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The effect of inorganic salts on bacterial omega-3 PUFA production

Ahmed Abd Elrazak^{1*}, Alan C. Ward², Jarka Glassey³

(1) Botany Department, Faculty of Science, Mansoura University, Egypt

(2) School of Biology, Newcastle University, UK

(3) School of Chemical Engineering and Advanced Materials, Newcastle University, UK

*Corresponding Author Contacts

Dr Ahmed Abd Elrazak

Lecturer of Microbial Biotechnology

Faculty of Science

Mansoura University

Aldakahlia, Egypt, 35111

e-mail: Ahmed_bt@mans.edu.eg

Abstract

The increase demand of Omega-3 in the market and the challenges facing its conventional supplies led to an increase interest to microbial Omega-3 sources. This research concentrates on the statistical role of some metal ions on the biosynthesis and productivity of eicosapentanoic acid (essential omega-3 element) in bacterial isolate, *Shewanella* 717. A Plackett-Burman design was applied to screen the main effect of all metal salts entrenched in the Artificial Sea Water medium components. Four salts in particular in addition to the interaction among them were highlighted as having a statistically significant effect upon the growth and/or EPA production. A subsequent Central Composite Design was performed to determine the exact optimum concentration of each of the chosen variables which was found to be 2.5 g/l, 1.8 g/l, 1.2 g/l and 23 g/l, for Na₂HPO₄, MgSO₄, KCl and NaCl respectively. All the experiments were performed with the minimal amount of carbon and nitrogen to eliminate any potential masking effect. A bioreactor batch run was operated and the ions uptake was monitored, using EDAX[®] Electron microscopy, concluding that the process of microbial omega-3 production could be a phosphate limited process. Optimising the concentration of the tested metal ions led to a remarkable increase in the omega-3 productivity resulted in a 30, 9 and 10 times increase in yield, concentration and percentage to the total fatty acids respectively even though the carbon and nitrogen were kept constant all over the research work.

Key Word: *Omega-3, Shewanella, Bioreactor, Metal ion uptake*

1. Introduction

Due to their potential application in pharmaceutical, nutraceutical and cosmetic industries, polyunsaturated fatty acids (PUFAs) have been in the spotlight over the last few years. They are classified as essential nutrients due to their extraordinary physiological and therapeutic impact on human health.

Long chain PUFAs, especially eicosapentaenoic acid (EPA, C20:5 ω 3) were shown to be some of the most bioactive molecules, from a nutritional and medical prospective. EPA was shown to be effective in the prevention and treatment of tumours, increased cholesterol levels, inflammatory diseases, coronary heart disease, blood platelet aggregation and even its ingestion potentially improves brain function (Pérez-López *et al.*, 2014). In addition, PUFAs were reported to play a vital role in treating Crohn's disease, psoriasis, rheumatoid arthritis, ulcerative colitis, cystic fibrosis and lupus (Adarme-Vega *et al.*, 2012).

Despite their vital impact on the human health, the current sources of EPA are considered to be unsustainable and scientists still strive to find a sustainable source of EPA to satisfy the increase in market demand (Adarme-Vega *et al.*, 2014). Microorganisms were shown to be one of the most suitable sources either as natural (El Razak *et al.*, 2014a) or genetically engineered producers (Zhixiong *et al.*, 2013). One of the main advantages of bacterial PUFAs is their high stability and the absence of heavy metal contaminations which are considered the most significant disadvantages of fish oils (the traditional source of EPA).

Several reports in literature described potential routes for microbial PUFA production. For example, El razak *et al.* (2014b) isolated a new bacterial isolate considered to be a potential high EPA producer. Isolate *Shewanella* 717 was able to produce up to 350 mg/l, representing 25 % of the total fatty acids, under optimum conditions within a bioreactor environment (Abd Elrazak *et al.*, 2013b). In addition, Pérez-López *et al.* (2014) succeeded in optimising the production conditions at pilot scale level to produce EPA that represents up to 36% of the total fatty acids from the marine diatom *Phaeodactylum tricornutum*.

Whilst a number of these reports concentrated on the optimisation of cultivation conditions or major medium components, the effect of metal ions and inorganic salts was rarely investigated. The effect of

inorganic salts on the ability of different microorganisms to synthesise EPA was previously reported in the marine bacterium, SCRC-2738, isolated from mackerel intestines (Akimoto *et al.*, 1991), the fungus *Mortierella ramanniana* (Hansson and Dostálek, 1988), and a bacterial isolate *Vibrio* 560 (Abd Elrazak *et al.*, 2013a), where it was concluded that the complete absence of the metal ions, except for NaCl, slightly reduced growth and may lead to the complete loss of the ability to synthesise EPA.

This research concentrates on the potential role of metal ions on the biosynthesis of EPA in a high producing isolate *Shewanella* 717. A structured design of experiments was carried out to explore the main effects and the significance of the effect of each metal ion upon the EPA production by this isolate.

2. Materials and Methods

2.1 Production strain

The isolate under investigation was purified from a deep sea core sediment and fluff sample obtained from the Dove Marine laboratory, Newcastle University. After the initial screening, the isolate was found to be able to produce EPA in relatively high amounts (El razak *et al.*, 2014b). The isolate was identified as *Shewanella* sp. 717 (Genbank accession no. **JX203388**).

2.2 Seed Culture Cultivation Conditions

The isolate under investigation was cultivated on Artificial Sea Water (ASW) medium at 12.7°C in a shaker incubator (150 rpm) for 48hrs. The ASW medium contained peptone 3.5 g/l; yeast extract 3.5 g/l; NaCl 30 g/l; MgCl₂ 6.08 g/l; MgSO₄ 5.16 g/l; Fe₂(SO₄)₃ 0.03 g/l; CaCl₂ 0.147 g/l; KCl 0.75 g/l; Na₂HPO₄ 0.89 g/l; NH₄Cl 5.0 g/l (Lang *et al.*, 2005).

2.3 Screening Plackett-Burman Design

The eight different inorganic salts included in the ASW medium (NaCl; MgCl₂; MgSO₄; Fe₂(SO₄)₃; CaCl₂; KCl; Na₂HPO₄; NH₄Cl) were screened to establish the significance of their effect on the growth and productivity of the isolate under investigation using a Plackett-Burman (PB) design of experiments.

The basal medium used in the PB contained 1 g/l of yeast extract in addition to 10 g/l of sodium chloride. The addition of NaCl was required due to the inhibition of growth of the isolate in the absence of NaCl. A dummy factor was introduced to the design in order to investigate the standard error of the design.

The experiment was conducted on a 'Presence/Absence' principle where the minimum values for each variable (except for NaCl) represented their complete absence from the medium and the maximum values represented the concentration of each metal salt in the ASW (Table 1).

This screening experiment allowed an objective comparison of the significance and the main effect of each factor on the amount of EPA produced by the isolate under investigation. The main effects of each variable were calculated using equation 2.1

$$E_{(Xi)} = \frac{2[\sum Y_i^+ - \sum Y_i^-]}{N} \quad (2.1)$$

where $E_{(Xi)}$ is the effect of the tested variable and Y_i^+ and Y_i^- are the responses at maximum and minimum levels, respectively. The significance level (P-value) of each variable was determined using the Student's t test (equation 2.2)

$$t_{(Xi)} = \frac{E_{(Xi)}}{SE} \quad (2.2)$$

where (SE), the standard error of variables, is calculated as the square root of the variance of an effect. Any variable with $P < 0.1$ was considered to be significant at 90% level of confidence.

The relationship between the response and the significant variables within Plackett-Burman can be expressed using a first order polynomial order equation (equation 2.3)

$$Y = \beta_0 + \sum \beta_i X_i \quad (i=1, 2, \dots, k) \quad (2.3)$$

where Y is the response variable, β_0 is the model intercept, β_i is the regression coefficient for each corresponding variable, X_i is the corresponding variable and k is the number of variables (Montgomery, 2004).

2.4 Optimising Central Composite Design

The screening of the effects of different inorganic salts indicated four of them (Na_2HPO_4 , KCl, NaCl and MgSO_4) to be the most statistically significant while the effect of the other metal ion salts were not statistically significant.

A set of Central Composite Design (CCD) experiments was performed using the four significant salts with 5 levels to determine the optimum composition and establish the significance of the interactions between the tested variables. The levels tested are summarised in Table 2:

The CCD provided an indication of the main effect of each factor in addition to the significance of the interactions between them. A second order polynomial model was generated for the prediction of EPA production and to identify the optimum growth conditions (equation 2.4):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_{ii} + \sum \beta_{ij} X_{ij} \quad (2.4)$$

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 interaction. An analysis of variance (ANOVA) was carried out using the Design Expert 8.0 statistical package (StatEase, Inc, Minneapolis, MN, USA) and Minitab 15.

2.5 Bioreactor Cultivation

An Ez-Control Applicon bioreactor (working volume 2 litres), was used to perform batch culture experiments to measure the growth and inorganic salts uptake over the cultivation time. The bioreactor was equipped with pH, temperature and dissolved oxygen measurement and control. Air flow rate was kept constant (2 L/m) and the agitation speed was adjusted to maintain the dissolved oxygen concentration at the desired values. Samples were taken at regular intervals for offline determination of biomass, OD and amount of EPA. The optimum cultivation conditions were previously reported to be DO = 40.8% , pH= 7.1 and incubation temperature= 12.7°C (Abd Elrazak *et al.*, 2013b). The run was carried out with temperature and DO controlled while pH was uncontrolled as it would be difficult to distinguish between the tested ions and the ions added in the acid and alkali solutions for pH control.

2.6 Monitoring the uptake of metal ions by the isolate 717

The uptake of elements by isolate 717 was investigated in a bioreactor batch cultivation experiment. The growth conditions were 12.7°C and 40.8% DO while pH was monitored but not controlled. The cultivation medium contained 2.5 g/l Na₂HPO₄, 1.2 g/l KCl, 23 g/l NaCl, 1.8 g/l MgSO₄, 1 g/l peptone and 1 g/l yeast extract as determined by the performed CCD experiment.

Samples were collected at regular time intervals, centrifuged at 6000g for 10 minutes and the supernatants were freeze dried. The residual amount of elements at each sampling point was quantified using energy-dispersive X-ray spectrometry (EDAX[®]) and elemental analysis for carbon, nitrogen and hydrogen.

2.6.1 EDAX[®] *Electron microscopy*

The freeze dried supernatant samples were examined using a Scanning Electron Microscopy [XL30 ESEM-FEG (ESEM)], (Electron Microscopy Services, Newcastle University) which is equipped with an EDAX[®], energy-dispersive X-ray spectrometer, (RONTEC system with Quantax software) with a liquid nitrogen-cooled anti-contamination device in place at all times. The electron microscope was operated at 25 kV in low vacuum mode. This technique was used to monitor the uptake of sodium, sulphur, chlorine, magnesium, potassium and phosphate elements.

Three readings from different regions were collected, showing no change in the readings, indicating a homogenous distribution of the elements across the samples.

2.6.2 *Elemental Analyser*

This technique was used to quantify the amount of hydrogen, nitrogen and carbon in the freeze dried supernatant samples. All samples were tested using a Carlo Erba 1108 Elemental Analyser controlled with CE Eager 200 software, run in accordance with the manufacturer's instructions and weighed using a Mettler MX5 Microbalance. Two readings were taken for each sample with a maximum standard deviation less than 0.3%.

2.7 *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 in water bath for 2 hours in sealed glass tubes. The tubes were cooled at room temperature for 30 minutes, then 1 ml distilled water was added and the tubes were vortexed. To extract the FAME 1 ml hexane was added and vigorously vortexed. The tubes were kept until two layers formed. The upper layer was transferred into a clean tube and dried under nitrogen. A known volume of hexane was added in addition to a known volume and concentration of the internal standard (Watanabe *et al.*, 1996). After culturing the bacteria in either a shake flask or a bioreactor with a suitable media, the biomass was harvested and treated to enable further extraction of lipid from the biomass with a suitable solvent. PUFAs were extracted from whole intact cells in order to avoid oxidation resulting from cell disruption. The extract was then methylated and the resulting Fatty Acid Methyl Esters (FAME) were analysed using gas chromatography (GC) as described in section 2.8.

2.8 *FAME profiling*

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

FAMEs were prepared as described in section 2.7 and analysed using a GC with flame ionisation detector (FID) on a Hewlett-Packard 5890 series 2 chromatograph equipped with a DB-23 J&W column; 30 m x 0.25 μ m film thickness using helium as the carrier gas. The recommended GC ramp temperature conditions (60°C for 1 min, 140°C at an increase of 20°C/min and held for 3 min, 190°C at increase of 10°C/min and held for 3 min and then increase of 10°C/min to 220°C for 60 min (Jostensen and Landfald, 1997), were also modified and programmed with 220°C held for 8 minutes. Preliminary experiments with the unmodified and modified methods on the same apparatus, gave identical results. As a result the modified method was used as it reduced the overall run time. Supelco-37 FAME reference sample was used as a positive control for the determination of the EPA and DHA presence, and the peak area determined.

3. Results

3.1 Screening Plackett-Burman Design

The matrix and the obtained responses are summarised in Table 3, and the full statistical analysis is presented in Table 4. The ANOVA table (Table 4) shows that Na_2HPO_4 and Fe_2SO_4 were the most significant variables affecting the EPA concentration produced by of the tested isolate; but in reverse directions. Na_2HPO_4 (+ve main effect) showed a significant increase in the concentration of produced EPA; while Fe_2SO_4 showed a significant negative influence (-ve main effect) on the same response. Fe_2SO_4 had a negative impact on all the calculated responses, while MgSO_4 , Na_2HPO_4 , KCl and NaCl were found to have a statistically significant positive effect on either EPA concentration and/or EPA yield.

3.2 Central Composite Design

During the screening the effect of inorganic salts was tested and four of them (Na_2HPO_4 , MgSO_4 , KCl and NaCl) were found to have the most significant positive effect on EPA production, as a result they were taken further and optimised via a CCD approach (as described in section 2.4).

This experiment was carried out with the minimum amount of yeast extract in the basal medium as the presence of high concentrations of carbon or nitrogen may mask the effect of the elements either on growth or EPA productivity. Table 5 summarise the performed matrix and the obtained responses for the Central Composite Optimisation Design and the interaction between the variables were summarized graphically in the form of 3D responses (S1, S2 and S3). To analyse the results and reveal the main effect of each factor, the interactions between them and the quadratic effects of each metal ion, an ANOVA table was produced (Table 6).

Second order polynomial models were generated, using coded units, to predict the optimum elemental concentration for each response (equations 3.1, 3.2, 3.3).

$$\text{EPA yield.} = 11.21 + 2.70A - 0.49B - 0.30C - 1.22D - 1.28AD - 1.19A^2 - 2.41C^2 \quad (\text{eq.3.1})$$

$$\text{EPA conc.} = 14.28 + 6.44A - 1.32B + 0.53C - 0.003D + 2.11AC - 2.39C^2 - 6.87AB^2 \quad (\text{eq.3.2})$$

$$\text{EPA \%} = 14.14 + 2.20A - 0.74B - 0.058C - 0.78D - 1.07AD - 1.54A^2 - 2.30C^2 + 1.28D^2 - 2.47AB^2 \quad (\text{eq.3.3})$$

where, A, B, C and D represent Na_2HPO_4 , KCl, NaCl and MgSO_4 respectively.

A validation experiments were conducted with the proposed optimum combination and the actual values were compared to the predicated values as shown in Table 7.

3.3 Monitoring the uptake of elements by the isolate 717

The optimum inorganic salt combination for maximum productivity was determined to be 2.5 g/l Na_2HPO_4 , 1.2 g/l KCl, 23 g/l NaCl and 1.8 g/l MgSO_4 .

A medium with this suggested concentration in addition to 1 g/l peptone and 1 g/l yeast extract was used to carry out an experiment at a bioreactor scale, as described in section 2.5.

Figures 1 and 2 show the time trajectories of each residual element in the medium (measured as described in section 2.6.1 and 2.6.2) in relation to the growth (OD), EPA yield and percentage. **Figure 1** shows the relationship between the elements present in relatively high concentrations, so called macro-elements, (reported in g/l of medium) including Cl and Na.

Elements with relatively low concentrations (also reported in g/l for direct comparison, however with remarkably lower numerical values), the so called micro-elements, were plotted against growth and EPA productivity in **Figure 2**.

Figure 3 shows the relationship between the growth and EPA productivity and the concentration of residual carbon and nitrogen (in elemental form) elements in the medium.

4. Discussion

The increase demand of Omega-3 in the market and the challenges facing its conventional supplies led to an increase interest to microbial Omega-3 sources. Toward achieving a successful process development of microbial omega-3, all the variables affecting their biosynthesis should be addressed. This research concentrates on the statistical role of some metal salts on the biosynthesis and productivity of EPA in bacterial isolate, *Shewanella* 717, which previously identified as a potential high producer.

Statistical approaches were applied and expected to be a successful way to explore and reveal the effect of such metals on the product under investigation. In addition, all the experiments were concluded in with the minimal amount of carbon and nitrogen to eliminate any potential masking effect also to make the exploration of the metal ion effect more recognizable.

Various inorganic salts are required not only for the growth of bacteria, but they also affect PUFA production. This was demonstrated by Akimoto *et al.* (1991) when they studied the effect of salts, on the basis of One Factor At A Time (OFAT), upon the production of EPA by marine bacteria, SCRC-2738, isolated from mackerel intestines. The optimum salt concentration identified in that study was 2% (w/v) NaCl, 0.15% (w/v) KCl and 0.16% (w/v) MgCl₂ which lead to the doubling of the amount of EPA (up to 51.9 mg/l) produced by the isolate under investigation.

Hansson and Dostálek (1988) reported that the addition of Cu²⁺ and Zn²⁺ to the cultivation media enhanced the ability of the fungus *Mortierella ramanniana* to produce γ -Linolenic acid while the addition of Mg²⁺ did not show any significant effect on the isolate productivity. The addition of 5 mg/l of Mn²⁺ to the cultivation media of *Mortierella ramanniana* var. *ramanniana* resulted in the highest percentage of GLA of the total fatty acids (13.3%) (Dyal *et al.*, 2005).

Although isolate 717 was able to grow well in the complete absence (except for residual concentrations from yeast extract) of all of the tested salts (except for NaCl), i.e. to grow in the presence of sodium chloride and yeast extract only, the amount of EPA produced decreased dramatically (trial number 5, Table 3), suggesting that the tested salts could have a significant role in the biosynthesis of EPA.

The effect of CaCl₂ on EPA produced by isolate 717 was statistically significant for a negative effect, especially on EPA yield (mg/l), which is similar to the observed effect of the same variable on isolate SCRC-2738, where Akimoto *et al.* (1991) reported that the addition of CaCl₂ to the isolate media sharply decreased the amount and

percentage of EPA, although it showed a positive effect on the amount of biomass produced. Iron showed a significant negative effect on all EPA calculated responses for the isolate 717. This result was not completely consistent with the result obtained by Chiou *et al.* (2001) as ferrous ions were found to enhance PUFA production when added in the cultivation media of *Marchantia polymorpha*.

Ammonium chloride showed no significant effect on any of the EPA calculated responses for isolate 717 and the same effect was reported on *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1991). For the diatom *Nitzschia laevis*, the addition of ammonium chloride in the growth media lead to a remarkable decrease in the amount of EPA produced (Cao *et al.*, 2008). The significant effect of MgSO_4 on isolate 717 was found on EPA concentration (mg/l) which is mostly related to increasing biomass rather than increasing EPA itself, within the cells. MgSO_4 also showed a positive effect on the amount of biomass produced without any significant effect on the amount of EPA produced by the isolate *Phaeodactylum tricornutum* (Yongmanitchai and Ward, 1991).

The effect of Na_2HPO_4 on the ability of isolate 717 to produce EPA was in line with the same positive effect of Na_2HPO_4 on the isolate *Phaeodactylum tricornutum* to produce EPA (Yongmanitchai and Ward, 1991). This positive effect was suggested to be due to the effect of the inorganic phosphate.

Four metal ions were investigated further based on the results reported in the screening Plackett-Burman experiment: Na_2HPO_4 , as it demonstrated a significant positive effect on EPA production, in terms of concentration and yield; KCl as it was found to be the only statistically significant factor which enhanced the percentage of EPA; MgSO_4 as the most significant factor affecting EPA concentration positively; and NaCl as the isolate 717 cannot grow in its complete absence. The same effect of NaCl was previously reported by Yoon *et al.* (2004), as *Shewanella gaetbuli* could not grow in the complete absence of NaCl.

The effect of these chosen salts were tested in a separate CCD experiment, in the presence of the minimal amount of yeast extract used as a basal medium (as described in section 2.4). The low concentration of yeast extract eliminated the potential masking of the element effect due to high carbon and/or nitrogen concentration in the medium. The ANOVA table from the CCD (Table 6) indicated that Na_2HPO_4 is the only variable with its main effect to be statistically significant on all EPA responses (P-values for EPA yield, concentration and percentage were 0.0285, 0.0001 and 0.0943, respectively) indicating that it could be the most significant salt affecting the biosynthesis of EPA by isolate 717. This hypothesis is supported by the fact that it was the only ion with a significant main effect on the EPA percentage of the total fatty acid suggesting that Na_2HPO_4 significantly induces the synthesis of EPA more

than the other ions tested. In addition to the main effect, the Na_2HPO_4 quadratic effect was also found to have a significant effect on the EPA yield (P-value 0.0465) and EPA% (P-value 0.0211) but not on EPA concentration.

KCl was found to have a significant effect in terms of the main effect on EPA concentration (mg/l) only, (P-value, 0.0791), while its main effect on the other responses was found to be insignificant. Neither the KCl quadratic effect, nor any of its two way interactions were found to have any significant effect on any of the EPA responses.

The quadratic effect of NaCl was estimated as significant on all EPA responses (for EPA yield P-value was 0.0073, for EPA concentration P-value was 0.0274 and for EPA% P-value was 0.0179). Also, NaCl was found to be involved in a significant interaction with Na_2HPO_4 (P-value, 0.0253) on EPA concentration.

MgSO_4 showed a significant main effect (P-value, 0.0604) on EPA yield. In addition to the significant main effect, its interaction with Na_2HPO_4 was significant (P-value, 0.0840) on the same response. The R^2 of the design was calculated to be 98%.

In an OFAT study, testing the effect of inorganic salts on EPA production by the isolate SCRC-2738, Akimoto *et al.* (1991) reported that MgSO_4 was found to be insignificant on the EPA percentage and thus it was discarded from the optimum medium. A similar result was obtained from the present CCD experiment, confirming that the main effect of that salt is insignificant (P-value, 0.2771) on EPA percentage. However, the interaction between MgSO_4 and Na_2HPO_4 (P-value, 0.0977) and the quadratic effect (P-value, 0.0947) were significant indicating that its effect should not be ignored. The one factor at a time approach however does not allow the identification of such effects.

Significant effect of Mg^{2+} ions on the ability of the microorganisms to produce PUFAs could be due to its effect on ATP citrate lyase and malic enzyme which influence the supply of acetyl CoA and NADPH for lipogenesis in microbial cells (Muhid *et al.*, 2008).

For isolate 717, the optimum NaCl concentration for the highest EPA production was approximately 20 g/l which is similar to the optimum concentration for EPA production by isolate SCRC-2738 (Akimoto *et al.*, 1991). Growing isolate SCRC-2738 in the bioreactor on the modified media resulted in 1.2 times higher EPA concentration than growing under the same conditions on ASW media. Relatively low salinity was also preferred by the isolate *Nitzschia laevis* for maximum EPA production (Cao *et al.*, 2008). The effect of NaCl and KCl on the EPA contents could be due to controlling the fluidity of the cellular membrane (Akimoto *et al.*, 1991). Also, the variation in the cellular membrane composition could be an adaptation concentration response towards the salt (Watanabe and Takakuwa, 1987).

Bivalent metal ions such as Mg^{2+} are usually required as cofactors. Acetyl coenzyme A carboxylase, catalysing the conversion of acetyl coenzyme A to malonyl coenzyme A via a carboxylation step, generally requires such bivalent metal ions; however exceeding the optimum concentration could have an inhibiting effect on the process (Guchhait *et al.*, 1974). The optimum inorganic salt combination for maximum productivity was determined to be 2.5 g/l Na_2HPO_4 , 1.2 g/l KCl, 23 g/l NaCl and 1.8 g/l $MgSO_4$.

For the purpose of monitoring the elemental uptake by isolate 717, a medium with this suggested concentration plus 1 g/l peptone and 1 g/l yeast extract was used to carry out an experiment at a bioreactor scale, as described in section 2.5. As expected all investigated elements showed a remarkable concentration reduction during the exponential growth of isolate 717 due to high uptake (**Figures 1, 2 and 3**). In stationary phase all elements, except phosphate, were present in the cultivation medium in relatively high concentrations, suggesting that phosphate may be limiting the growth of isolate 717 in this medium. Also, the highest amount of EPA was produced following phosphate depletion suggesting that phosphate starvation may trigger an increased rate of EPA biosynthesis. However further experimental work is required to confirm this hypothesis. The C/N ratio profile was relatively low when EPA was at its maximum.

As a final conclusion our results indicated that some inorganic salts, independently of the effect of carbon and nitrogen, may play a vital role in the biosynthesis of EPA and must be optimised on each process development step. This research shades the light, for the first time, on the possible role of the interaction between ions on the biosynthetic pathway of EPA.

Four salts in particular were highlighted in this research as having a statistically significant effect upon the growth and/or EPA production. The validation cultivation carried out under these conditions (using a low concentration of yeast extract) resulted in 15 mg/g and 21 mg/l of EPA representing 15% of the total fatty acids, compared to the 0.48 mg/g and 2.61 mg/l of EPA representing 1.8% of the total fatty acids when grown in basal medium. The results of the analysis of the uptake of elements during the bioreactor cultivation indicated that the process of EPA production may be a phosphate limited process which may be helpful in developing a successful fed-batch strategy.

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List of Tables

Table 1: Investigated metal ions and their levels used in the Plackett-Burman screening design for the bacterial isolate 717.

Variables	Code	unit	Minimum level (-)	Maximum level (+)
<i>NH₄Cl</i>	<i>X₁</i>	g/l	0	5
<i>Na₂HPO₄</i>	<i>X₂</i>	g/l	0	0.89
<i>KCl</i>	<i>X₃</i>	g/l	0	0.75
<i>CaCl₂</i>	<i>X₄</i>	g/l	0	0.15
<i>Fe₂(SO₄)₃</i>	<i>X₅</i>	g/l	0	0.03
<i>MgCl₂</i>	<i>X₆</i>	g/l	0	6
<i>MgSO₄</i>	<i>X₇</i>	g/l	0	5
<i>NaCl</i>	<i>X₈</i>	g/l	10	30
<i>Dummy</i>	<i>X₉</i>	g/l	Distilled Water	Bi-Distilled Water

Table 2: Metal ions and their levels investigated via CCD for maximum EPA production by isolate 717.

Variables	Levels				
	-2	-1	0	+1	+2
<i>Na₂HPO₄</i> (g/l)	0.05	0.79	1.52	2.26	3
<i>KCl</i> (g/l)	0.05	0.79	1.52	2.26	3
<i>NaCl</i> (g/l)	10	16.25	22.50	28.75	35
<i>MgSO₄</i> (g/l)	1	4.50	8.00	11.50	15

Table 3: The matrix and the responses for the Plackett-Burman design for the isolate 717 where, X_1 represents NH_4Cl , X_2 represents Na_2HPO_4 , X_3 represents KCl , X_4 represents CaCl_2 , X_5 represents $\text{Fe}_2(\text{SO}_4)_3$, X_6 represents MgCl_2 , X_7 represents MgSO_4 , X_8 represents NaCl .

Run	Variables								Responses		
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	+	-	+	-	-	-	+	+	3.53	9.01	13.55
2	+	+	-	+	-	-	-	+	8.76	5.32	7.06
3	-	+	+	+	-	+	+	-	7.52	9.93	10.43
4	+	+	-	+	+	-	+	-	3.67	7.25	5.91
5	-	-	-	-	-	-	-	-	0.48	2.61	1.80
6	-	+	-	-	-	+	+	+	5.37	13.04	7.36
7	+	-	+	+	-	+	-	-	0.94	0.69	9.34
8	-	-	+	+	+	-	+	+	0.22	3.14	4.65
9	-	+	+	-	+	-	-	-	3.75	0.95	6.21
10	+	+	+	-	+	+	-	+	4.03	5.77	6.45
11	+	-	-	-	+	+	+	-	0.26	6.51	1.26
12	-	-	-	+	+	+	-	+	1.54	1.17	2.09

Table 4: Estimated Effects, Coefficients and P-values for the tested variables for the isolate 717. P-values of the significant factors are shown in bold underline.

Variables	EPA yield. (mg/g)				EPA conc. (mg/l)				EPA %			
	Effect	Coefficient	T- Value	P- Value	Effect	Coefficient	T- Value	P- Value	Effect	Coefficient	T- Value	P- Value
NH_4Cl (X_1)	0.383	0.191	0.36	0.743	0.620	0.310	1.04	0.374	1.840	0.920	1.53	0.170
Na_2HPO_4 (X_2)	4.352	2.176	5.90	<u>0.000</u>	3.191	1.596	4.44	<u>0.007</u>	1.790	0.895	1.49	0.181
KCl (X_3)	-0.016	-0.008	-0.02	0.989	-1.064	-0.532	-1.79	0.172	4.192	2.096	3.48	<u>0.010</u>
$CaCl_2$ (X_4)	0.871	0.436	0.82	0.473	-1.728	-0.864	-2.40	<u>0.061</u>	0.476	0.238	0.34	0.754
Fe_2SO_4 (X_5)	-2.189	-1.094	-2.97	<u>0.018</u>	-2.635	-1.318	-3.67	<u>0.015</u>	-3.831	-1.916	-3.18	<u>0.015</u>
$MgCl_2$ (X_6)	-0.128	-0.064	-0.12	0.912	1.474	0.737	2.05	<u>0.096</u>	-0.375	-0.188	-0.27	0.805
$MgSO_4$ (X_7)	0.178	0.089	0.17	0.878	5.395	2.698	7.51	<u>0.001</u>	1.704	0.852	1.23	0.308
$NaCl$ (X_8)	1.140	0.570	1.55	0.161	1.587	0.794	2.21	<u>0.078</u>	1.038	0.519	0.75	0.510

Table 5: Central Composite Design of variables (in coded units) with EPA production as responses to test the effect of metal ions on EPA

productivity by the isolate 717

Run	Variables				Responses		
	Na ₂ HPO ₄	KCl	NaCl	MgSO ₄	EPA yield (mg/g)	EPA conc. (mg/l)	EPA %
1	0	0	0	-2	11.83	9.41	12.94
2	-1	-1	+1	-1	10.28	12.35	13.18
3	-1	+1	+1	+1	8.19	11.88	14.32
4	+1	-1	-1	+1	6.11	12.63	10.68
5	+1	+1	-1	+1	4.81	11.84	9.45
6	0	0	-2	0	2.76	1.52	4.98
7	-1	+1	-1	+1	9.49	13.25	13.49
8	-2	0	0	0	2.11	4.35	3.02
9	0	0	0	0	13.46	15.88	15.25
10	-1	-1	-1	+1	15.61	18.14	14.19
11	-1	-1	+1	+1	8.13	11.04	13.56
12	0	+2	0	0	6.82	9.11	10.36
13	+2	0	0	0	9.82	21.56	8.38
14	0	0	0	0	12.21	16.46	14.65
15	0	0	0	0	12.57	14.93	14.11
16	-1	+1	+1	-1	8.46	9.69	12.82
17	0	0	+2	0	3.99	8.86	2.76
18	+1	-1	+1	-1	7.31	13.43	14.04
19	0	0	0	+2	2.38	5.71	3.11
20	0	-2	0	0	8.71	14.42	14.59
21	+1	-1	+1	+1	6.65	17.72	15.58
22	0	0	0	0	12.71	15.37	15.35
23	+1	+1	-1	-1	9.91	10.75	16.88
24	+1	-1	-1	-1	8.056	6.42	13.65
25	0	0	0	0	13.28	14.91	14.61
26	0	0	0	0	13.53	13.73	15.28
27	-1	-1	-1	-1	6.98	19.44	13.31
28	-1	+1	-1	-1	9.29	9.12	11.36
29	+1	+1	+1	+1	3.51	10.79	9.13
30	+1	+1	+1	-1	9.76	16.45	12.55

Table 6: ANOVA table for the CCD experiment. P-values of the significant factors are shown in bold underline.

Variables	EPA yield (mg/g)			EPA conc.(mg/l)			EPA %		
	Sum of	F	p-value	Sum of	F	p-value	Sum of	F	p-value
	Squares	Value	Prob > F	Squares	Value	Prob > F	Squares	Value	Prob > F
<i>A-Na₂HPO₄</i>	43.88	5.53	<u>0.0285</u>	330.38	26.69	<u>0.0001</u>	29.13	3.09	<u>0.0943</u>
<i>B-KCl</i>	5.81	0.73	0.4018	41.95	3.39	<u>0.0791</u>	13.06	1.38	0.2534
<i>C-NaCl</i>	1.86	0.23	0.6329	5.79	0.47	0.5011	0.069	0.003	0.9328
<i>D-MgSO₄</i>	31.24	3.94	<u>0.0604</u>	0.004	0.005	0.9954	11.78	1.25	0.2771
<i>AB</i>	1.80	0.21	0.6513	21.69	1.44	0.2499	0.85	0.068	0.7985
<i>AC</i>	1.39	0.17	0.6901	71.27	5.76	<u>0.0253</u>	0.049	0.003	0.9510
<i>AD</i>	26.09	3.29	<u>0.0840</u>	1.15	0.077	0.7859	24.43	2.85	<u>0.0977</u>
<i>BC</i>	0.048	0.003	0.9411	3.97	0.26	0.6154	2.96	0.24	0.6342
<i>BD</i>	14.74	1.75	0.2070	1.04	0.069	0.7966	3.10	0.25	0.6262
<i>CD</i>	7.60	0.90	0.3580	4.70	0.31	0.5853	3.43	0.27	0.6086
<i>A²</i>	35.48	4.47	<u>0.0465</u>	14.31	0.95	0.3461	59.16	6.27	<u>0.0211</u>
<i>B²</i>	8.37	0.99	0.3357	0.083	0.003	0.9420	3.26	0.26	0.6179
<i>C²</i>	69.98	8.82	<u>0.0073</u>	69.07	5.58	<u>0.0274</u>	62.87	6.66	<u>0.0179</u>
<i>D²</i>	15.42	1.83	0.1974	14.20	0.94	0.3478	27.27	2.99	<u>0.0947</u>

Table 7: The predicted and the actual values under the optimum metal ions concentrations for maximum EPA production by the isolate 717

Media				Predicted Values			Actual Values		
Na ₂ HPO ₄	KCl	NaCl	MgSO ₄	EPA yield	EPA conc.	EPA	EPA yield	EPA conc.	EPA %
(g/l)	(g/l)	(g/l)	(g/l)	(mg/g)	(mg/l)	%	(mg/g)	(mg/l)	
2.56	1.21	22.92	1.80	15.61	19.44	16.57	14.6 (±0.98)	17.11 (±3.2)	17.48 (±0.3)

Figure Legends

Figure 1 Time trajectory of the residual macro-elements (Cl and Na) in relation to the growth (OD) and EPA yield (mg/g) and percentage

Figure 2 Time trajectory of the residual micro-elements (S, Mg, K and P) in relation to the growth (OD) and EPA yield (mg/g) and percentage

Figure 3 Time trajectory of the residual carbon and nitrogen elements and C/N ratios in relation to the growth (OD) and EPA yield (mg/g) and percentage