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Defined salt formulations for the growth of *Salinispora tropica* strain NPS21184 and the production of salinosporamide A (NPI-0052) and related analogs

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Abstract Salinosporamide A (NPI-0052) is currently produced by a marine actinomycete, *Salinispora tropica*, via a saline fermentation process using a non-defined, commercially available synthetic sea salt, Instant Ocean. In order to control the consistency of the production of NPI-0052 and related analogs, two chemically defined salt formulations were developed to replace Instant Ocean. A chemically defined sodium-chloride-based salt formulation with similar sodium and chloride contents as in Instant Ocean was found to support higher production of NPI-0052 and a better metabolite production profile for downstream processing than Instant Ocean. A chemically defined sodium-sulfate-based salt formulation with low chloride concentration at 17 mM was found to support a similar NPI-0052 and metabolite production profile as Instant Ocean. The sodium-sulfate-based formulation is a robust formulation for large-scale production process due to its reduced corrosiveness in fermentation as compared with the saline fermentation utilizing Instant Ocean or the sodium-chloride-based salt formulation. The production of NPI-0052 in both chemically defined salt formulations was successfully scaled-up to a 42-l fermentor, indicating that these salt formulations can be used for large-scale manufacturing process.

Keywords Salinosporamide A · NPI-0052 · *Salinispora tropica* · Saline fermentation · Salt formulation

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Introduction

Salinosporamide A (NPI-0052) is a novel, potent proteasome inhibitor (Chauhan et al. 2005, 2006; Groll et al. 2006) isolated from the marine actinomycete *Salinispora tropica* (Feling et al. 2003). It possesses a broad spectrum of activities against various tumors in animal models (Chauhan et al. 2005, 2006; Cusack et al. 2006). It is currently undergoing Phase I clinical studies for the treatment of patients with various cancers (Chauhan et al. 2006). Based on the observations (Mincer et al. 2002; Maldonado et al. 2005) that *S. tropica* requires sea water type media for growth, the preclinical supply and the current clinical supply of NPI-0052 have been prepared by a saline fermentation process of *S. tropica* growing in a medium containing high concentration of sodium chloride (~24 g/L). A commercially available synthetic sea salt, Instant Ocean, has been incorporated into seed and production media to support good yield of NPI-0052 at ~270 mg/L (Reed et al. 2007; Tsueng and Lam 2007; Tsueng et al. 2007).

Like many other complex medium components, the composition of Instant Ocean is non-defined. The supply of Instant Ocean is also subject to lot-to-lot variances. The above two factors might affect the reproducibility of the production profile of NPI-0052 during manufacturing of the clinical supply of NPI-0052. In order to maintain a reproducible production profile for NPI-0052, we decided to develop our own salt formulation with defined chemical composition. The original saline fermentation media employed in the production of NPI-0052 is highly corrosive to the stainless steel fermentors and the processing equipments associated with the manufacturing of NPI-0052 due to the high chloride content. Furthermore, we developed a chemically defined salt formulation with a greatly reduced

level of chloride. This low chloride salt formulation significantly improves the operating process for the manufacturing of NPI-0052.

Materials and methods

Microorganism

The producing strain, *S. tropica* NPS21184, is a single colony isolate of the wild type strain, CNB476. Strain CNB476 was isolated from a sediment sample collected from Cross Harbor, Abaco, Bahamas (Jensen et al. 1991). Strains CNB476 and NPS21184 were deposited with the American Type Culture Collection and assigned the accession numbers PTA-5275 and PTA-6685, respectively.

Growth media and salt formulation

Seed medium SD2 consists of the following ingredients per liter of deionized water: 8 g of glucose (Sigma), 6 g of Hy Soy (Kerry Biosciences), 6 g of yeast extract (USB), and supplemented with either 30 g of Instant Ocean (Aquarium Systems) or one of the two salt formulations described below.

Production medium SHY consists of the following ingredients per liter of deionized water: 10 g of starch (USB), 4 g of Hy Soy, 4 g of yeast extract, 1 g of $CaCO_3$ (Sigma), 0.04 g of $Fe_2(SO_4)_3$ (Aldrich), 0.1 g of KBr (Fisher), and supplemented with either 30 g of Instant Ocean or one of the two salt formulations described below.

Salt formulation I is a sodium-chloride-based formulation and has different compositions for seed and production media due to the presence of different ion contents in the seed and production media. For addition to the seed medium, salt formulation I consists of the following ingredients per liter of deionized water: 24 g of NaCl, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.69 g of KCl, 0.43 g of $CaCO_3$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 85.8 mg KBr, 21.5 mg H_3BO_3, 15.5 mg $SrCl_2$, 2.6 mg NaF, and 208 μg of $CoCl_2 \cdot 6H_2O$. For addition to the production medium, salt formulation I consists of the following ingredients per liter of deionized water: 24 g of NaCl, 0.69 g of KCl, 0.43 g of $CaCl_2 \cdot 2H_2O$, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 2.6 mg of NaF and 52 μg of $CoCl_2 \cdot 6H_2O$.

Salt formulation II is a sodium-sulfate-based formulation and has the same compositions of salt formulation I except replacing sodium chloride by sodium sulfate (10 g/l to 20 g/l).

Culturing conditions for shake flask and fermentors

To prepare inoculum for shake flask culture, a frozen stock culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of seed medium SD2 supplemented with either Instant Ocean or one of the two salt formulations described above. The first seed culture was incubated at 28°C and 250 rpm on a rotary shaker. After 3 days, 5-ml aliquots of the first seed culture were inoculated into 500-ml Erlenmeyer flasks containing 100 ml of the same seed medium. The second seed culture was incubated at 28°C and 250 rpm on a rotary shaker for 2 days. Five-milliliter aliquots of the second seed culture was then transferred to 500-ml Erlenmeyer flasks containing 100 ml of production medium SHY supplemented with either Instant Ocean or one of the two salt formulations described above. After 1 day of incubation at 28°C and 250 rpm, 2 g of Amberlite XAD-7 resin (Sigma) was added to the production cultures. The production cultures were further incubated at 28°C and 250 rpm for an additional 5 days to complete the production cycle of salinosporamides.

For preparation of fermentor cultures, 20-ml aliquots of the second seed culture were inoculated into 2.8-l Fernbach flasks containing 400 ml seed medium. The third seed culture was incubated at 28°C and 250 rpm for 2 days. Two hundred milliliters and 1.5 l of the third seed cultures were inoculated into a 7.5-l fermentor containing 4 l production medium and a 42-l fermentor containing 26 l production medium, respectively. For preparation of production medium containing sodium chloride-based salt formulation I, all medium components except sodium chloride were sterilized at 121°C for 20 min. After cooling to lower than 30°C, the filter-sterilized (via a 0.2 μm filter) sodium chloride solution was added to the fermentor. No special precaution was taken for the production medium containing sodium-sulfate-based salt formulation. All medium components, including sodium sulfate, were sterilized at 121°C for 20 min. After incubating the fermentor cultures at 28°C and 250 to 300 rpm for 38 to 40 h, Amberlite XAD-7 resin was added to the fermentor cultures at a final concentration of 20 g/l. The production cultures were further incubated for an additional 4 days to complete the production cycle of salinosporamides.

Growth analysis

The growth of the culture was determined by centrifuging 10 ml of culture in a 15-ml centrifuge tube at 3,000 rpm for 15 min in a Beckman centrifuge (Allegra model 6). The growth of the culture was expressed as percent packed cell volume defined as the volume of packed cell/volume of culture × 100%.

Extraction and analytical methods

The production of salinosporamides in the fermentation was monitored by Agilent HP1100 high-performance liquid

chromatography (HPLC) using an ACE C-18 reversed-phase column (4.6×150 mm) and a solvent system consisting of water (0.01% TFA) as solvent A and acetonitrile (0.01% TFA) as solvent B. The elution profile was as follows: 100% solvent A for 1 min, followed by a linear gradient to 35% solvent A in 7 min, held at 35% solvent A for 11 min, followed by a linear gradient to 100% solvent B in 8 min, held at 100% solvent B for 9 min at a flow rate of 1.5 ml/min with the detector wavelength set at 210 nm and column temperature at 35°C. The fermentation extract for the HPLC analysis was prepared by extracting production culture (3.5 ml) with an equal volume of ethyl acetate for 1 h. A 1-ml aliquot was evaporated to dryness under a stream of nitrogen and redissolved in 320 μl of dimethyl sulfoxide. Five microliters of the extract was used for HPLC analysis.

Inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) has been successfully applied for trace element analysis (Cubadda et al. 2006; Boulyga et al. 2007) and was applied in this study. ICP-DRC-MS was performed by Applied Speciation (Tukwila, WA) on a Perkin-Elmer ELAN DRC II spectrometer. Aliquots of each sample were introduced into a radio frequency plasma where energy-transfer processes cause desolvation, atomization, and ionization. The ions were extracted from the plasma through a differentially pumped vacuum interface and travel through a pressurized chamber (DRC) containing a specific reactive gas which preferentially reacted with interfering ions of the same target mass to charge ratios (*m/z*). A solid-state detector detected ions transmitted through the mass analyzer, on the basis of their mass-to-charge ratio (*m/z*), and the resulting current is processed by a data handling system. Different reaction gases and settings were applied depending on the target analyte and projected interference. Comparison of the different isotopes, reaction gases, and reaction gas settings allowed for interference monitoring and selection of optimum instrument settings depending on each sample matrix type and element.

Results

Comparison of salinosporamide production in defined salt formulations and synthetic sea salt formulation in shake flask cultures

An initial salt formulation was generated from 23 ions based on reported ionic compositions of Instant Ocean (Bidwell and Spotte 1985) and natural sea water (Pilson 1998). Additional evaluations of these ions were performed in order to simplify the composition of the salt formulation, allowing us to eliminate ions unnecessary for growth, resulting in the current sodium-chloride-based salt formulation I described in the "Materials and methods" section. Since sulfate is known to be significantly less corrosive than chloride to the stainless steel fermentor (Schweitzer 1995; Sedriks 1996; Tsaur et al. 2005), we replace sodium chloride with sodium sulfate in the salt formulation II.

The maximal production of salinosporamides in media containing the non-defined synthetic sea salt formulation (Instant Ocean) and two chemically defined salt formulations was examined in shake flask cultures. The growth yield of *S. tropica* strain NPS21184 was similar in all three media as determined by packed cell volume (5% to 6%, Table 1). The maximal titers of salinosporamides in these three media are reported in Table 1. The compositions of the two salt formulations are the same except that salt formulation I is sodium-chloride-based (411 mM) while salt formulation II is sodium-sulfate-based (106 mM). The sodium-chloride-based salt formulation I supported a better production (22% higher) of NPI-0052 than Instant Ocean. The production of the two analogs, NPI-0047 (75% less) and NPI-2065 (33% less), in salt formulation I is lower than in Instant Ocean. While the production of NPI-0052 was similar in sodium-sulfate-based salt formulation II and Instant Ocean formulation, the production of NPI-0047 (43% less) and NPI-2065 (56% less) is lower in salt formulation II than in Instant Ocean formulation.

Comparison of ions present in different media by ICP-DRC-MS analysis

In order to identify the important ions in the media that may be responsible for improving the production of NPI-0052 and reducing the production of NPI-0047 and NPI-2065, the three media were subject to ICP-DRC-MS analysis and the results for seven ions were reported in Table 2. The sodium and chloride concentrations were similar in media containing salt formulation I and Instant Ocean, between

Table 1 Production of NPI-0052, NPI-0047 and NPI-2065 in production media containing various salt compositions

Media	NPI-0052 (mg/L)	NPI-0047 (mg/L)	NPI-2065 (mg/L)	Growth yield (% packed cell volume)
Synthetic sea salt (Instant Ocean)	228	12.7	7.8	6
Chemically defined salt formulation I (NaCl-based, 411 mM)	279	3.2	5.2	6
Chemically defined salt formulation II (Na_2SO_4-based, 106 mM)	217	7.3	3.4	5

Table 2 ICP-DRC-MS analysis of ions (mM) in media

Media	Na	Cl	K	Ca	Mg	Co	S
Synthetic sea salt (Instant Ocean)	363	406	16	7.6	56	8.5×10^{-5}	37
Chemically defined salt formulation I (411 mM NaCl-based)	426	507	15	1.2	0.64	4.5×10^{-4}	3.3
Chemically defined salt formulation II (106 mM Na_2SO_4-based)	222	17	16	Not determined	0.49	3.0×10^{-4}	124

360 to 500 mM. The potassium concentrations were essentially the same in the three media, between 15 mM to 16 mM. As expected, the chloride concentration in the medium containing salt formulation II was very low at 17 mM, about 4% to 5% the concentration present in the salt formulation I and Instant Ocean. It is also expected to observe the different concentrations of sulfur present in the three media. The calcium and magnesium concentrations are significantly higher in Instant Ocean than the two salt formulation media, by six folds, and 72 to 94 folds, respectively. Conversely, the cobalt concentration was 3.5 to 5.3 folds higher in the two salt formulation media than the Instant Ocean medium.

Examination of sodium chloride concentrations in the defined salt formulation for the production of NPI-0052 in shake flask cultures

The concentrations (0 to 513 mM) of sodium chloride in salt formulation I in shake flask culture were examined in order to determine the optimal sodium chloride concentration used in salt formulation I. The results are summarized in Table 3. No growth of *S. tropica* strain NPS21184 was observed when sodium chloride was excluded in the salt formulation, indicating that the organism requires certain amounts of sodium chloride for growth. At 86 mM sodium concentration, *S. tropica* strain NPS21184 achieved maximum growth yield as determined by the packed cell volume (6%) although only a small amount of NPI-0052 was detected (5.4 mg/l). Maximal production of NPI-0052 (288 mg/l to 295 mg/l) was detected at sodium chloride concentrations of 257 mM to 411 mM in salt formulation I.

Table 3 Effect of sodium chloride concentrations on the production of NPI-0052, NPI-0047 and NPI-2065 in shake flask culture

NaCl concentration	NPI-0052 (mg/L)	NPI-0047 (mg/L)	NPI-2065 (mg/L)	Growth yield (% packed cell volume)
0	0	0	0	0
86 mM	5.4	0.5	0	6
171 mM	235	14	3.9	6
257 mM	288	6.4	6.2	6
342 mM	286	1.2	6.2	5
411 mM	295	2.1	6.2	5
513 mM	223	1.4	4.2	5

Increasing the sodium chloride concentration over 500 mM decreased the production of NPI-0052.

Examination of sodium sulfate concentrations on the production of NPI-0052 in a 7.5-l fermentor

The concentrations (105 mM to 221 mM) of sodium sulfate in salt formulation II in fermentor cultures (5 l medium in a 7.5-l fermentor) were examined in order to determine the optimal sodium sulfate concentration used in salt formulation II (Table 4). The growth yield of *S. tropica* strain NPS21184 was similar at the three concentrations sodium sulfate tested and was the same as the growth yield obtained in the sodium-chloride-based salt formulation. The maximal production of NPI-0052 (221 mg/l) was detected at the highest sodium sulfate tested, at 141 mM. The production of NPI-0047 in the fermentor culture was significantly higher (78%) than in the shake flask culture (Table 1).

Comparison of salinosporamide production in defined salt formulations and Instant Ocean in a 42-l fermentor

The scale up production of salinosporamides in the media containing Instant Ocean, salt formulation I and II was examined in a 42-l fermentor. The maximal titers of salinosporamides from this fermentor study are reported in Table 5. The optimal concentrations of sodium chloride in salt formulation I and sodium sulfate in salt formulation II as determined by experiments described above were used for this study. The concentration of Instant Ocean used in this study is the optimal concentration for supporting the production of NPI-0052 in shake flask culture determined from an earlier study (data not shown). The production of NPI-0052 in the 42-l fermentor has the same trend as the shake flask; with salt formulation I (sodium-chloride-based) produced the highest amount of NPI-0052, followed by Instant Ocean and salt formulation II (Tables 1 and 5). While the production of NPI-0047 was similar in both shake flask and fermentor culture in Instant Ocean and salt formulation I, the production of NPI-0047 was significantly higher in salt formulation II media in fermentor culture than shake flask culture, a trend that was also observed in the small fermentor study (Table 4). The production of NPI-2065 was slightly higher in the fermentor culture than in the shake flask culture (Tables 1 and 5) but with a similar trend of production.

Table 4 Effect of sodium sulfate concentrations on the production of NPI-0052, NPI-0047 and NPI-2065 in a 7.5-l fermentor

Na_2SO_4	NPI-0052 (mg/L)	NPI-0047 (mg/L)	NPI-2065 (mg/L)	Growth yield (% packed cell volume)
70 mM	105	9.4	3.4	5
106 mM	177	13	4.6	6
141 mM	221	12	5.8	6

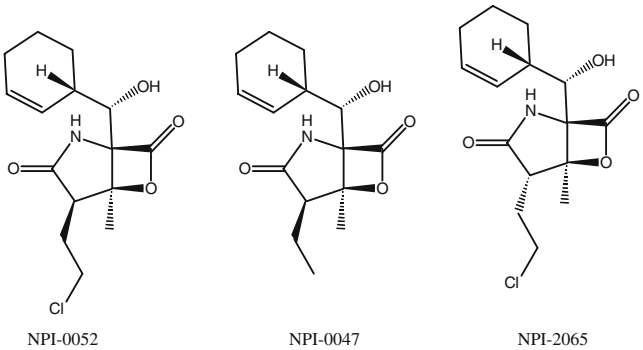

Fig. 1 Structure of *NPI-0052*, *NPI-0047*, and *NPI-2065*

Discussion

We successfully developed two chemically defined salt formulations to replace the undefined synthetic sea salt, Instant Ocean, for the production of NPI-0052 by *S. tropica* strain NPS21184. Salt formulation I is a sodium-chloride-based medium that supported 22% higher production of NPI-0052 than the Instant Ocean medium. The metabolite production profile in salt formulation I was better than Instant Ocean due to the reduced production of NPI-0047 and NPI-2065, two structurally related analogs that interfere with the purification of NPI-0052 (Macherla et al. 2005; Reed et al. 2007). The structures of NPI-0052, NPI-0047, and NPI-2065 are shown in Fig. 1. The concentrations of the major ions, sodium, chloride, and potassium, of salt formulation I are similar to that of Instant Ocean, as determined by ICP-DRC-MS analysis. The concentrations of calcium, magnesium, cobalt, and sulfur are significantly different between the two salt formulations. The concentrations of calcium, magnesium, and sulfur in salt formulation I are six to 88 folds lower than in Instant Ocean, and the concentration of cobalt ion is 5.3-folds higher in salt formulation I than in Instant Ocean. Salt formulation I fulfills the requirements of having a defined composition, supporting reproducible production of a high titer of NPI-0052 and reducing levels of the undesired critical related analogs NPI-0047 and NPI-2065.

Table 5 Production of NPI-0052, NPI-0047 and NPI-2065 in a 42-l fermentor containing various salt compositions

Media	NPI-0052 (mg/L)	NPI-0047 (mg/L)	NPI-2065 (mg/L)	Growth yield (% packed cell volume)
Synthetic sea salt (Instant Ocean)	257	10.6	9.9	6
Chemically defined salt formulation I (411 mM NaCl-based)	274	2.9	7.0	6
Chemically defined salt formulation II (141 mM Na_2SO_4-based)	229	15.6	6.4	6

The sodium-sulfate-based salt formulation II also supported a high level of production of NPI-0052 and acceptable levels of NPI-0047 and NPI-2065, similar to the production titers in Instant Ocean in shake flask culture. The chloride concentration in salt formulation II (17 mM) is about 30-fold less than in salt formulation I and Instant Ocean as determined by ICP-DRC-MS analysis (Table 2). The sulfate concentration in salt formulation II (124 mM) is five and 38 folds higher than Instant Ocean and salt formulation I, respectively. Chloride ion has been identified as the key ion for causing corrosion in stainless steel fermentors and processing equipments (Sedriks 1996; Cruz et al. 1998). Sulfate ion is not only significantly less corrosive than chloride ion to stainless steel (Schweitzer 1995; Sedriks 1996; Tsaur et al. 2005) but it can act as an inhibitor to crevice corrosion to stainless steel (Sedriks 1996). Not only is the chloride concentration in salt formulation II (17 mM) lower than the saline fermentation media (~450 mM), it is significantly lower than some of the media for the commercial production of erythromycin (86 mM), tylosin (68 mM), and daunorubicin (171 mM) by terrestrial actinomycetes (Vandamme 1985). The manufacturing of NPI-0052 using a medium containing the salt formulation II can be considered to be equivalent to a terrestrial fermentation process, eliminating the special operational parameters necessitated by a high saline fermentation process (unpublished information). This is also the rationale for performing the optimization of sodium chloride concentration in shake flask culture (Table 3) and the optimization of sodium sulfate in fermentor culture (Table 4). Significant additional efforts and expenses are required when dealing with the use of high sodium-chloride-based media in stainless steel fermentors and subsequent decontamination and cleaning. Salt formulation II is a robust medium for use in large-scale manufacturing of NPI-0052 due to its low corrosive property. While the production of food products for use in aquaculture by

growing the microalgae *Schizochytrium* and *Thraustochytrium* in a low chloride medium has been described (Barclay 2002), this study represents the first reported production of bioactive secondary metabolite with pharmaceutical application by a marine actinomycete in a low chloride medium.

The scale up production of NPI-0052 in both salt formulation I and II was successfully carried out in a 42-l stainless steel fermentor. This finding supported the concept that both salt formulations can be used for large-scale production of NPI-0052. While the titers of NPI-0052 and NPI-2065 in the fermentor cultures were similar with the shake flask culture, the titer of NPI-0047 was higher in the fermentor culture than in the shake flask culture. The above observation suggests that *S. tropica* strain NPS21184 has different metabolic profiles in shake flask and fermentor cultures. Further evaluation of operating parameters for the fermentor culture is required to lower the production of NPI-0047.

During the investigation of sodium chloride concentrations in salt formulation I to support the production of NPI-0052, we found that 86 mM sodium chloride present in the salt formulation is adequate to support the maximal growth of *S. tropica* strain NPS21184 while no growth of *S. tropica* was observed if the salt formulation was not supplemented with any sodium chloride. The minimal sodium chloride concentration in the salt formulation to support maximal growth of *S. tropica* is therefore between 0 and 86 mM. It is important to investigate the minimal sodium chloride growth requirement for this novel marine actinomycete since there is very limited information available on the nutritional growth requirement of this class of marine actinomycete that is rich in the production of secondary metabolites (Fenical and Jensen 2006; Udwary et al. 2007).

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