasks with 50 ml of previously optimised production media (data not shown) at a temperature determined by the design of experiments for 2 days in an orbital shaker incubator at 160 rpm. The composition of the production medium was 6.5 g/l casein, 4.25 g/l Hy-Soy, 2.2 g/l Na_2HPO_4, 10 g/l NaCl, 5 g/l $MgSO_4$ and 1 g/l KCl. Final biomass from each flask was collected into a 50-ml Falcon centrifuge tube and centrifuged at 6,000 rpm for 15 min. The cell pellets were transferred into a 1.5-ml screw-cap tube and freeze-dried overnight.

Fatty acid methyl ester (FAME) preparations

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

FAME profiling and measurement

The fatty acid profile analysis was performed by growing the isolate in 50 ml of ASW at 20 °C in an orbital shaking incubator at 160 rpm for 24 h. Subsequent measurement of FAME concentration was carried out using cells grown in the optimised production media. In all cases the protocol below was followed.

Fatty acid methyl ester profiling was performed by gas chromatography (GC) with flame ionization detection (FID) on a Hewlett-Packard 5890 series 2 chromatograph equipped with an SGE forte-BPX70 column (30 m × 0.22 mm i.d., 0.25 μm film thickness; SGE Analytical Science) with helium as the carrier gas. The GC temperature was held at 210 °C for 30 min. Nonadecanoic acid (C19:0) (Sigma Aldrich, UK) was used as an internal standard and its peak area was used to determine the amount of each fatty acid based on the areas of all peaks and the known concentration of the standard added.

GC–MS analysis was performed on an Agilent 7890A GC system in split mode, injector at 280 °C, linked to an Agilent 5975C MSD operating at 70 eV, source temperature 230 °C, quad temperature 150 °C, multiplier voltage 1,800 V, interface temperature 310 °C, controlled by a HP Compaq computer using Chemstation software. The sample (1 μl) in hexane was injected using an HP7683B autosampler 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 polysiloxane (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 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).

Bioreactor cultivations

An Ez-Control Applicon bioreactor (working volume 2 l) was used to perform batch bioreactor cultivations using the production medium (see section on "Growth in production medium"). After sterilisation, the bioreactor was set up according to the manufacturer's instructions. Then, 100 ml of the seed culture, grown in the production medium at 20 °C in an orbital shaking incubator at 160 rpm for 24 h, was added to the vessel. Culture conditions were set up depending on the trial combination from the central composite design (CCD) matrix (Table 3). Samples were taken at 6-h intervals for 3 days and OD_{600} measurements were taken.

For the temperature shock, the cells were grown at DO 40 %, pH 7, as recommended by the CCD experiment, and 25 °C (above this temperature this isolate does not produce any EPA—data not shown) and at the mid exponential phase the temperature was reduced to 10 °C. Samples were taken at 6-h intervals over the entire cultivation (48 h) except during the induction process (reducing the temperature) when samples were taken at 30-min intervals.

For the anaerobic growth, the cultivation process was carried out at 10 °C and an agitation rate of 100 rpm. Samples were taken at 6-h intervals for 3 days and OD_{600} measurements were performed. The anaerobic conditions were maintained by sparging nitrogen through the vessel from the start of the cultivation at 2 l/min.

Response surface methodology (RSM)

A Box–Wilson CCD was used to estimate the optimum levels of each variable. The CCD matrix included five levels for each variable, six centre points and star points to estimate the curvature. Table 1 illustrates the levels of each variable under investigation.

A second-order polynomial model was developed to predict the optimum conditions for EPA production:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_{ii} + \sum \beta_{ij} X_{ij}$$

where β_i are the regression coefficients for each factor, β_{ii} are the regression coefficients for square effects and β_{ij} are the regression coefficients for interactions. Analysis of variance (ANOVA) was carried out using Design Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA).

Thermal stability of the bacterial oil

The total bacterial lipids were extracted using the method of Bligh and Dyer [22], where for each 1 ml of bacterial suspension 3.75 ml of a mixture of chloroform/methanol (1:2) was added and vortexed for 10–15 min. Then 1.25 ml of chloroform was added with mixing for 1 min followed by addition of 1.25 ml of 1 M NaCl. This is a modification from the original procedure where distilled water was used.

Table 1 Variables and their levels used for the CCD experiment

Variables	−2	−1	0	1	2
Dissolved oxygen (%)	10	20	40	60	70
pH	5	6	7	8	9
Temperature (°C)	10	15	20	25	30

The modification was introduced to block the binding of some acidic lipids to denatured proteins [23] with mixing for an extra minute before centrifugation. After centrifugation, two layers were obtained. The upper layer was discarded and the lower layer was collected with a Pasteur pipette. The lower layer (containing total lipids) was dried under nitrogen gas, and the lipid extract was re-dissolved in a known volume of hexane.

The thermal stability of the total fatty acids, including EPA, produced by isolate 717 was tested by thermogravimetric analysis (TGA). TGA is a routine analytical tool to study the thermal behaviour of different materials including oils [24]. The thermal stability of the oil was monitored as a function of mass loss with respect to temperature. The TGA studies were performed on a Pyris STA 6000 model under flowing helium at a constant rate of 30 ml/min at a temperature ramp rate of 10 °C/min from 30 to 1,000 °C. The TGA system consists of an electronic microbalance and a ceramic container containing a platinum crucible suspended in a furnace. The sample's initial mass, temperature and final mass loss were logged by a computerised control unit. Thermal stability of the bacterial oil was compared to that of fish oil from Menhaden (*Brevoortia tyrannus*) and fish liver oil from *Gadus morrhua* (Sigma Aldrich, UK), whereas sunflower oil was used as a reference.

Electron microscopy

The presence of heavy metals in isolate 717 biomass was examined using a scanning electron microscope (XL30 ESEM-FEG, Electron Microscopy Services, Newcastle University) which is equipped with an energy-dispersive X-ray spectrometer (EDAX®, RONTEC system with Quantax software) with a liquid nitrogen-cooled anti-contamination device in place at all times. The electron microscope was operated at 20 kV in low vacuum mode. The accumulation of heavy metals in the bacterial biomass was tested by growing the isolate in the bioreactor under the optimum conditions, collecting two different biomass samples by centrifugation, and scanning three different areas for each sample.

Results and discussion

FAME profile

The fatty acid profile of isolate 717 contains n-16:0 and n-16:1ω7 as dominant fatty acids (Table 2). Isolate 717 was confirmed as an EPA producer, in addition to producing DHA in trace amounts (<0.3 % of the total fatty acids).

Table 2 Fatty acid profile for isolate 717 when grown in ASW media at 20 °C for 48 h in a shake flask (see Sect. "Materials and methods")

Fatty acid	Percentage
n-13:0	2.5
n-14:0	3.7
n-15:0	1.1
n-16:0	27.9
n-16:1ω7c	37.1
n-17:0	5.8
n-17:1ω8	8.4
n-18:0	3.1
n-18:1ω9	1.9
n-20:5ω3	7.6
n-22:6ω3	0.3

The fatty acid profile lacks 12-oxo-5,8,10-heptadecatrienoic acid and hexadeca-4,7,10,13-tetraenoic acid fatty acids, which are present in fish oil and were found reduce the efficacy of chemotherapy in cancer patients [25].

Optimising the operating conditions

A set of 20 bioreactor cultivations with six centre points and five levels for each variable was carried out to explore a wide range of operating conditions and to test the interactions among them.

Optimising the bioreactor growth conditions was expected to significantly increase the amount of produced EPA, as previous reports indicated that the EPA biosynthesis, especially the percentage of EPA in the total fatty acids, is affected more by the environmental parameters than the nutrient composition and availability [26].

The matrix and the responses in terms of biomass, EPA yield (mg/g) and percentage of EPA in the total fatty acids are summarised in Table 3. These results confirm that EPA is not just a growth-related product but is significantly affected by the growth conditions independent of their effect on growth. For example, trial number 12 resulted in the maximum biomass but not the maximum EPA yield.

The results of ANOVA of the main effects of each factor, interactions among them and the quadratic effect are shown in Table 4. The model *F* values for each of the response variables and the model *P* values (Table 4) all imply that the models are significant with very low chance (0.05–5.2 %) that a model *F* value is large owing to noise.

At the 90 % level of confidence, any factor with a *P* value less than 0.1 can be considered as statistically significant. The smaller the *P* value, the higher the significance of the corresponding variable [27]. The *P* values of

each variable shown in Table 4 clearly show that temperature is the most significant growth factor affecting the growth and EPA production, whereas pH was found to have a significant effect on EPA yield only. Lee et al. [28] similarly reported that temperature and pH have the most significant effect on the EPA productivity of the isolate *Shewanella* sp. KMG427.

Dissolved oxygen (DO) also shows a significant effect on all the responses; this behaviour could be related to the antioxidant capability of EPA as discussed by Nishida et al. [5] for exogenous addition of H_2O_2.

Unlike one-factor-at-a-time experiments, statistically designed experiments are able to test the effect of the interactions among the factors in addition to the square terms which evaluate the quadratic effect of the variables. Table 4 indicates that the interaction between the temperature and DO has a significant effect on EPA yield, whereas the interaction between the DO and pH showed a significant effect on the biomass concentration. In addition the quadratic effect of pH had a significant impact on the amount of biomass and EPA production, whereas the quadratic effect of temperature was significant only in terms of the EPA yield.

The significant interaction between the temperature and the DO in relation to the EPA yield may be partially due to the fact that the solubility of oxygen in water significantly increases at lower temperatures [29], which may lead to a higher production of EPA as protection against the potential harmful effects of oxygen.

The effects of DO, the quadratic terms and the interaction between the investigated environmental factors upon the EPA production have not been previously explored in the literature. These can be modelled with a second-order polynomial (in coded units with *A* representing DO, *B* representing pH and *C* representing temperature):

Table 3 Matrix and responses for the CCD experiment (the levels for each variable are shown in Table 1)

Run	Variables			Responses		
	Dissolved oxygen	pH	Temperature	EPA yield (mg/g)	Dry weight (g/l)	EPA %
1	−1	−1	−1	40.8	6.9	12.2
2	0	0	0	32.1	4.5	14.5
3	1	−1	−1	7.4	2.5	16.3
4	0	0	0	33.7	4.4	15.4
5	0	0	0	32.2	4.9	14.7
6	2	0	0	18.2	0.8	20.6
7	0	0	0	31.7	4.8	13.8
8	0	0	2	0.0	0.4	0.0
9	0	2	0	13.3	1.5	10.4
10	−1	1	−1	41.3	5.3	16.1
11	1	−1	1	9.9	2.0	9.7
12	−2	0	0	21.4	9.1	2.9
13	1	1	1	13.8	5.2	12.3
14	1	1	−1	14.0	6.8	19.1
15	0	0	0	33.3	4.9	13.7
16	−1	−1	1	3.0	3.6	7.9
17	0	−2	0	1.6	0.2	1.5
18	0	0	−2	39.4	7.9	23.3
19	0	0	0	32.2	5.1	14.4
20	−1	1	1	16.9	3.1	11.8

Table 4 Analysis of variance (ANOVA) of the CCD experiment for the calculated responses at 90 % confidence level

Variable	EPA yield (mg/g)			Dry weight (g/l)			EPA %		
	Sum of squares	*F* value	*P* value Prob >*F*	Sum of squares	*F* value	*P* value Prob >*F*	Sum of squares	*F* value	*P* value Prob >*F*
A	167.22	4.44	0.0614*	21.46	6.26	0.0313*	147.85	8.70	0.0145*
B	136.26	3.62	0.0864*	6.59	1.92	0.1956	52.14	3.07	0.1103
C	834.78	22.15	0.0008*	26.49	7.73	0.0195*	296.89	17.48	0.0019*
AB	4.68	0.12	0.7319	11.75	3.43	0.0938*	1.50	0.09	0.7728
AC	568.88	15.10	0.0030*	2.81	0.82	0.3867	0.02	1.05E−03	0.9748
BC	9.50	0.25	0.6264	1.12E−03	3.26E−04	0.9860	0.01	8.01E−04	0.9780
A^2	89.50	2.37	0.1543	1.63	0.48	0.5060	12.60	0.74	0.4093
B^2	1081.56	28.70	0.0003*	15.77	4.60	0.0576*	65.22	3.84	0.0785*
C^2	267.46	7.10	0.0237*	0.037	0.01	0.9190	0.58	0.03	0.8574
Model *F* value	10.47			2.98			3.78		
Model *P* value	0.0005			0.0520			0.0251		

A dissolved oxygen, *B* pH, *C* temperature

* Statistically significant ($P < 0.1$)

$$\text{EPA yield}\,(\text{mg/g}) = 32.99 - 3.52A + 3.34B - 7.41C - 0.75AB + 8.21AC + 1.09BC - 1.97A^2 - 10.48B^2 - 3.23C^2$$

$$\text{Dry weight}\,(\text{g/l}) = 4.69 - 1.26A + 0.73B - 1.32C + 1.19AB + 0.58AC - 0.012BC + 0.27A^2 - 1.27B^2 - 0.038C^2$$

$$\text{EPA}\,\% = 15.30 + 3.31A + 2.06B - 4.42C - 0.42AB - 0.046AC - 0.041BC - 0.74A^2 - 2.57B^2 - 0.15C^2$$

The interactions between the factors can be visualised in 3D surface response graphs shown in Figs. 1 and 2 for interactions that were shown to have a statistically significant effect at least on one of the response variables (reflected by the *P* values shown in Table 4).

Figure 1 shows the optimum range of pH for all three responses at a neutral level (pH 7). The DO optimum range differs between the responses with the optimum DO for the biomass being relatively low (10–20 %), whereas for the EPA yield it was relatively high (30–45 %) and 45–65 % for percentage of EPA in the total fatty acids. High DO enhanced the biosynthesis of EPA, specifically the proportion of EPA in the total fatty acids. The high level of EPA at high DO may be due to enhanced biosynthesis of EPA as a response to high DO concentration in the surrounding environment.

In previously reported studies EPA was found to a have a protective role as an antioxidant, and an EPA-deficient mutant of the isolate *Shewanella marinintestina* IK-1 was found to be more sensitive to the exogenous addition of H_2O_2 and showed a remarkable decrease in the amount of cells recovered from the cultures treated with H_2O_2. In addition, protein carbonylation was enhanced only in EPA-deficient cells when they were treated with 0.01 mM H_2O_2 under bacteriostatic conditions. These results confirmed that EPA has a protective effect on the bacterial cells [5, 30] and appear to support the results obtained in this research.

The interaction between DO and temperature (Fig. 2) showed that the optimum range of DO was relatively low for high biomass but relatively high for EPA expressed either as yield or percentage of the total fatty acids.

The optimum temperature range for both biomass and EPA yield is between 13 and 16 °C, whereas for the EPA percentage the analysis indicates that the lower the temperature the higher the EPA percentage. This observation is in line with the theory suggesting that EPA is required to sustain the fluidity of the plasma membrane when the organism grows in extreme low temperature environments [31]. The isolate 717 completely loses the ability to produce EPA when incubated at 30 °C (trial number 8 with 40 % DO level maintained). The same observation was previously reported for different bacteria, as they were unable to produce EPA at temperatures above 25 °C [32]. EPA was also proven to play an important role in bacterial membrane organisation and cell division, especially at low temperature. The lack of EPA resulted in the cells of *Shewanella livingstonensis* Ac10 forming multi-nucleoid filaments leading to reduced growth [7].

Model validation

Using the mathematical models and the interaction 3D surface response graphs, we estimated the optimum conditions for each response variable individually (i.e. single-objective optimisation) as summarised in Table 5. Subsequently, three cultivations were carried out in triplicates under these conditions and the actual values of response variables were compared to the predicted ones (Table 5).

These results confirm that a neutral pH is optimal for both the growth and productivity of isolate 717. A relatively high DO and low temperature were desirable for the highest EPA yield, whereas a relatively low DO was desirable for the highest biomass concentration.

As the optimum ranges of the growth and the EPA productivity diverged, an additional multi-objective optimisation was carried out to maximise all the calculated responses simultaneously (Table 6). As expected, there was a slight reduction in each of the response variables compared to the cultivations performed at optimum conditions for the individual response variables. However, this reduction was only slight in the case of the EPA yield (44.2 mg/g compared to 45.2 mg/g, respectively), confirming a rather robust window of bioprocess operation for this response variable as indicated by the curvature of the response surfaces in Figs. 1 and 2.

Temperature shock

The maximum amount of EPA was obtained at the late exponential phase and the start of the stationary phase (data not shown). Low temperature proved to enhance the EPA production; thus in order to avoid intensive cooling requirements during the entire cultivation, an experiment was carried out to test the feasibility of a short-term temperature shock (Fig. 3).

The highest amount of EPA was obtained 4 h after reaching the new temperature set point. Applying this operating policy will limit the requirement of intensive cooling to around 4 h prior to harvesting and still result in a 40 mg/g EPA yield representing 22 % of the total fatty acids compared to 44.22 (±3) mg/g EPA and 22.5 (±0.9) % EPA when applying the low temperature over the whole fermentation process (12.7 °C).

Production under anaerobic conditions

To test the ability of the isolate to grow and produce EPA under totally anaerobic conditions, a batch cultivation was

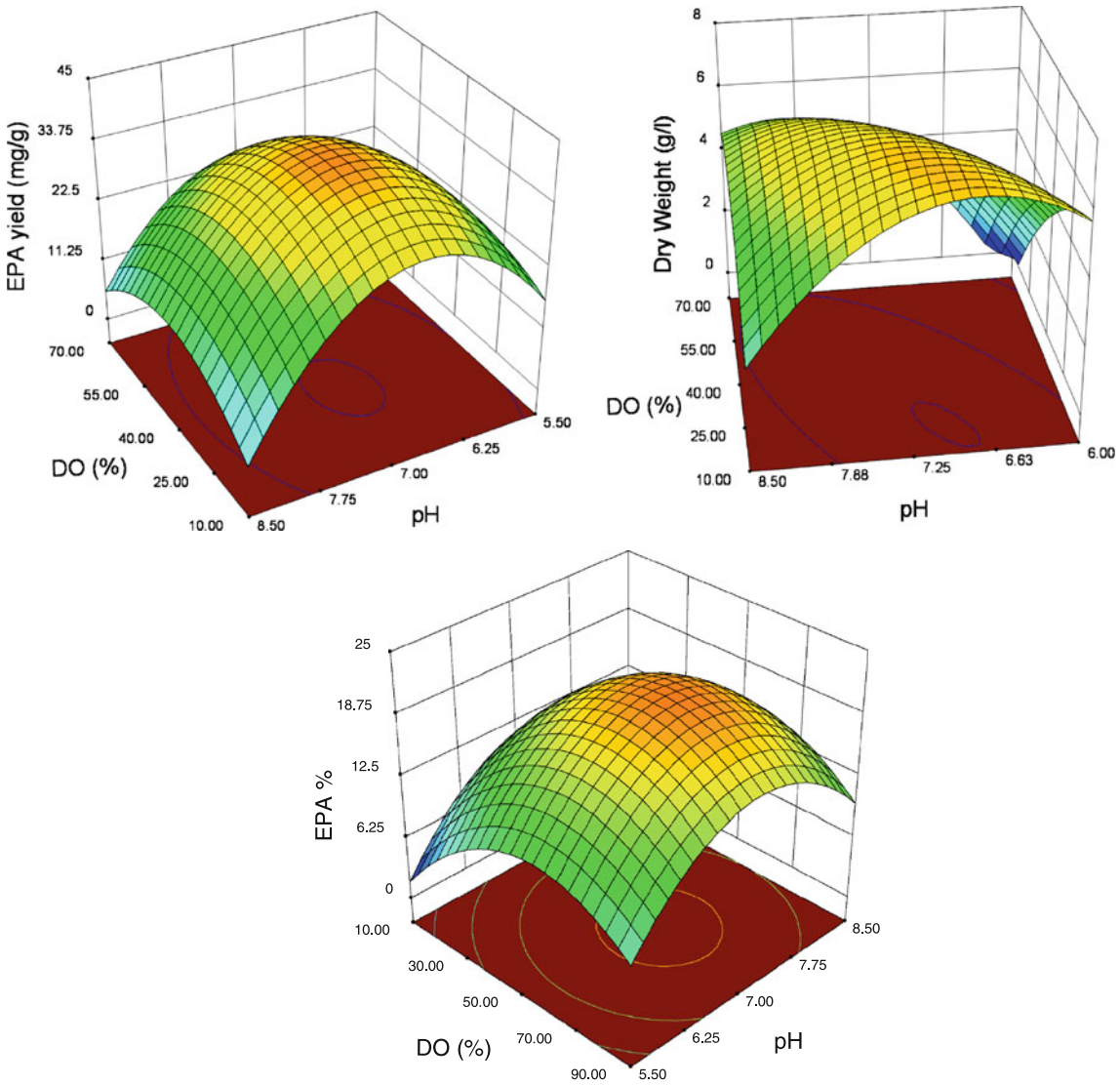

Fig. 1 Three-dimensional surface plot showing the effect of the interaction between pH and DO, at the optimum temperature for each response, on the calculated responses: EPA yield (mg/g), dry weight (g/l) and EPA % of the total fatty acids

carried out in the complete absence of oxygen. The EPA yield was 11.64 mg/g biomass (compared to 45 mg/g of EPA under the optimum conditions) representing approximately 8 % of the total fatty acids (compared to 26 % of the total fatty acids under the optimum conditions). The total amount of biomass obtained under these conditions was 4.3 (±0.4) g/l. This indicates that even in the complete absence of oxygen, this isolate is able to produce EPA, albeit at significantly reduced levels.

Comparison of productivity

The isolate 717 can be considered a high EPA producer, producing 45 mg/g of EPA (350 mg/l production medium) representing 25 % of the total fatty acids when growing under optimum culture conditions within the bioreactor. This represents twice the EPA percentage compared to the recombinant *Escherichia coli* cells carrying EPA genes and capable of producing EPA up to 12 % of the total fatty acids. The amount of EPA in the recombinant *E. coli* increased from 3 to 12 % by introducing a high-performance catalase gene, *vktA* [33]. In addition isolate 717 is able to produce much higher yields of EPA than previously reported high producers such as *Shewanella* sp. NJ136 (14.2 mg/g) [34] and the diatom *Nitzschia laevis* (280 mg/l) [35]. In each case, the cultivations reported in the literature, and used here for comparison, were carried out under conditions identified by respective research teams as optimum for the stated organism, thus providing the maximum obtainable production rate.

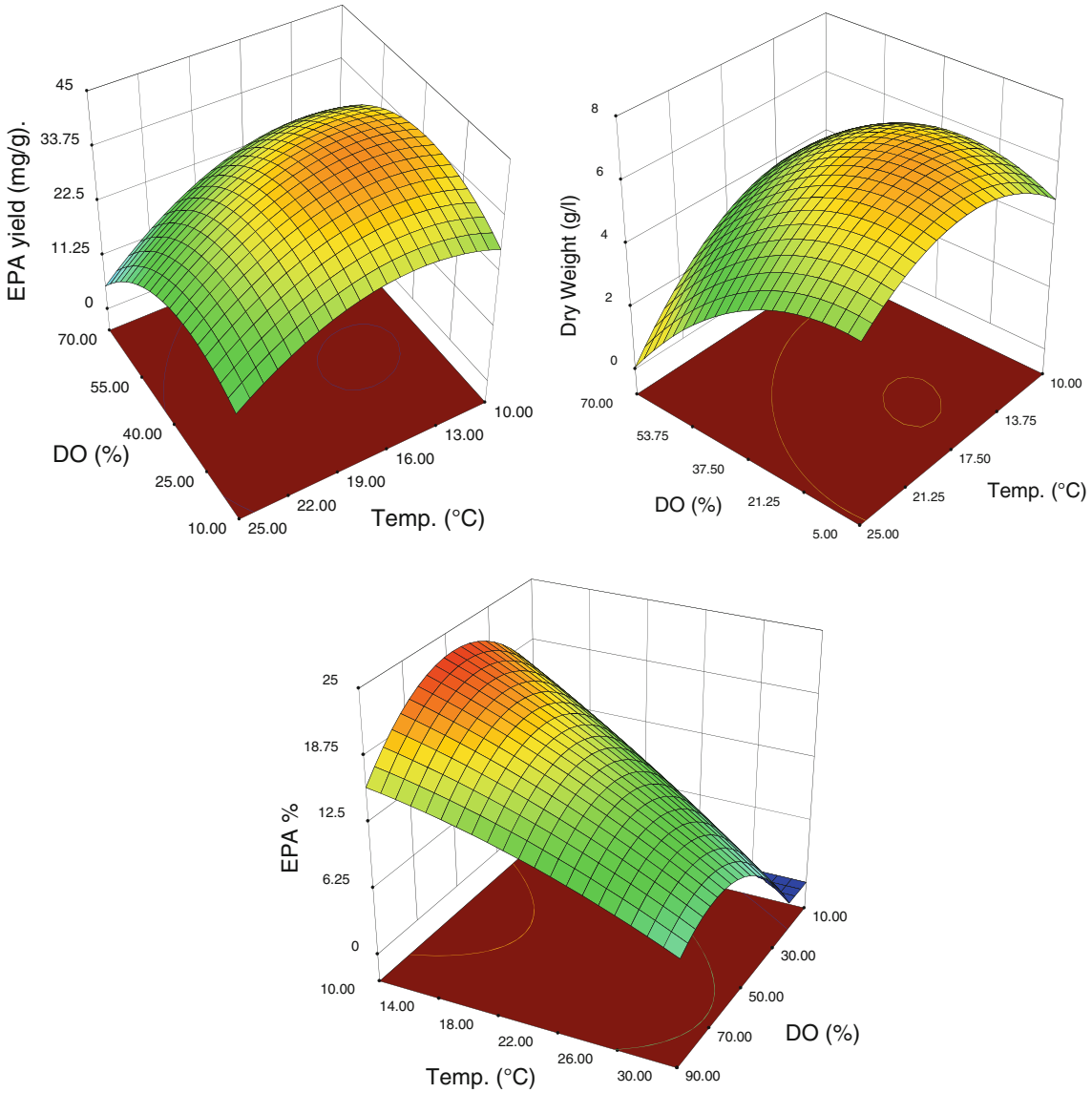

Fig. 2 Three-dimensional surface plot showing the effect of the interaction between temperature and DO on the calculated responses: EPA yield (mg/g), dry weight (g/l) and EPA % of the total fatty acids

Table 5 Optimum growth condition combinations for each response in addition to the predicted and the actual values for each response variable

Target	Variables			Predicted values	Actual values
	DO (%)	pH	Temperature (°C)		
Maximum EPA yield (mg/g)	35	7.1	14	55.7	45.2 (±3.0)
Maximum dry weight (g/l)	15	7	14.6	13.4	9.8 (±0.6)
Maximum EPA %	50	7.07	9.2	29.8	25.6 (±0.8)

Table 6 Optimum culture conditions combination

Variable			Predicted value			Actual value		
DO (%)	pH	Temperature (°C)	Dry weight (g/l)	EPA amount (mg/g)	EPA %	Dry weight (g/l)	EPA amount (mg/g)	EPA %
40.8	7.1	12.7	7.6	37.9	21.5	6.2 (±0.8)	44.2 (±3.0)	22.5 (±0.9)

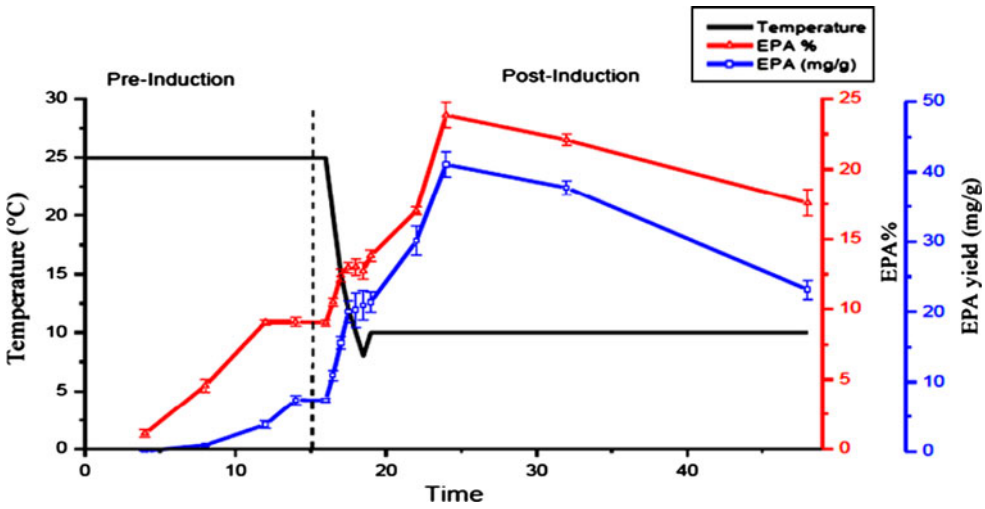

Fig. 3 EPA profile in response to the temperature shock

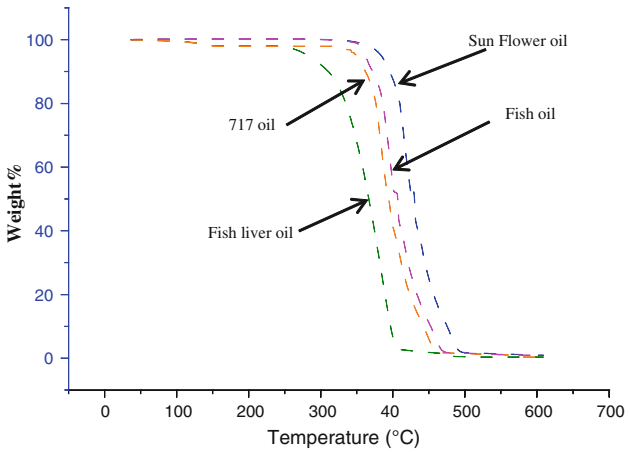

Fig. 4 Weight loss over time at different temperature to compare the stability of isolate 717 oil against fish and fish liver oil, with sunflower oil used as a reference

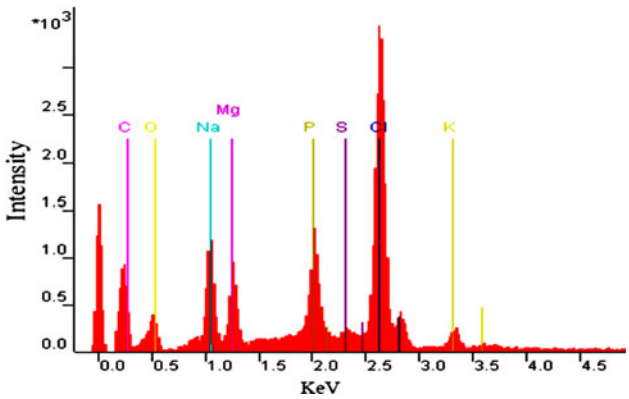

Fig. 5 EDX spectrum for isolate 717 biomass showing the metal ions content

Bacterial oil thermal stability

Thermal stability of fish oil is an important criterion in terms of oil purification, handling and utilisation operations and as an indicator of oil quality [36, 37]. Bacterial oil stability was tested against the fish and fish liver oil, with sunflower oil as a baseline reference, showing a competitive stability compared to fish-derived oils (Fig. 4, where 'weight %' indicates the loss of mass due to thermal degradation compared to the initial mass, as described in Sect. "Materials and methods"). The bacterial oil, including EPA, produced by isolate 717 appears to be more stable than fish liver oil and comparable to the fish oil. The additional lack of the bad odour means that bacterial PUFA could be used as an omega-3 supplement in particular for applications requiring high temperature exposures.

Testing for presence of heavy metals

Energy-dispersive X-ray spectroscopy (EDX) is a useful technique for detecting heavy metal [38]. A preliminary screening of the isolate biomass using EDX indicated undetectable levels of heavy metals under the tested conditions (Fig. 5). This promising initial result indicates that purification costs associated with the removal of heavy metal ions may be avoided during bacterial oil manufacture.

Conclusions

Response surface methodology is a useful technique to optimise the culture growth conditions for different bioprocesses including EPA production. Temperature, DO and pH proved to have a significant effect on EPA production, either as a main effect, two-way interactions or quadratic effect. The effect of DO during the cultivation process and

the interaction between the variables was reported for the first time.

Although high oxygen levels increased the amount of EPA, the ability of the isolate to produce EPA in the complete absence of oxygen suggests that its biosynthesis does not require oxygen, and the role of oxygen could be enhancing the process as an inducer. The fact that the anaerobic cultivation was carried out at low temperature (10 °C) suggest that the amount of EPA produced (11.64 mg/g, representing 8 % of the total fatty acids) could be the amount of EPA required by the isolate to sustain the fluidity of plasma membrane under extreme cold conditions, whereas the high amount of EPA produced during the aerobic cultivation processes could be due to the interaction between DO and temperature.

The lack of the unpleasant odour, the temperature stability, absence of heavy metal contamination and the composition (with no 12-oxo-5,8,10-heptadecatrienoic acid and hexadeca-4,7,10,13-tetraenoic acid fatty acids to interfere with chemotherapy) of the bacterial oil further increase the commercial attractiveness of PUFAs produced from bacterial sources.

Bioaccumulation of heavy metals within the fish fats is one of the main health risks resulting from consuming fish and fish products [39], leading to higher purification expenditure. As expected no accumulation of such compounds in the bacterial biomass was detected, thus potentially leading to reduced purification costs.

Microbial EPA could be extracted and supplied directly to the food and pharmaceutical industries or the microbial biomass could be introduced as poultry feed and as a fish supplement in aquaculture manufacturing [40] or by incorporating microbial PUFAs into higher plants, such as cereals, by solid-state fermentation [41] reducing the unsustainable exploitation of non-food fish species for fishmeal and oil supplements.

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