Query

Q1. Please confirm if the forename and surname of all the authors are presented appropriately in the author byline here.

Q2. Please provide expansion for abbreviations “HPLC and MALDI-TOF” both in abstract and in the text.

Q3. References “Shimoni et al. (1998), Todokoro et al. (2015), and Lee and Chen (1997)” are cited in the text but not provided in the list. Please check and provide the references in the list or delete the citation.

Q4. As per journal specification, numbered reference style is followed. So the Name-and-Date reference citations “Meiwes et al. (1990), Nakouti and Hobbs (2014), Tóth et al. (2009), Emri et al. (2013), and Szigeti et al. (2014)” have been replaced with numbered reference citations to match with reference list. Please check and confirm.

Q5. Please check whether “variance analysis” can be changed to “analysis of variance” in the sentence that begins with “The effects of the carbon...”

Q6. Please provide expansion for “DP” in sentence that begins with “External calibration was applied...”

Q7. Figures 2 and 3 have been changed to Figures 1 and 2 and also the citations “Figures 2 and 1” have been changed to “Figures 1 and 2” in the text. Please check and amend if necessary.

Q8. The value “715 ± 34 99 ± 2 mg/l” seems incorrect in the sentence that begins with “In MOPS buffered media....” Please check and amend if necessary.

Q9. Table citations were not in sequential order. So citations have been renumbered as per style. Please check and amend if necessary.

Q10. Please provide expansion for “DF” in Table IV.

Q11. Please check the suggested placement of footnote “a” in Table IV, amend if necessary.

Q12. Please note that reference and its citation for the mention “Mortazavi and Akbarzadeh” in the sentence that begins with “Furthermore, Mortazavi and Akbarzadeh reported...” are not provided. Kindly check and provide.

Q13. Reference [28] is missing both in the list and in the text. So we have renumbered the subsequent references both in the list and the text. Please check and confirm.

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OPTIMIZATION OF DESFERRIOXAMINE E PRODUCTION BY *STREPTOMYCES PARVULUS*

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Siderophores are produced by a number of microbes to capture iron with outstandingly high affinity, which property also generates biomedical and industrial interests. Desferrioxamine E (DFO-E) secreted by streptomycetes bacteria can be an ideal candidate for iron chelation therapy, which necessitates its cost-effective production for *in vitro* and animal studies. This study focused on the optimization of DFO-E production by *Streptomyces parvulus* CBS548.68. Different combinations of various carbon and nitrogen sources as well as the addition of 3-morpholinopropane-1-sulfonic acid (MOPS) markedly affected DFO-E yields, which were attributed, at least in part, to the higher biomass productions found in MOPS-supplemented cultures. In MOPS-supplemented glucose and sodium glutamate medium, DFO-E productions as high as $2{,}009 \pm 90$ mg/l of culture Q2 medium were reached. HPLC analysis demonstrated that a simple two-step purification process yielded DFO-E preparations with purities of ~97%. MALDI-TOF mass spectrometry analysis showed that purified DFO-E always contained traces of desferrioxamine D2.

Keywords: *Streptomyces parvulus*, desferrioxamine E, MOPS, Na-glutamate

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Introduction

Siderophores are iron chelators produced by many microorganisms to sequester iron from the environment [1]. These compounds bind iron with remarkably high affinity and, hence, they are pivotally important in supporting the growth of microbes and are also required in the invasion of host organisms by many pathogens [2]. Although iron is an abundant element on Earth, its bioavailability is considerably limited in the environment by the low solubility of Fe^{3+} ($\sim 10^{-24}$ mol/l) [3–5].

Some soil bacteria like the Gram-positive *Streptomyces* species produce commercially important antibiotics and other metabolites like iron-chelator siderophores, e.g., desferrioxamine B (DFO-B), a product of *Streptomyces pilosus*, which is employed to treat iron overload in humans [6]. Iron chelators are routinely used for the medication of the patients with iron overloads like in the case of β-thalassemia or sickle cell disease [7, 8]. Several studies have demonstrated that DFO-B is able to mitigate oxidative cell damages in various biological systems through disrupting iron-mediated redox cycling reactions [9–11]. Hartley et al. [11] also showed that DFO-B itself can act as a radical scavenger by donating an electron or hydrogen atom from the hydroxamate center. Therefore, siderophores of both bacterial and fungal origins are considered in the prevention and treatment of oxidative-stress-mediated diseases, e.g., ischemia–reperfusion injury diabetes, inflammation, and atherosclerosis [12–16]. Not surprisingly, there is also an increasing body of evidence that these siderophores are able to target cancer cells selectively [17, 18].

In a recent study published by Todokoro et al. (2015), a promising new application was described for the natural hexadentate iron chelator deferriferrichrysin, fermented by the rice-saccharifying fungus *Aspergillus oryzae* as a food-grade antioxidant [19]. Furthermore, Shimoni et al. (1998) demonstrated that the hexadentate cyclic siderophore desferrioxamine E (DFO-E, also produced by various streptomycetes) possessed significant antioxidant effects in linoleic acid and also in combined linoleic acid–β-carotene emulsions.

These results suggest that various siderophores of microbial origin, including DFO-E, could be candidate drugs to medicate oxidative-stress-or iron-excess-mediated diseases in humans. Because today's knowledge on the optimization of microbial siderophore productions is still rather uneven, further studies are definitely needed to introduce new fermentation processes for the more cost-effective production of microbial siderophores like DFO-E, e.g., for *in vitro* and animal studies [20, 21].

Materials and Methods

Chemicals

D-Glucose monohydrate, agar, K_2HPO_4, NaCl, methanol, chloroform, acetonitrile (HiPerSolv CHROMANORM, isocratic grade for HPLC), and glycerol were purchased from VWR BDH Prolabo (VWR, Radnor, PA, USA); yeast extract and $FeCl_3 \cdot 6H_2O$ from Alfa Aesar (Ward Hill, MA, USA); L-aspartic acid (L-Asp), Amberlite XAD-2, Na-L-glutamate (monosodium salt monohydrate), 3-morpholinopropane-1-sulfonic acid (MOPS), and $ZnSO_4 \cdot 7H_2O$ from Sigma-Aldrich (St. Louis, MO, USA); anhydrous $MgSO_4$, Silica Gel 60, and soluble starch from Merck (Darmstadt, Germany); $CaCO_3$ and K_2SO_4 from Reanal (Budapest, Hungary); and malt extract from Oxoid (Basingstoke, UK), while anhydrous $CaCl_2$ was bought from Scharlau (Barcelona, Spain).

Production and harvesting of spores

S. parvulus CBS 548.68 was cultured on glucose–yeast extract–malt extract (GYM) *Streptomyces* medium (pH 7.2), containing 4.0 g/l glucose, 4 g/l yeast extract, 10 g/l malt extract, 2.0 g/l $CaCO_3$, and 12 g/l agar. After incubation at 28 °C for 9 days, spores were harvested by agitation in sterile 0.9% NaCl solution supplemented with 0.1% Tween-80. Spore suspensions were passed through sterile cotton wool and centrifuged at $12{,}000 \times g$ for 2 min at room temperature, washed with distilled water, centrifuged again, and stored in 20% glycerol at −20 °C. Colony-forming units (CFU) were determined by streaking a limited dilution of spores on GYM *Streptomyces* medium and incubated at 28 °C for 5 days.

Siderophore production

All glasswares were rinsed with 36% HCl and were washed subsequently with distilled water to remove any residual iron. Siderophore production was carried out in glass Erlenmeyer flasks (250 ml) containing 50 ml aliquots of production medium, which were inoculated with 1.9×10^8 CFU spores from glycerol stock, and were incubated at 28 °C and at 4.2-Hz shaking frequency. The basic siderophore production medium (pH 7.5) contained 5 g/l L-Asp or Na-L-glutamate monosodium salt monohydrate (Na-L-Glu), 5 g/l L-lysine monohydrochloride (L-Lys), 2 g/l K_2HPO_4, 2 g/l K_2SO_4, 1 g/l NaCl, 0.1 g/l anhydrous $CaCl_2$, 0.2 g/l anhydrous $MgSO_4$, 0.1 g/l $ZnSO_4 \cdot 7H_2O$, and 2.5%

carbon source (glucose, glycerol, or soluble starch). The buffered siderophore production medium contained the same ingredients as the basic medium, but it was also supplemented with 21 g/l MOPS and autoclaved for 15 min at 121 °C. Carbon sources were autoclaved separately as 25% stock solutions. In order to determine the siderophore productions by different cultures, 1.0 ml of production medium samples were taken at 72-, 96-, and 120-h incubation times after inoculation. Samples were centrifuged at 14,000 × *g* for 5 min at room temperature, and then, supernatants from each flasks (500 µl) were transferred to 1.5-ml Eppendorf tubes and supplemented with 20 µl of 10% $FeCl_3 \cdot 6H_2O$ to saturate all produced siderophores with iron before HPLC analysis. The effects of the carbon source, Q5 nitrogen source, and MOPS were evaluated with variance analysis (ANOVA), and Tukey's difference test using the R 2.3.1 software (The R Development Core Team; http://www.r-project.org/) was adapted to describe the response surface (Lee and Chen 1997).

HPLC analysis

HPLC measurements were carried out with a HP 1090 liquid chromatograph, equipped with autosampler and diode array detector. Ferrioxamines were separated on a mixed-phase column (Luknova Hyper SCX 4.6 mm × 100 mm, 5 µm), in which both the cation exchange and hydrophobic interactions contribute to the separation. The 10-µl injected sample was separated by isocratic elution with 1:4 acetonitrile: 0.5 M NaCl solvent mixture at 0.5 ml/min flow rate. Siderophores were detected at $\lambda = 435$ nm, and Agilent ChemStation was used to evaluate the chromatograms. Commercially available, ferrated DFO-E was used as standard.

Purification of siderophores with column chromatography

Culture media of *S. parvulus* ($V = 1{,}000$ ml, incubation time = 96 h) were centrifuged at 4,000 × *g* for 20 min at 4 °C, and the supernatants were supplemented with 2 g/l of $FeCl_3 \cdot 6H_2O$ to reach iron complexation of all siderophores. Ferrated siderophores were isolated first using Amberlite XAD2 resin and were purified further with Silica Gel 60 chromatography with a chloroform: methanol:water (17.5:6:1) solvent mixture as the mobile phase. Organic solvents were evaporated with a Buchi Rotavapor R-3000 equipment. Following that, the ferrated DFO-E was dissolved in distilled water and analyzed with HPLC, and the two fractions detected with HPLC were analyzed with MALDI-TOF mass spectrometry (MS).

MALDI-TOF MS

HPLC fractions of the purified siderophores were analyzed with MALDI-TOF MS in positive-ion mode using a Bruker Biflex MALDI-TOF mass spectrometer equipped with delayed-ion extraction. Desorption/ionization of the sample molecules was performed with a $\lambda = 337$ nm nitrogen laser, and 2,5-dihydroxy benzoic acid was used as matrix. Spectra from multiple (at least 100) laser shots were summarized using 19-kV accelerating and 20-kV reflectron voltage. External calibration was applied using the $[M + Na]^+$ peaks of cyclodextrins DP 6–8 with *m*/*z*: 995 Da, *m*/*z*: 1,157 Da, and *m*/*z*: 1,319 Da, respectively.

Response surface methodology (RSM)

The optimal medium composition for DFO-E production was determined using the RSM with two selected variables (Na-L-Glu and L-Lys concentrations) with the central composite design and full-factorial design consisting of two-factor-two-level pattern with 11 design points (nine combinations with three replications of the center points). In accordance with our preliminary experiments, DFO-E yields depended highly on L-Lys and Na-L-Glu concentrations. Two plausible variables, glucose and MOPS concentrations, were omitted from the analysis since neither increasing glucose concentration from 25 g/l to 50 g/l nor decreasing to 10 g/l, or decreasing MOPS concentration from 21 g/l to 15 g/l or 10 g/l did not affect the DFO-E yields considerably, while decreasing either L-Lys or Na-L-Glu from 5 g/l to 2.5 g/l reduced DFO-E yields by 20%–40% (data not shown). A full second-order polynomial model was obtained by a multiple regression technique for two factors using the R 2.3.1 software.

Results

Identification of DFO-E produced by S. parvulus

When culture media supplemented with Fe(III) were subjected to HPLC analysis, four clearly distinguishable peaks were identified in each fermentation medium. Two of them, observed at ~3.5-min and ~4.4-min retention times, had the same retention values as the DFO-B and DFO-E standards, respectively (data not shown).

HPLC and MALDI-TOF MS analysis of the purified DFO-E

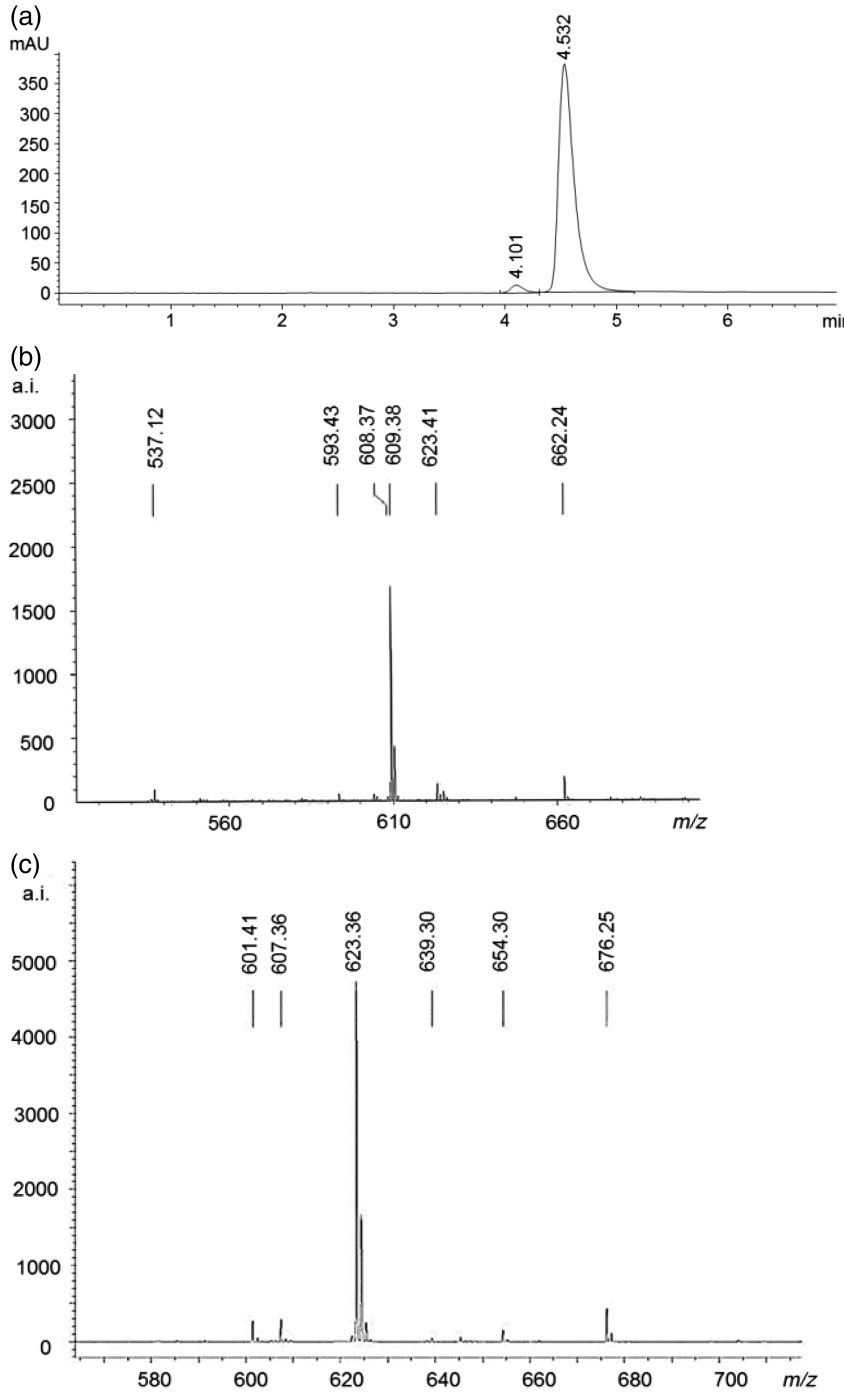
The purity of DFO-E prepared from the culture supernatant of *S. parvulus* after Amberlite XAD and Silica Gel purification was checked with HPLC, and approximately 97% of the siderophore content was DFO-E (~4.5-min retention time, Peak 2 in Figure 1A). A minor siderophore peak always appearing at 4.1-min retention time in all preparations (Peak 1 in Figure 1A) was identical to desferrioxamine D2 (DFO-D2). The chemical structures of DFO-E and DFO-D2 were verified by MALDI-TOF MS analyses (Figure 1B and C).

Influence of MOPS as well as various carbon and nitrogen sources on biomass and siderophore yields

In unbuffered culture media, DFO-E production reached its maximum at 96-h incubation time after inoculation, and it remained constant or even slightly decreased following that (Figure 2). The highest DFO-E production was detected in the glucose + Na-L-Glu medium followed by glucose + L-Asp and glycerol + Na-L-Glu media, while the glycerol + L-Asp medium showed slightly decreased yields. Starch was not a favored carbon source either in L-Asp- (96 ± 3 mg/l) or in Na-L-Glu- (99 ± 2 mg/l) supplemented media, which may indicate that the positive effect of Na-L-Glu is dependent on the nature of carbon source (Figure 2). On the other hand, MOPS supplementation markedly increased DFO-E yields in the presence of all carbon and nitrogen sources and their combinations tested (Figure 2).

In MOPS-buffered media, DFO-E production reached its peak at 120 h after inoculation, and the most beneficial effects of MOPS on DFO-E yields were recorded in the presence of starch, where 7- to 9-fold increases in DFO-E productions were recorded when compared with unbuffered systems (from 96 ± 3 mg/l to 882 ± 179 mg/l in starch + L-Asp and from 99 ± 2 mg/l to 715 ± 34 99 ± 2 mg/l in starch + Na-L-Glu media). The maximum DFO-E yield (1,390 ± 65 mg/l) was detected in MOPS-buffered glucose + Na-L-Glu medium (Figure 2).

The final pH of the fermentation media for all tested combinations (including media with MOPS) was slightly acidic (pH 5.3–6.2) except starch + L-Asp (pH 8.1) and starch + Na-L-Glu (pH 7.1). Dry cell masses correlated well with yields with the exception of unbuffered starch + L-Asp and starch + Na-L-Glu media (Figure 2 and Table I). Surprisingly, the final biomass gains found in MOPS-buffered starch + L-Asp and MOPS-buffered glucose-Na-L-Glu media were comparable (7.9 g/l and 8.9 g/l, respectively), but the DFO-E yields recorded in these systems differed considerably. In MOPS-buffered Na-L-Glu supplemented



Q7 **Figure 1.** HPLC and MALDI-TOF MS analysis of the purified DFO-E

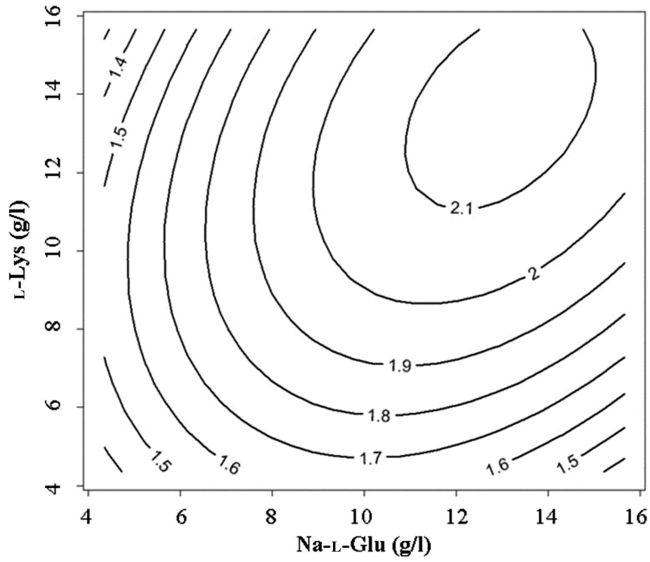


Figure 2. Fitted regression surface showing Na-L-Glu and L-Lys dependence of DFO-E production

Q9 **Table I.** Effect of MOPS, carbon source, and nitrogen source on dry cell mass. MOPS supplementation markedly increased dry cell mass compared with the unbuffered media

DFO-E production medium	Dry cell mass at 72-h incubation (g/l)	Dry cell mass at 96-h incubation (g/l)	Dry cell mass at 120-h incubation (g/l)
Glucose + aspartic acid	2.2 ± 0.2	2.3 ± 0.2	2.4 ± 0.2
Glucose + aspartic acid + MOPS	4.2 ± 0.1	4.7 ± 0.2	4.7 ± 0.2
Glycerol + aspartic acid	2.6 ± 0.1	2.6 ± 0.1	2.7 ± 0.1
Glycerol + aspartic acid + MOPS	5.4 ± 0.5	6.2 ± 0.6	6.4 ± 0.4
Starch + aspartic acid	2.2 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
Starch + aspartic acid + MOPS	4.7 ± 0.2	6.3 ± 0.5	7.9 ± 0.3
Glucose + glutamic acid	5.2 ± 0.1	5.3 ± 0.1	5.5 ± 0.1
Glucose + glutamic acid + MOPS	5.5 ± 0.3	8.1 ± 0.2	8.9 ± 0.1
Glycerol + glutamic acid	3.5 ± 0.3	3.5 ± 0.2	3.7 ± 0.2
Glycerol + glutamic acid + MOPS	5.8 ± 0.5	8.1 ± 0.2	8.4 ± 0.4
Starch + glutamic acid	2.3 ± 0.1	2.3 ± 0.1	2.4 ± 0.1
Starch + glutamic acid + MOPS	6.0 ± 0.3	6.2 ± 0.3	7.9 ± 0.2

Note: Results are shown as mean values of three independent experiments with standard deviations.

Table II. ANOVA of DFO-E production data

Variable	Difference in DFO-E production (mg/l)	Adjusted p values
Effect of carbon source		
Glycerol–glucose	−175.75	<0.000001
Starch–glucose	−372.99	<0.000001
Starch–glycerol	−197.24	<0.000001
Effect of nitrogen source		
Glutamic acid–aspartic acid	202.18	<0.000001
Effect of MOPS		
Without MOPS–with MOPS	−441.32	<0.000001
Effect of sample taking		
72–120 h	−178.13	<0.000001
96–120 h	−35.49	0.26
96–72 h	142.64	<0.000001

Note: According to ANOVA, the effects of carbon source, nitrogen source, and the presence of MOPS were significant. There was no significant difference between 96-h and 120-h sample taking, but at 72 h, the DFO-E concentrations were significantly lower than at 120 h or at 96 h.

media, all the glucose, glycerol, and starch carbon sources resulted in high growth rates, but siderophore productions varied again within a relatively wide range (Figure 2 and Table I).

According to ANOVA, the effects of carbon sources, nitrogen sources, and the presence of MOPS on DFO-E productions were significant (Table II). On the other hand, there was no significant difference between taking samples at 96-h or 120-h incubation times, but DFO-E concentrations recorded at 72-h incubation time were significantly lower than those found at 120-h or 96-h incubations. According to the Tukey's difference test, the DFO-E contents of media containing glucose, glutamic acid, and MOPS incubated for 120 h were significantly higher (adjusted $p < 0.05$) than those found in other cultures at any incubation times tested, except the glucose, glutamic acid, and MOPS containing cultures when incubated for 96 h (Supplementary Material).

Optimization of Na-L-Glu and L-Lys concentrations in DFO-E production using RSM

To describe the optimal medium for DFO-E production with *S. parvulus*, the response surface was studied as a function of Na-L-Glu and L-Lys concentrations as described earlier [22]. Glucose and MOPS levels in the fermentation media were set to the concentrations (25 g/l for glucose and 21 g/l for MOPS) used before for the optimization of the carbon and nitrogen sources. The levels for initial Na-L-Glu and L-Asp concentrations as well as experimental data for the

Table III. Experimental data of the central composite and full-factorial design

	Na-L-Glu (g/l)	L-Lys (g/l)	Coded values[a]		DFO-E (g/l)
Trial	X_1	X_2	Z_1	Z_2	Y
1	6	6	−1	−1	1.7
2	6	14	−1	1	1.6
3	14	6	1	−1	1.7
4	14	14	1	1	2.0
5	15.66	10	1.414	0	2.0
6	4.34	10	−1.414	0	1.5
7	10	15.66	0	1.414	2.1
8	10	4.34	0	−1.414	1.6
9	10	10	0	0	2.1
10	10	10	0	0	2.0
11	10	10	0	0	2.0

Note: The glucose content of the medium was set to 25 g/l, while MOPS to 21 g/l, and DFO-E production was quantified after 120 h of cultivation.
[a] $Z_i = (X_i - 10)/4$.

two-factor-two-level response surface analysis are summarized in Table III. Three repeats were included at the center of the design, and the total number of the test runs was 11. Regression coefficients, their t values, and determination coefficient (R^2) for the full second-order polynomial model of DFO-E production are presented in Table IV. The regression model for DFO-E production was significant ($p = 0.01912$) with a satisfactory value of R^2 (0.89). The estimated DFO-E production at optimal Na-L-Glu (12.96 g/l) and L-Lys (13.60 g/l) concentrations was 2,130 mg/l, which corresponded well with the measured $2{,}009 \pm 90$ mg/l DFO-E production ($n = 3$) at 13 g/l Na-L-Glu and 14 g/l L-Lys concentrations.

Q10 **Table IV.** Regression of coefficients, p values, determination coefficient (R^2), and F-statistic of a full second-order polynomial model for DFO-E production

Q11

Term[a]	Coefficient	p value
b0	2.033	4.524×10^{-7}
b1	0.138	0.01383
b2	0.113	0.02871
b12	0.100	0.11637
b11	−0.154	0.01777
b22	−0.104	0.06578
$R^2 = 0.89$		
F-statistic: 8.124 (DF 5 and 5), p value = 0.01912		

[a] $Y = \mathrm{b0} + \mathrm{b1}Z_1 + \mathrm{b2}Z_2 + \mathrm{b11}Z_{12} + \mathrm{b12}Z_1Z_2 + \mathrm{b22}Z_{22}$, where Y is DFO-E (g/l) production, and Z_1 and Z_2 are Na-L-Glu and L-Lys content of the media (given in coded values), respectively.

Discussion

There is a plethora of evidence that the types of carbon sources and nitrogen sources as well as other culture media supplements (salts and precursors) greatly influence secondary metabolite biosyntheses, e.g., siderophore [20, 23–25]. In good accordance with this, different combinations of carbon sources, nitrogen sources, and MOPS resulted in various DFO-E yields during the production of this hexadentate cyclic siderophore by *S. parvulus*.

In unbuffered systems, i.e., when MOPS was not supplemented, the gains in biomasses recorded in starch + L-Asp and starch + Na-L-Glu media were similar to those found in glucose + L-Asp and glycerol + L-Asp media, but DFO-E yields were considerably higher in the latter cases (Table I). The lower DFO-E yields detected in unbuffered starch systems may be explained by the neutral or even slightly alkaline pH of the fermentation media (pH 7.1 for the starch + Na-L-Glu and pH 8.1 for the starch + L-Asp media). It is worth noting that even in the presence of MOPS, the final pH values measured in most tested culture media were slightly acidic (pH 5.3–6.2), and it is well established that hydroxamate-type siderophores are most stable under acidic conditions [26]. Szigeti et al. [27] also found that triacetylfusarinin C production by the opportunistic human pathogenic fungus *Aspergillus fumigatus* also favored acidic conditions. Because culture media underwent acidification even in the presence of MOPS and DFO-E yields were not affected by decreasing MOPS concentration from 21 g/l to 10 g/l, we can conclude that the positive effects of MOPS on DFO-E yields cannot be attributed solely to its buffering effects in the culture media.

The addition of MOPS is likely to facilitate DFO-E productions through supporting higher biomass productions (Table I) because dry cell masses correlated well with DFO-E yields when siderophore productions in MOPS-buffered and not buffered media were compared (Figure 2 and Table I). On the other hand, the improved yields could not be explained exclusively with the increased biomass gains because various DFO-E productions were observed in MOPS-buffered Na-L-Glu media supplemented with different carbon sources (glucose, glycerol, and starch), meanwhile the biomasses measured at 120-h incubation times were quite similar (Table I). Hence, further experiments are needed to shed light on the mechanism(s) through which MOPS stimulates DFO-E production in *S. parvulus*.

Considering the beneficial effects of the nitrogen sources on siderophore yields, the positive effect of L-Lys on DFO production is well known because L-Lys, the precursor of DFOs, greatly enhances their productions [20, 21]. As shown above, Na-L-Glu also stimulated the biomass production and the biosynthesis of

DFO-E by *S. parvulus* in both MOPS-buffered and unbuffered systems with the exception of media containing starch as carbon source (Figure 2 and Table I). In the absence of MOPS, starch-containing media supplemented with either L-Lys or Na-L-Glu as nitrogen sources provided us with comparable biomass and DFO-E yields (Figure 2 and Table I). These data indicated that both siderophore yields and biomass gains were influenced by the carbon source as well, and the beneficial effect of Na-L-Glu was carbon source dependent. Similar phenomena were also observed when MOPS was also added to the culture media (Figure 2).

After optimizing the Na-L-Glu (14 g/l) and L-Lys (13 g/l) concentrations in glucose (25 g/l) and MOPS (21 g/l) containing fermentation medium, *S. parvulus* CBS548.69 provided us with DFO-E in considerable yields (~2 g/l). Following that high purity grade (~97%), DFO-E preparations were obtained in a simple two-step purification scheme relying on the capture of DFO-E by XAD resin and silica gel chromatography (Figure 1A–C). MALDI-TOF MS demonstrated that purified DFO-E always contained traces of DFO-D2 (Peak 1 in Figure 1A).

There are plenty of experimental data available on the optimization of the production of hydroxamate-type hexadentate siderophores of microbial origin, including compounds produced by either fungi (coprogen, ferrichrysin, and triacetylfusarinin C) [22, 27, 29] or bacteria [23, 30]. Considering yields, maximum coprogen productions by the filamentous fungus *Neurospora crassa* varied between 200 mg/l and 250 mg/l, meanwhile triacetylfusarinin C production by *A. fumigatus* reached as high as 2.9 g/l [22, 27, 29]. Todokoro et al. [19] demonstrated that ferrichrysin production by *A. oryzae* can even reach 2.8 g/l. Q12 Furthermore, Mortazavi and Akbarzadeh reported on almost 2 g/l DFO-B yields with *S. pilosus* ATCC 19797, and Chiani et al. [25] published a 5 g/l DFO-B production with the same streptomycete by adding $Na_2HPO_4 \cdot 12H_2O$, NaH_2PO_4, $MgSO_4 \cdot 7H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCl_2 \cdot 2H_2O$, NaCl, $MnSO_4$, NH_4Cl, KH_2PO_4, and K_2HPO_4 to the medium.

Not surprisingly, although production data for DFO-B [25, 28, 31] are abundant because this siderophore is widely used for the treatment of iron overload in humans, similar data for the optimization of DFO-E production are scarcely available [20, 21]. Nevertheless, the highest DFO-E yield was reported by Meiwes et al. [21], which reached 12 g/l in a fed-batch fermentation system with *Streptomyces olivaceus* TÜ 2718. Although this yield is six times higher than the highest DFO-E production by *S. parvulus* recorded in this study, there are arguments for considering *S. parvulus*, when future DFO-E production technologies will be developed, at least at laboratory scale. For example, the *S. parvulus* CBS 548.68 strain used in this study is commercially available and the shaking flask method reported here may be scaled up simply in bench-top bioreactors [22]. Importantly, the favorable effects of MOPS on DFO-E production can be tested

and, hopefully, also exploited in any other chemically defined fermentation media currently applied in siderophore productions [22, 27, 29].

We hope that our results will enable us to carry out further experiments to reveal the complexometric properties, cytotoxicity, and physiological/pharmacological characteristics of this DFO-E. Large-scale production of various siderophores would make many potential applications of these iron chelators possible in the future, e.g., in managing human diseases like atherosclerosis, imaging fungal infections, or preparing siderophore–antibiotic conjugates [16, 32, 33]. Other possible applications of siderophores include the prevention of post-harvest diseases in stored crops, fruits, and vegetables or using siderophores as food-grade antioxidants or in heavy metal bioremediation technologies [19, 34–36]. Therefore, we need further studies to set up efficient and profitable technologies for the bulk production of these promising compounds.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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