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A low-sodium-salt formulation for the fermentation of salinosporamides by *Salinispora tropica* strain NPS21184

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Abstract In this paper, we described the development of a potassium-chloride-based-salt formulation containing low sodium concentrations (5.0 to 11 mM) to support the growth of *Salinispora tropica* strain NPS21184 and its production of salinosporamide A (NPI-0052). The sodium present in the media was essentially derived from the complex nitrogen sources Hy Soy, yeast extract, and peptone used in the media. We demonstrated that good growth rate and yield of *S. tropica* strain NPS21184 were detected in both agar and liquid media containing the potassium-chloride-based-salt formulation with sodium concentration as low as 5.0 mM, significantly less than the critical seawater-growth requirement concentration of 50 mM sodium for a marine microorganism. We also observed good production of NPI-0052 (176 to 243 mg/l) by *S. tropica* strain NPS21184 grown in production media containing the potassium chloride-based-salt formulation. The production of deschloro analog, salinosporamide B (NPI-0047), was significantly lower in the low-sodium-salt-formulation medium than in the high-sodium-salt-formulation media. We demonstrated that while *S. tropica* strain NPS21184 is a novel marine actinomycete that requires high salt content for growth, it does not require sodium-chloride-based seawater-type media for growth and production of NPI-0052.

Keywords Salinosporamide A · NPI-0052 · *Salinispora tropica* · Low-sodium-salt formulation · Non-saline fermentation · Non-seawater-growth requirement

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Introduction

Salinosporamide A (NPI-0052) is a novel, potent proteasome inhibitor (Chauhan et al. 2005; Chauhan et al. 2006; Groll et al. 2006) isolated from the marine actinomycete *Salinispora tropica* (Feling et al. 2003) that is currently undergoing phase I clinical studies for the treatment of patients with various cancers (Chauhan et al. 2006). A chemically defined salt formulation with a reduced level of chloride has been developed for the fermentation of *S. tropica*, which significantly enhances the operating protocol for the manufacturing of NPI-0052 (Tsueng et al. 2008). While *S. tropica* can grow and produce secondary metabolites in this low-chloride medium, the medium is still high in sodium, thus seawater-like in content.

The specific requirement of sodium has been identified as a primary characteristic of marine microorganisms, although the quantity, stability, and uniqueness of the sodium requirement have been topics of much debate (Macleod 1965). Indeed, the requirement of sodium is not unique to marine microorganisms, and several terrestrial non-marine prokaryotes and unicellular eukaryotes have been found to require it for growth (Hutner 1972). In addition, the requirement for sodium may be induced by alterations to the microorganisms' environment (O'Brien et al. 1969). The presence of a primary sodium pump has been suggested to be one of the many criteria for the definition of marine bacteria (Oh et al. 1991), while others have suggested the use of salt ranges required for the microorganisms' optimal growth as a criterion (Imhoff 2001). Regardless of the confounding definition of marine microorganisms and their relationship to sodium, we report here the growth and production of *S. tropica* strain NPS21184 in a non-seawater-based medium with sodium concentration as low as 5.0 mM.

Materials and methods

Microorganism, growth analysis by packed cell volume (PCV), culturing conditions for shake flask, extraction of cultures, HPLC analysis, and inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS) analysis were described in the preceding paper (Tsueng et al. 2008).

Growth media, wash medium, and salt formulation

The composition of salt formulation II was described in the preceding paper (Tsueng et al. 2008). Salt formulation III is a potassium-chloride-based formulation and has different compositions for seed and production media because of the presence of different ion contents in the seed and production media. In the seed medium, salt formulation III consists of the following ingredients per liter of deionized water: 30 g of KCl, 4.29 g of $MgSO_4 \cdot 7H_2O$, 0.43 g of $CaCO_3$, 0.43 g of $CaCl_2 \cdot 2H_2O$, 85.9 mg of KBr, 21.5 mg of H_3BO_3, 15.5 mg of $SrCl_2$, 2.6 mg of NaF, and 208 μg of $CoCl_2 \cdot 6H_2O$. In the production medium, salt formulation III consists of the following ingredients per liter of deionized water: 30 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 $CoCl_2 \cdot 6H_2O$.

Seed medium Lo.BNA consists of the following ingredients per liter of deionized water: 10 g of starch (USB), 4 g of Hy Soy (Kerry Biosciences), 4 g of yeast extract (USB), 1 g of $CaCO_3$ (Sigma), and 40 mg of $Fe_2(SO_4)_3$ (Aldrich) and supplemented with salt formulation II with 15 g/l of Na_2SO_4 (EM Science).

Production medium SHY.KcMC consists of the following ingredients per liter of deionized water: 10 g of starch, 4 g of Hy Soy, 4 g of yeast extract, 1 g of $CaCO_3$, 40 mg of $Fe_2(SO_4)_3$, 100 mg of KBr (Fisher) and supplemented with salt formulation III.

Seed medium SD2 supplemented with 30 g/l of Instant Ocean was described in the preceding paper (Tsueng et al. 2008).

Seed medium A1.Kc4C consists of the following ingredients per liter of deionized water: 10 g of starch, 2 g of peptone (USB), 4 g of yeast extract and supplemented with salt formulation III. Agar medium A1. Kc4C has the same composition as the seed medium A1. Kc4C, with addition of 17 g/l of agar (Difco) as solidifying agent.

Wash medium A1.K consists of the following ingredients per liter of deionized water: 10 g of starch, 2 g of peptone, 4 g of yeast extract, and 30 g of KCl (Sigma). Agar medium A1.K has the same composition as the wash medium A1.K, with addition of 17 g/l agar (Difco) as solidifying agent.

Seed medium SHY.KCD consists of the following ingredients per liter of deionized water: 10 of starch, 4 g of Hy Soy, 4 g of yeast extract, 1 g of $CaCO_3$, 40 mg of $Fe_2(SO_4)_3$ and supplemented with salt formulation III with the omission of KBr.

Sterile inoculation loops were used to transfer washed cells to agar plates containing 20 ml of agar media A1.K and A1.Kc4C. After inoculation, the edges of the agar plates were wrapped with parafilm to reduce evaporation. The agar plates were incubated at 28°C for 4 weeks to observe growth.

Results

Production of salinosporamides by *S. tropica* strain NPS21184 grown in production medium containing 16 mM sodium ion determined by ICP-DRC-MS analysis

S. tropica strain NPS21184 was first grown in seed medium Lo.BNA containing sodium-sulfate-based (15 g/l sodium sulfate) salt formulation II described in Tsueng et al. (2008). The second seed culture was then inoculated into production medium SHY.KcMC containing KCl-based salt formulation III. The only known source of sodium salt in the above production medium is NaF (2.6 mg/l) that supplies 0.06 mM sodium ion to the production medium. The other known source of the sodium ion was derived from 5% seed inoculum (containing 15 g/l sodium sulfate), which contributed an estimated 10.6 mM sodium ion to the production medium after inoculation, significantly more than the sodium ion derived from NaF. The total sodium concentration present in the production medium was calculated to be ~10.7 mM. We observed good growth yield (6% PCV) and production of NPI-0052 (239 mg/l) at day 6 of the production cycle (Table 1). The production of NPI-0047 was low at 0.5 mg/l (0.2% of the concentration of NPI-0052, Table 1).

The actual concentrations of sodium and the other key ions present in the seed medium, production medium, and production medium after inoculation with 5% seed medium

Table 1 Production of salinosporamides by *Salinispora tropica* strain NPS21184 grown in production medium containing 16 mM sodium ion determined by ICP-DRC-MS analysis

Culture age (days)	Titer (mg/l)		
	NPI-0052	NPI-0047	NPI-2065
3	109	0.7	0.9
4	187	1.1	2.5
5	226	0.3	3.5
6	239	0.5	4.0

Table 2 ICP-DRC-MS analysis of key ion concentration (mM) in different media

Media	[Na]	[Cl]	[K]	[Mg]	[Co]	[S]	[Ca]	[Fe]
Lo.BNA[a]	215	17	13	19	1.2×10^{-3}	143	3.8	0.17
SHY.KcMC[b]	11	477	468	0.75	3.7×10^{-4}	1.7	4.1	0.12
SHY.KcMC[b] after inoculation of 5% Lo.BNA[a]	16	453	411	1.6	3.9×10^{-4}	8.8	3.8	0.12
A1.K[c]	5.0	368	441	0.21	5.1×10^{-5}	<0.42	0.14	0.008
A1.Kc4C[b]	5.0	379	458	20	1.0×10^{-3}	30	2.7	0.009
A1 in deionized water	5.2	Not detected[d]	4.2	0.19	6.0×10^{-5}	0.39	0.08	0.008
SHY.KCD[b]	9.3	463	498	20	1.0×10^{-3}	22	3.6	0.12

[a] Containing sodium-sulfate-based-salt formulation II (Tsueng et al. 2008)
[b] Containing potassium-chloride-based-salt formulation III ("Materials and methods" section)
[c] Containing 30 g/l of KCl ("Materials and methods" section)
[d] Below detection limit

were determined by ICP-DRC-MS analysis. The results are reported in Table 2. The sodium concentrations in the production medium SHY.KcMC (11 mM) and production medium after inoculation (16 mM) determined by ICP-DRC-MS analysis were 6 to 10 mM higher than the calculated values, indicating that there are additional sources of sodium present in the media. The potassium concentration in the production medium SHY.KcMC was 468 mM (Table 2), similar to the sodium concentration in sea water (~450 mM; Pilson 1998). The low chloride concentration in the sodium-sulfate-based-salt formulation II medium observed in the preceding paper (Tsueng et al. 2008) was confirmed by ICP-DRC-MS analysis at 17 mM (Table 2). The concentrations of the other key ions (Mg, Co, S, Ca, and Fe) were in good agreement in the three media determined by ICP-DRC-MS analysis (Table 2).

Growth of washed cell of *S. tropica* strain NPS21184 in agar media containing low sodium ion (5.0 mM by ICP-DRC-MS analysis)

To examine the growth yield of *S. tropica* strain NPS21184 in the agar medium containing low level of sodium, strain NPS21184 was first grown in the SD2 seed medium supplemented with 30 g/l of Instant Ocean. After growth (3% PCV) was established, the seed culture was centrifuge washed twice with wash medium A1.K containing 30 g/l KCl and no discrete sodium salt to reduce carryover of the sodium ion from the first seed culture into the subsequent fermentations. Even though no discrete sodium salt was added to wash medium A1.K, ICP-DRC-MS analysis demonstrated that wash medium A1.K contained 5.0 mM sodium ion (Table 2). After the second wash, the washed cell was inoculated onto two agar media, A1.K and A1.Kc4C (supplemented with KCl-based-salt formulation III). The sodium, potassium, and chloride concentrations in A1.K and A1.Kc4C are essentially the same by ICP-DRC-MS analysis (Table 2). Growth of strain NPS21184 was observed on agar medium A1.Kc4C containing 5.0 mM sodium after 1 week of incubation, and excellent growth was achieved after 3 weeks of incubation (Table 3). After 3 weeks of incubation, the growth of strain NPS21184 was transferred to fresh agar medium A1.Kc4C. Good growth of strain NPS21184 was also observed on the agar medium A1.Kc4C of the second transfer (Table 3 and Fig. 1). No growth of strain NPS21184 was observed on agar medium A1.K.

Growth of washed cell of *S. tropica* strain NPS21184 and production of salinosporamide A in low sodium, seed, and production media containing trace amount of sodium ion (5.0 to 11 mM by ICP-MS analysis)

The above washed cell was also used to inoculate into the second seed medium A1.Kc4C to examine the production of NPI-0052 by *S. tropica* strain NPS21184 grown in the low-sodium-liquid medium. After 2 days of incubation, the second seed culture was inoculated into the third seed medium A1.Kc4C to further reduce the carryover effect from the first seed culture. The third seed culture was inoculated into production medium SHY.KcMC containing 11 mM sodium ion (Table 2). Maximal production of NPI-0052 was observed at 176 mg/l at day 6 (Table 4). Low level of NPI-0047 production was again observed (0.6 mg/l, 0.34% of NPI-0052 production) in the medium contain-

Table 3 Growth of *Salinispora tropica* strain NPS21184 grown on agar media A1.Kc4C and A1.K

Agar medium	Week 1	Week 2	Week 3	Week 4
A1.Kc4C (first transfer)	++	++	+++	+++
A1.K (first transfer)	–	–	–	–
A1.Kc4C (second transfer)	++	++	+++	+++

– No growth, −/+ poor growth, + fair growth, ++ growth, +++ very good growth

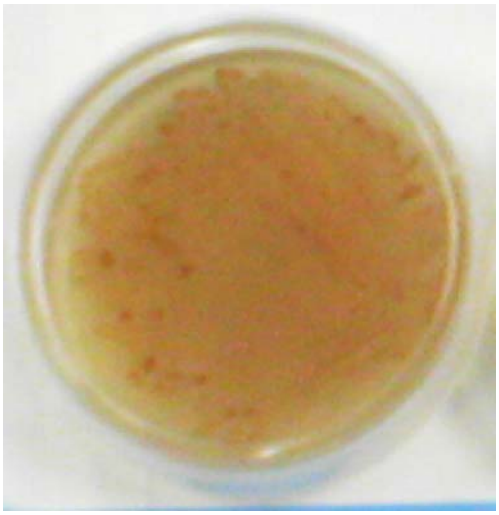

Fig. 1 Growth of washed cell of *S. tropica* strain NPS21184 on agar medium A1.Kc4C containing 5.0 mM sodium ion after 3 weeks of incubation (second transfer)

ing low concentration of sodium (Table 4). Good growth yield (6% PCV) was also observed in the production culture. We did not observe any growth of *S. tropica* strain NPS21184 in the production medium supplemented only with 30 g/l of KCl.

Growth of *S. tropica* strain NPS21184 and production of NPI-0052 in media containing low concentration of sodium ion (9.3 to 11 mM by ICP-DRC-MS analysis)

S. tropica strain NPS21184 was grown in seed (SHY.KCD) and production (SHY.KcMC) media containing low levels of sodium ion, 9.3 and 11 mM, respectively (Table 2). Media SHY.KCD and SHY.KcMC were supplemented with KCl-based-salt formulation III. Strain NPS21184 was grown well in the seed (5% PCV) and production (6% PCV) media containing low sodium concentration. Good production of NPI-0052 (243 mg/l) and low level of NPI-0047 (0.9 mg/l, 0.37% of NPI-0052 production) were again detected in the low-sodium medium (Table 5).

Discussion

In the preceding paper (Tsueng et al. 2008), we reported the development of two sodium-based chemically defined salt formulations to replace the synthetic sea salt, Instant Ocean, for the production of NPI-0052 by *S. tropica* strain NPS21184. In this paper, we developed a potassium-based-salt formulation containing 0.06 mM sodium ion derived from NaF (2.6 mg/l) for supporting the production of NPI-0052. In our first investigation, we found that carryover of sodium ion from the seed medium into the production medium supplemented with the KCl-based-salt formulation III was enough to support the growth of *S. tropica* strain NPS21184 and the production of NPI-0052 by strain NPS21184. The concentration of sodium in the production medium after inoculation was 16 mM, determined by the ICP-DRC-MS analysis (Table 2), about 5.3 mM higher than the amount of sodium ion present in the medium based on the calculation from the known amount of sodium salts from the seed medium (carryover effect) and NaF from the production medium. This suggested that there are other sources of sodium ions present from the other media components. Yeast extract and soy-bean-type products from the medium are known to provide micronutrients to the medium (MacLeod 1965; Miller and Churchill 1986). ICP-DRC-MS analysis of the yeast extract and Hy Soy confirmed the hypothesis that the extra sodium observed in the production medium was derived from yeast extract and Hy Soy. The production titer (239 mg/l) of NPI-0052 in the low-sodium medium (16 mM) is similar to the titer (~270 mg/l) in the high-sodium (~450 mM) saline-fermentation condition (Tsueng et al. 2008). The KCl-based-salt formulation III media consisted of ~450 mM potassium, the same concentration of sodium present in the seawater. It appears that potassium supplemented with the other ions in the salt formulation can replace majority of the sodium in the medium in supporting the growth of *S. tropica* strain NPS21184 and the production of NPI-0052 by strain NPS21184.

Table 4 Production of salinosporamides by washed cell of *Salinispora tropica* strain NPS21184 grown in production medium containing 11 mM sodium ion based on ICP-MS analysis

Culture age (days)	Titers (mg/l)		
	NPI-052	NPI-0047	NPI-2065
3	45	0	0.7
4	100	0	1.0
5	170	0.5	1.8
6	176	0.6	2.1

Table 5 Production of salinosporamides by *Salinispora tropica* strain NPS21184 grown in seed and production media containing 11 mM sodium ion determined by ICP-MS analysis

Culture age (days)	Titers (mg/l)		
	NPI-0052	NPI-0047	NPI-2065
3	88	0.2	0.4
4	188	0.5	2.4
5	211	0.7	2.8
6	243	0.9	3.7

A washed cell experiment was carried out to obtain an inoculum with low-sodium content before inoculating into agar and liquid media containing KCl-based-salt formulation III. Medium A1 supplemented with 30 g/l of KCl (Medium A1.K) was used as the wash medium to remove the sodium ion in the first seed culture before inoculating onto A1-based agar media and the second seed liquid medium. The inclusion of 30 g/l of KCl in the wash medium is to ensure that the medium has the proper ionic strength to prevent lysing of mycelia during the washing process. This also serves to maintain the similar potassium chloride content and ionic strength in the agar media and second seed culture after inoculation of the washed mycelia. Excellent growth rate and yield of *S. tropica* strain NPS21184 were observed in agar medium A1.KcMc after inoculation of the washed mycelia and the subsequent transfer of the growth from the first agar culture to the second agar culture. Observation of the growth in the second agar culture of *S. tropica* strain NPS21184 on A1.Kc4C agar medium indicated that *S. tropica* strain NPS21184 can be grown in agar medium containing sodium concentration as low as 5.0 mM (Table 2). Because the only known sodium salt in medium A1.Kc4C is NaF (2.6 mg/l, 0.06 mM sodium), the rest of the sodium was derived from yeast extract and peptone in the medium. ICP-DRC-MS analysis on yeast extract and peptone (A1 medium in deionized water) confirmed the presence of 5.2 mM sodium (Table 2). It is interesting to observe that no growth of *S. tropica* strain NPS21184 was detected in agar medium supplemented only with 30 g/l KCl (A1.K) but not the other ions present in salt formulation III. Because the concentrations of sodium, potassium, and chloride are very similar in both A1.K and A1.Kc4C agar media, the presence of other ions such as magnesium and calcium are important to support the growth of *S. tropica* strain NPS21184.

We also examined the use of the low-sodium washed cell as inoculum for the liquid culture. Again, growth of *S. tropica* strain NPS21184 was detected in the second and third seed cultures growing in medium A1.Kc4C and the production medium SHY.KcMC, indicating that *S. tropica* strain NPS21184 can be grown in liquid media containing low sodium concentrations of 5.2 to 11 mM. We did not observe any growth of *S. tropica* strain NPS21184 in seed medium simply supplemented with KCl alone, confirming the observation in the agar culture that the presence of other ions such as magnesium and calcium are important to support the growth of *S. tropica* strain NPS21184. Production of NPI-0052 at 176 mg/l was detected in the production medium SHY.KcMC (Table 4). The lower production of NPI-0052 detected in this fermentation condition may be attributed to the use of washed cell as inoculum and/or A1-based medium as the seed medium.

The growth of *S. tropica* strain NPS21184 and the production of NPI-0052 were examined in the seed and production media containing salt formulation III and low sodium concentration of 9 to 11 mM. Good growth yield (5% to 6% PCV) was observed in both seed and production cultures, confirming the ability of *S. tropica* strain NPS21184 to grow in medium containing low sodium concentration of 9 to 11 mM. The growth yield of *S. tropica* strain NPS21184 in the low-sodium media is similar to the growth yield in the high-sodium (360 to 500 mM) saline-fermentation media at 5% to 6% PCV (Tsueng et al. 2008). Excellent production titer of NPI-0052 at 243 mg/l was detected in the above fermentation conditions using both low-sodium seed and production media (Table 5). We also observed a trend that the relative production of NPI-0047 to NPI-0052 in the low-sodium-salt-formulation medium (0.2% to 0.3%) is lower than in the high-sodium-salt-formulations media (1.2% to 3.4%; Tsueng et al. 2008).

Mincer et al. (2002) and Maldonado et al. (2005) reported that *S. tropica* requires seawater for growth, particularly the requirement of sodium ion for growth. However, no specific concentration of sodium-growth requirement for *S. tropica* was reported in these publications. Based on the observation that marine bacteria can grow at one-tenth of the optimal concentration of sodium in seawater after a sufficiently long incubation period (MacLeod 1965), an accepted definition of the seawater-growth requirement for a marine microorganism is the requirement for growth at a sodium concentration of 50 mM, i.e., ~10% of the sodium content in seawater. It is clear from the above study that *S. tropica* strain NPS21184 can be grown at good growth rate and yield in media containing sodium concentration as low as 5.0 mM, ~1% of the sodium content in seawater, by supplementing the media with proper concentrations of potassium and a mixture of other ions present in salt formulation III. Because majority of sodium ion present in the media are derived from the medium components, yeast extract and peptone, we have not yet established the lowest sodium concentration required for supporting the growth of *S. tropica* strain NPS21184. To determine the lowest concentration of sodium for supporting the growth of *S. tropica* strain NPS21184, a defined medium is needed with medium components devoid of sodium. Nonetheless, we established that *S. tropica* strain NPS21184 does not require seawater-type media for growth based on the observation that it can be grown in both agar and liquid media containing 5.0 mM sodium, significantly less that the 50 mM sodium concentration growth requirement. At present, the observations made in this study apply only to the strain we have examined and not to other strains of *S. tropica*. *S. tropica* strain NPS21184 is a single colony isolate derived from *S. tropica* strain CNB476. The natural

variant *S. tropica* strain NPS21184 may represent a single variant that lost the seawater-growth requirement property during isolation. To confirm the seawater-growth requirement of the species of *S. tropica* and the genus of *Salinispora*, the growth of additional *Salinispora* strains in media supplemented with salt formulation III should be examined. Even though *S. tropica* strain NPS21184 does not require seawater for growth, it is indeed a novel marine actinomycete that requires a specific combination of salts to maintain a high enough ionic strength in the medium for growth.

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