Optimization of Medium for the Production of a Novel Aquaculture Probiotic, Streptomyces sp. A1 Using Central Composite Design of Response Surface Methodology

Chau, Ngo Thi Tuong
Ha Noi University of Science, Viet Nam National University

Matsumoto, Masaru
Institute of Tropical Agriculture, Kyushu University

Miyajima, Ikuo
Institute of Tropical Agriculture, Kyushu University

https://doi.org/10.5109/1434376

出版情報：九州大学大学院農学研究院紀要. 59 (1), pp.25-32, 2014-02-28. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **59** (1), 25–32 (2014)

Optimization of Medium for the Production of a Novel Aquaculture Probiotic, *Streptomyces* sp. A1 Using Central Composite Design of Response Surface Methodology

Ngo Thi Tuong CHAU[1]*, Masaru MATSUMOTO[2] and Ikuo MIYAJIMA[2]

Institute of Tropical Agriculture, Kyushu University, Fukuoka 812–8581, Japan
(*Received October 28, 2013 and accepted November 11, 2013*)

A strain, *Streptomyces* sp. A1 isolated from shrimp pond sediments has been identified as an aquaculture probiotic antagonistic to pathogenic *Vibrio harveyi* V7. In the present study, the different carbon and nitrogen sources and growth factors in a mineral base medium were optimized for enhanced biomass production and antagonistic activity, following response surface methodology (RSM). Accordingly, the minimum and maximum limits of the selected variables were determined and a set of fifty experiments programmed employing central composite design (CCD) of RSM for the final optimization. The response surface plots of biomass production showed similar pattern with that of antagonistic activity, which indicated a correlation between the biomass and antagonism. The optimum concentrations of the carbon and nitrogen source, and growth factors for both biomass and antagonistic activity were starch (14.62 g/l), casein (0.93 g/l), NaCl (15.6 g/l), DL–α–alanine (0.233 g/l), and vitamin B_6 (0.023 g/l).

Key words: aquaculture probiotic, central composite design, response surface methodology, *Streptomyces* sp. A1, *Vibrio harveyi* V7

INTRODUCTION

Vibriosis has been recognized as the major systemic bacterial disease of shrimp and prawn larvae in hatcheries (Karunasagar *et al*., 1994; Singh *et al*., 1998). Administration of antibiotics, although recognized as one of the management measures, leads to multiple antibiotic resistance among aquaculture pathogens (Karunasagar *et al*., 1994; Abraham *et al*., 1997; Tendencia *et al*., 2001) and the same is likely to be transferred to human pathogens too. Therefore, as an alternative strategy in the management of vibrios, application of biological control agents, especially antagonistic probiotics has been suggested (Verschuere *et al*., 2000; Irianto *et al*., 2002). As potential probiotic strains in shrimp culture, actinomycetes have many following advantages: (1) the production of antimicrobial and antiviral agents (Austin, 1989; Oskay *et al*., 2004); (2) the degradation of complex biological polymers, such as starch and protein (Barcina *et al*., 1987), lignocellulose, hemicellulose, pectin, keratin, and chitin (Williams *et al*., 1984) which shows the potential to involve in mineralization and nutrient cycles in the culture ponds and in feed utilization and digestion once getting colonized into the host intestine; (3) the competition for nutrients, particularly iron in marine microbes (Kesarcodi *et al*., 2008); (4) the mostly non–pathogenic to the target animals in aquaculture (Yang *et al*., 2007); and (5) the formation heat– and desiccation– resistant spores and the retention of viability during preparation and storage. However, reports on probiotics consisting of actinomycetes are meager.

Thua Thien Hue province (Viet Nam) with the largest Tam Giang–Cau Hai coastal lagoon system of Southeast Asia has many favorable conditions for development of shrimp culture. In fact, remarkable increase in shrimp culture helps to restructure the rural economy in a positive direction. However, in recent years, a rapid, large–scale and often unplanned increase in brackish water shrimp culture ponds have resulted in shrimp epidemic diseases. The shrimp diseases have mainly accounted for vibriosis caused by *Vibrio* spp.

In our previous study, indigenous *Streptomyces* sp. A1 strain isolated and identified as a novel antagonistic probiotic suitable for tropical shrimp aquaculture systems in Thua Thien Hue was found to inhibit a range of vibrios, especially severe pathogenic *Vibrio harveyi* V7 strain (Chau *et al*., 2011). However, to facilitate its application for improving the shrimp aquaculture, mass production of the probiotic was required, warranting immediate development of an appropriate bioprocess technology. The first step in any such process is to design a suitable medium with optimum carbon, nitrogen and other growth factors.

The response surface methodology (RSM) is a combination of statistical and mathematical techniques useful for optimization of bioprocesses, and it can be used to evaluate the effect of several factors that influence the responses by varying them simultaneously in limited number of experiments and thereby it can improve product yield and reduce process variability, time, cost etc. The methodology has been utilized successfully to optimize composition of microbiological media for the production bacteriocins (Leal–Sanchez *et al*., 2002), enzymes (Beg *et al*., 2003), antibiotics (Adinarayana *et al*., 2003), polysaccharides (Wang *et al*. 2004), and organic acids (Xiong *et al*., 2005).

[1] Ha Noi University of Science, Viet Nam National University, 334 Nguyen Trai, Thanh Xuan, Ha Noi, Viet Nam
[2] Institute of Tropical Agriculture, Kyushu University, Fukuoka 812–8581 Japan
* Corresponding author: (E–mail: ngotuongchau@hus.edu.vn).

In the present study, different carbon and nitrogen sources and growth factors were screened by conventional 'one–variable–at–atime method' and further optimized statistically by a fullfactorial central composite design (CCD) of the RSM in a mineral fraction of starch casein broth (SCB) medium for both biomass production of *Streptomyces* sp. A1 and its antagonistic activity to *Vibrio harveyi* V7.

MATERIALS AND METHODS

Actinomyces strain and inoculum preparation

The actinomyces strain, *Streptomyces* sp. A1, used in this study was previously described by Chau *et al.* (2011). The strain was grown in SCB medium supplemented with NaCl 1.5% (pH 7.6), at 35°C for 96 h. Cells then were harvested, washed twice in demineralized water, and resuspended in sterile saline (0.85% NaCl) and used as the inoculum.

Primary screening of nutrients for biomass production and antagonistic activity

Different C–sources (glucose, sucrose, maltose, lactose and starch; HiMedia, India) were screened as the sole source of carbon at concentrations of 8–13 g/l in mineral fraction of SCB medium (g/l) (K_2HPO_4 2, KNO_3 2, NaCl 2, $MgSO_4$. $7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4$. $7H_2O$ 0.01, pH 7.6). Ammonium chloride, ammonium nitrate, ammonium sulphate, casein and urea were screened as sole N–source at concentrations of 0.1–0.6 g/l in the same mineral–based medium with optimal C–source without nitrogen source. Sodium chloride was screened at concentrations of 0–34 g/l. Four amino acids (DL–α–alanine, DL–nor–leucine, L–histidine and L–lysine) (HiMedia, India) and casamino acid (BD Biosciences, USA) were screened as growth factors at concentrations of 0– 0.2 g/l and vitamins such as vitamin A, thiamine, pantothenic acid, pyridoxine and ascorbic acid (HiMedia, India) at concentrations of 0– 0.02 g/l.

The screening of nutrients in a pattern one–at–a–time for biomass production and antagonistic activity of *Streptomyces* sp. A1 and its antagonistic activity to *Vibrio harveyi* V7 were carried out in Erlenmeyer flasks (250 mL capacity) with 100 ml mineral fraction of SCB medium. Sugars, amino acids, sodium chloride and vitamins were filter sterilized using cellulose acetate membrane (0.22 μm pore size; Sartorius) and added to sterile mineral raction of SCB medium. Subsequently, pH was adjusted to 7.6 with sterile 1 M NaOH or 1 M HCl. All flasks were inoculated with the inoculum to a final concentration of approximate 10^8cfu per milliliter. Incubations were done in a temperature controlled rotary shaker (SI–600R, Jeiotech Scientific, Korea) at 35°C, 120 rpm for 96 hours obtained from a previous study (data not given).

Analysis of the sample

The activity against pathogenic *Vibrio harveyi* V7 of *Streptomyces* sp. A1 was determined using the double–layer agar method. The actinomycetes was inoculated on petri dishes containing 15 ml SCA and incubated at 35°C for 4 days. Then TCBS agar medium (HiMedia, India) was poured onto the basal layer containing actinomycetes colonies. *Vibrio harveyi* V7 were inoculated in flask containing 50 ml peptone alkaline (10 g peptone, NaCl 10 g, distilled water to 1 L, pH 8.5) at 35°C for 24 hours, then plated onto the top layer, respectively. The inhibition zones (halo zones) were measured after incubation at 35°C for 24 hours (You *et al.*, 2005).

The cultured suspension was left to stand for 30 min to allow the vegetative biomass (micro–colonies) of the *Streptomyces* sp. A1 to settle. The biomass was harvested using sterile filter papers and a vacuum filter, washed with sterile distilled water at least three times, dried at 35°C until reached a constant weight.

Experiment design and optimization using response surface methodology

By conventional 'one–variable–at–a–time' approach, the minimum and maximum limits of the variables were continuously determined and a set of fifty experiments was programmed employing response surface methodology (RSM). Central composite design (CCD) of the RSM was used for the final optimization experiment. CCD has three groups of design points: two level factorial or fractional factorial design points, axial points (sometimes called "star" points), and centre points. CCD's are designed to estimate the coefficients of a quadratic model. The experiments were done using the software Design Exprert 7.0 (Stat–Ease, USA). Finally validation was carried out in shake flasks under conditions predicted by the model.

Proliferation of probiotic using medium optimized by CCD of RSM

Streptomyces sp. A1 was cultivated in medium optimized by CCD of RSM sterilized for 15 min at 121°C in 250 mL Erlenmeyer flasks, on the incubated shaker (Jeiotech SI–600R, Korea) at 150 rpm, 30°C for 2 days. Then, this suspension were inoculated into the sterilized such fresh medium in a 10–L fermentor (Infors Labfors HT, Switzerland) with a ratio of 1: 10 (v/v) and proliferated at well–controlled optimal fermental conditions for 3 days. After that, the fementor was left to stand for 30 min to allow the vegetative biomass (micro–colonies) of the *Streptomyces* sp. A1 to settle. The biomass was harvested using sterile filter papers and a vacuum filter, washed with sterile distilled water at least three times, dried at 35°C for 30 min and weighted.

RESULTS

Screening of nutrients for biomass production and antagonistic activity

Starch, casein, NaCl, DL–α–alanine, and vitamin B6 appeared to be suitable nutrients for biomass production of *Streptomyces* sp. A1 and its antagonistic activity to pathogenic *Vibrio harveyi* V7 and chosen for the further study by a 'one–variable– at–a–time' method. The minimum and maximum limits of the variables were starch

0.6–29 g/l, casein 0–1.88 g/l, NaCl 1.4–30.8 g/l, DL–α–alanine 0–0.445 g/l and vitamin B_6 0–0.046 g/l.

Optimization of medium for biomass production and antagonistic activity

The most popularly used CCD of RSM was employed to maximize the biomass production and antagonistic activity. The interactive effect of nutritional factors on both biomass and activity was also investigated. The coded values of the independent variables along with the experimental values of biomass and antagonistic activity are given in Tables 1 and 2. The goodness of fit of the model was checked by coefficient of determination (R^2). R^2 was 0.8597 in the case of biomass and 0.9010 in the

Table 1. Central composite design matrix of the variables (g/l) along with the experimental values (n=3) of biomass (cell dry mass. g/l) and antagonistic activity (diameter of inhibition zone in mm)

Expt. No.	Starch	Casein	NaCl	DL–α–alanine	Vit.B_6	Biomass	Activity
1	0.6	0	1.4	0	0	2.19	0
2	29	0	1.4	0	0	2.35	0
3	0.6	1.88	1.4	0	0	2.40	0
4	29	1.88	1.4	0	0	2.47	0
5	0.6	0	30.8	0	0	2.28	0
6	29	0	30.8	0	0	2.37	0
7	0.6	1.88	30.8	0	0	2.33	0
8	29	1.88	30.8	0	0	2.28	0
9	0.6	0	1.4	0.455	0	2.42	0
10	29	0	1.4	0.455	0	2.26	0
11	0.6	1.88	1.4	0.455	0	2.61	0
12	29	1.88	1.4	0.455	0	2.52	0
13	0.6	0	30.8	0.455	0	2.55	0
14	29	0	30.8	0.455	0	2.63	0
15	0.6	1.88	30.8	0.455	0	2.32	0
16	29	1.88	30.8	0.455	0	2.18	0
17	0.6	0	1.4	0	0.046	2.63	0
18	29	0	1.4	0	0.046	2.18	0
19	0.6	1.88	1.4	0	0.046	2.51	0
20	29	1.88	1.4	0	0.046	2.51	0
21	0.6	0	30.8	0	0.046	2.27	0
22	29	0	30.8	0	0.046	2.37	0
23	0.6	1.88	30.8	0	0.046	2.17	0
24	29	1.88	30.8	0	0.046	1.77	0
25	0.6	0	1.4	0.455	0.046	2.25	0
26	29	0	1.4	0.455	0.046	2.65	0
27	0.6	1.88	1.4	0.455	0.046	2.44	0
28	29	1.88	1.4	0.455	0.046	2.62	0
29	0.6	0	30.8	0.455	0.046	2.43	0
30	29	0	30.8	0.455	0.046	2.56	0
31	0.6	1.88	30.8	0.455	0.046	2.33	0
32	29	1.88	30.8	0.455	0.046	1.67	0
33	0	0.94	16.1	0.2275	0.023	2.22	0
34	48.57	0.94	16.1	0.2275	0.023	1.84	0
35	14.8	0	16.1	0.2275	0.023	3.35	0
36	14.8	3.175	16.1	0.2275	0.023	3.24	0
37	14.8	0.94	0	0.2275	0.023	2.64	0
38	14.8	0.94	51.06	0.2275	0.023	2.17	0
39	14.8	0.94	16.1	0	0.023	3.42	8
40	14.8	0.94	16.1	0.7685	0.023	3.56	8.2
41	14.8	0.94	16.1	0.2275	0	3.45	8.8
42	14.8	0.94	16.1	0.2275	0.077	3.63	9.2
43	14.8	0.94	16.1	0.2275	0.023	4.91	18.2
44	14.8	0.94	16.1	0.2275	0.023	4.92	18.3
45	14.8	0.94	16.1	0.2275	0.023	4.88	19.7
46	14.8	0.94	16.1	0.2275	0.023	4.90	18.8
47	14.8	0.94	16.1	0.2275	0.023	4.91	19.8
48	14.8	0.94	16.1	0.2275	0.023	4.94	19.7
49	14.8	0.94	16.1	0.2275	0.023	4.89	19.2
50	14.8	0.94	16.1	0.2275	0.023	4.93	20.2

Table 2. Statistical analysis of experimental values (n=3) of biomass and antagonistic activity

Parameter	Biomass	Activity
Std. Dev.	0.048	2.95
R–Squared	0.8597	0.9010
Mean	2.9	3.76
Coefficient of Variation (%)	16.57	78.43

case of antagonistic activity. It could be expressed in percentage also, and it is interpreted as the percentage variability in the response in the given model. As per the model, sample variation of 85.97% for biomass and 90.10% for antagonistic activity was attributed to the independent variables (>80%). A higher value of correlation coefficient (R) indicated an excellent correlation between independent variables. For biomass, R–value was 0.9272 and for antagonistic activity 0.9492. The RSM gave the following regression equations for the biomass and antagonistic activity as a function of starch (a), casein (b), sodium chloride (c), DL–α–alanine (d) and vitamin B_6 (e). Final equations in terms of coded factors are:

$$\text{Biomass}=0.221+0.9290.E^{-003}a+0.103b+8.741E^{-003}c+0.331d+3.518e-3.488E^{-004}ab-1.382E^{-005}ac+1.773E^{-004}ad-6.602E^{-003}ae-7.922E^{-004}bc-0.013bd-0.202be+3.208E^{-004}cd-0.017ce-0.181de-3.003E^{-004}a^2-0.043b^2-2.498E^{-004}c^2-0.67d^2-63.95e^2$$

$$\text{Antagonistic activity}=0.302+0.589a+8.54b+0.598c+22.79d+211.74e-0.020a^2-4.546b^2-0.0186c^2-50.003d^2-4585.879\ e^2$$

The interaction between nutrients and their effect on biomass production and antagonistic activity of *Streptomyces* sp. A1 are presented in Figs 1 and 2. The response surface plot of biomass production showed similar pattern with that of antagonistic activity indicating a strong correlation between them. Optimum concentration of the carbon, nitrogen sources, NaCl and growth factors for both biomass and antagonistic activity, obtained from the regression equations, was starch (14.62 g/l), casein (0.93 g/l), NaCl (15.6 g/l), DL–α–alanine (0.233 g/l), and vitamin B_6 (0.023 g/l).

Validation of the model

The validation was carried out in shake flasks under optimum conditions of the media predicted by the model. At these conditions, the predicted biomass production was 4.81 g/l and antagonistic activity in terms of halo zone was 24.5 mm. The experimental values were 4.75 g/l for biomass production and 18 mm diameters for halo zone. The value of biomass of *Streptomyces* sp. A1 obtained was 17 g/l when proliferating with above medium optimized by CCD of RSM in 10–L fermenter (Infors Labfors HT, Switzerland) at well–controlled optimal fermental conditions (not given here) (Figs 3 and 4).

DISCUSSION

Streptomyces sp. A1 showed the activity against pathogenic strain *Vibrio* sp. V7 through production of inhibitory compounds was suggested as novel antagonistic probiotic for shrimp aquaculture. Their growth and the accumulation of antagonistic products are strongly influenced by culture medium (Chau *et al*., 2011). However, no defined medium has yet been established for mass production of any antagonistic probiotic used in aquaculture (Preetha *et al*., 2007). Each organism has its own requirement for maximum biomass and antagonistic compound production (Elibol, 2004). Therefore, this requirement necessitated the present study to optimize carbon and nitrogen sources and growth factors for *Streptomyces* sp. A1 so that a commercial production process could be enhanced. Among the medium components tested starch, casein, NaCl, DL–α–alanine and vitamin B6 can act as limiting factors and minor variations in their concentrations may alter the biomass production and antagonistic activity. The conventional method of single factor optimization by maintaining other factors involved at an unspecified constant level is not only tedious, but also can lead to misinterpretation of results, especially because the interactions between different factors is overlooked (Lotfy *et al*., 2007). However, central composite design (CCD) using response surface methodology (RSM) provides important information regarding the optimum level of each factors along with its interactions with other factors and their effects on product yield (Elibol, 2004). In the present study, response surface of biomass showed similar pattern with the response surface of antagonistic compound indicating a strong correlation between the biomass and antagonistic compound production. Composition of the medium obtained from the model for biomass production and antagonistic activity contained starch (14.62 g/l), casein (0.93 g/l), NaCl (15.6 g/l), DL–α–alanine (0.233 g/l), and vitamin B_6 (0.023 g/l). In comparision to the medium composition derived based on conventional 'one– at– a–time' method, in the above composition of the medium designed using response surface methodology, biomass production could be increased by 14% (4.20 g/l to 4.75 g/l) and antagonistic activity by 53% (11.7 mm to 18.0). Moreover, the result of the proliferation of probiotic using medium optimized by CCD of RSM in 10–L fermenter (Infors Labfors HT, Switzerland) at well–controlled optimal fermental conditions showed that biomass of *Streptomyces* sp. A1 increased by 3.5 times (4.75 g/l to 17 g/l) compared to proliferation in above experimental shake flasks. It may be due to cell growth and the biosynthesis of antagonistic products are not only impacted by medium but also by fermentation conditions such as

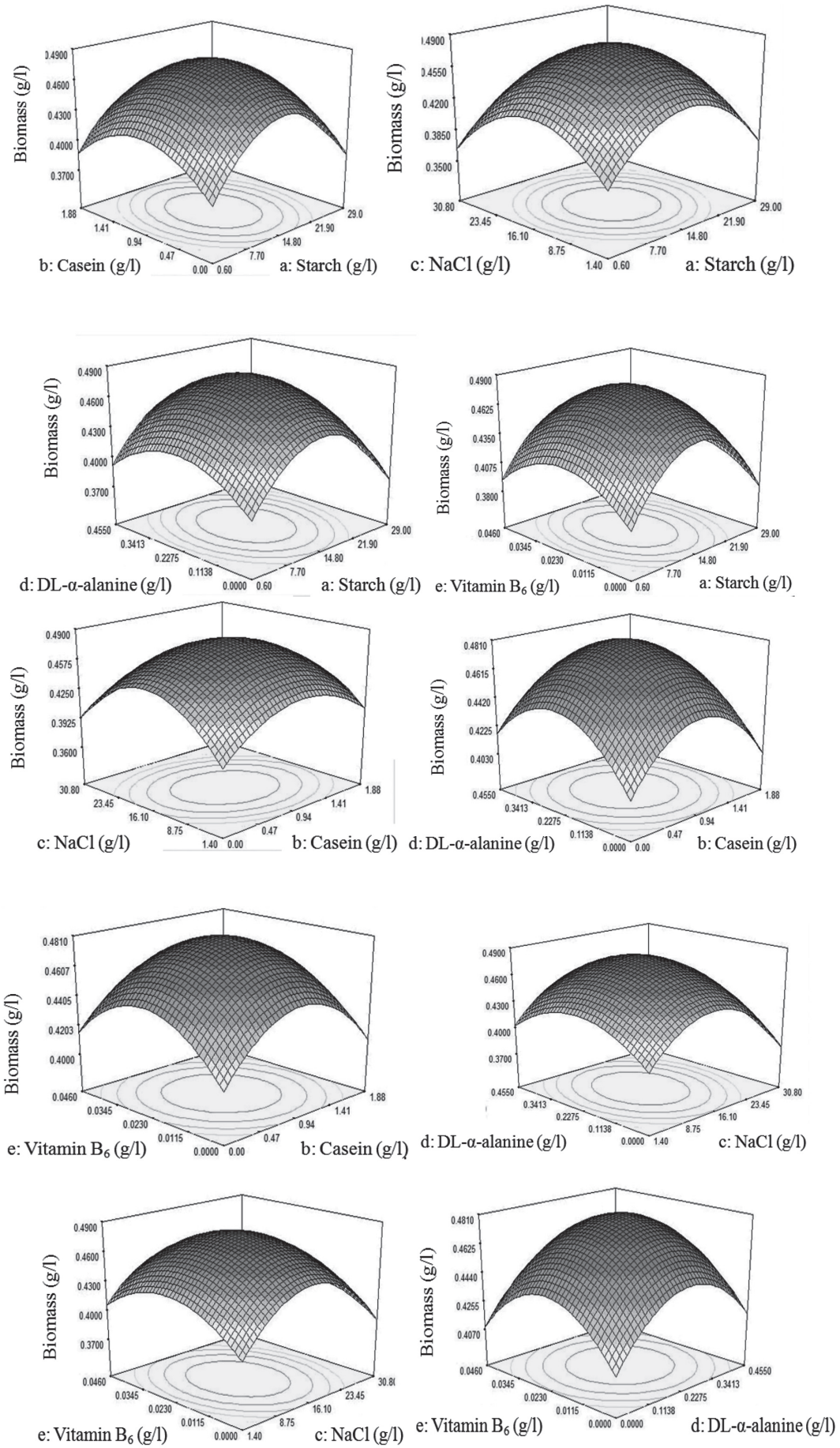

Fig. 1. Interaction of nutrients (starch, casein, NaCl, DL–α–alanine and vitamin B_6) on biomass production (g/l) of *Streptomyces* sp. A1.

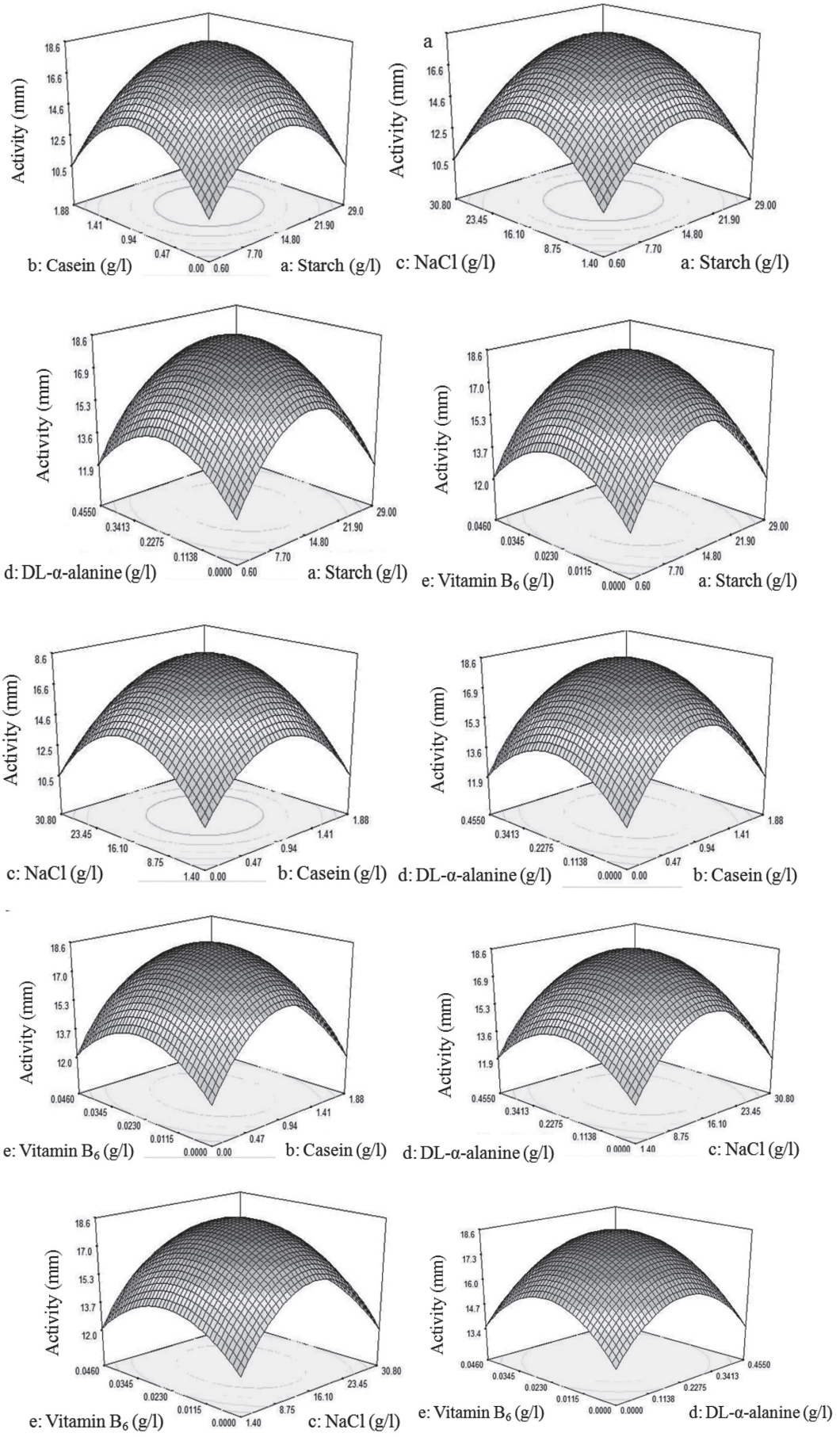

Fig. 2. Interaction of nutrients (starch, casein, NaCl, DL–α–alanine and vitamin B_6) on antagonistic activity (diameter of inhibition zone in mm) of *Streptomyces* sp. A1.

Fig. 3. Biomass of *Streptomyces* sp. A1 after 2 days in flasks on incubated shaker.

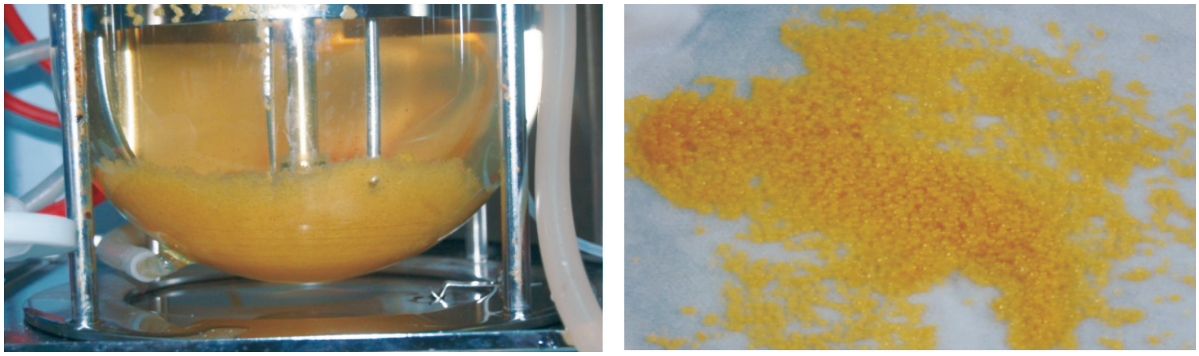

Fig. 4. Biomass of *Streptomyces* sp. A1 after 3 days in 10–L fermenter.

pH, temperature, agitation and oxygen availability to achieve high product yields. In conclusion, the ultilized method was proved to be a powerful tool for the optimization of medium for probiotic production from *Streptomyces* sp. A1, which was efficient, simple, time and cost saving. Furthermore, the information obtained is considered fundamental and useful for the development of antagonistic probiotic production in aquaculture on a large scale.

ACKNOWLEDGEMENTS

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number **106.03–2011.59**.

REFERENCES

Abraham, T. J., R. Manley, R. Papanippan and K. Dhevendran 1997 Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. *J. Aqua. Trop.*, **12**: 1–8

Adinarayana, K., P. Ellaiah, B. Srinivasulu, R. Bhavani Devi and G. Adinarayana 2003 Response surface methodological approach to optimize the nutritional parameters for neomycin production by *Streptomyces marinensis* under solid–state fermentation. *Process. Biochem.*, **38**: 1565–1572

Austin, B. 1989 Novel pharmaceutical compounds from marine bacteria. *J. Appl. Bacteriol.*, **67**(5): 461–470

Barcina, I., J. Iriberri and L. Egea 1987 Enumeration isolation and some physiological properties of actinomycetes from sea water and sediment. *Syst. Appl. Microbiol.*, **10**: 8–91

Beg, Q. K., V. Sahai and R. Gupta 2003 Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. *Process. Biochem.*, **39**: 203–209

Chau, N. T. T., X. H. Nguyen, L. T. N. Thuan, M. Matsumoto and I. Miyajima 2011 Identification and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. isolated from shrimp culture pond sediments in Thua Thien Hue–Viet Nam. *J. Fac. Agr., Kyushu Univ.*, **56**(1): 15–22

Elibol, M. 2004 Optimization of medium composition for actinorhodin production by *Streptomyces coelicolor* A3(2) with response surface methodology. *Process. Biochem.*, **39**: 1057–1062

Irianto, A. and B. Austin 2002 Probiotics in aquaculture. *J. Fish Dis.*, **25**: 633–642

Karunasagar, I., R. Pai, G.R. Malathi and I. Karunasagar 1994 Mass mortality of *Panaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture*, **128**: 203–209

Kesarcodi, W. A., H. Kaspar, M. J. Lategan and L. Gibson 2008 Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture*, **274**(1): 1–14

Lotfy W. A., Ghanem K. M. and EI–Helow E. R. 2007. Citric acid production by a novel *Aspergillus niger* isolate: Ⅱ. Optimization of process parameters through statistical experimental designs. *Bioresour. Technol.*, **98**: 3470–3477

Oskay, M., A. U. Tamer and C. Azeri 2004 Antibacterial activity of some actinomycetes isolated from farming soils of Turkey. *Afr. J. Biotechnol.*, **3**(9): 441–446

Preetha, R., N.S. Jayaprakash, R. Philip and I. S. B. Singh 2007 Optimization of medium for the production of a novel shrimp aquaculture probiotic, Micrococcus MCCB 104 using Central Composite Design. *Biotechnol. Bioprocess Eng.*, **12**: 548–555

Sanchez, M. V. L., R. J. Diaz, A. M. Barragan, A. G, Fernandez and J. L. R. Barba 2002 Optimization of bacteriocin production by

batch fermentation of *Lactobacillus plantarum* LPCO10. *Appl. Environ. Microbiol.*, **68**: 4465–4471

Singh, I. S. B., P. Lakshmanaperumalsamy and D. Chandramohan 1998 Bacterial flora of pond reared *Penaeus indicus* (Milne Edwards). *J. Aqua. Trop.*, **13**: 133–142

Tendencia, E. A. and L. D. de la Pena 2001 Antibiotic resistance of bacteria from shrimp ponds. *Aquaculture*, **195**: 193–204

Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete 2000 Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. R.*, **64**(4): 655–671

Williams, S. T., S. Lanning and E. M. H. Wellington 1984 Ecology of actinomycetes. *In* "The biology of actinomycetes", ed. By M. Goodfellow, M. Mordarski and S. T. Williams, Academic Press Inc., London, pp. 481–528

Wang, Y. X. and Z. X. Lu 2004 Statistical optimization of media for extracellular polysaccharide by *Pholiota squarrosa* (Pers. ex Fr.) Quel. AS 5.245 under submerged cultivation. *Biochem. Eng. J.*, **20**: 39–47

Xiong, C., C. Shouwen, S. Ming and Y. Ziniu 2005 Medium optimization by response surface methodology for poly–γ–glutamic acid production using dairy manure as the basis of a solid substrate. *Appl. Microbiol. Biotechnol.*, **69**: 390–396

Yang, J., L. Chen, L. Sun, J. Yu and Q. Jin 2007 VFDB 2008 release: an enhanced web–based resource for comparative pathogenomics. *Nucleic. Acids. Res.*, **36**: 539–542

You, J., L. X. Cao, G. F. Liu, S. N. Zhou, H. M. Tan and Y. C. Lin 2005 Isolation and characterization of actinomycetes antagonistic to pathogenic *Vibrio* spp. from nearshore marine sediments. *World. J. Microb. Biot.*, **21**(5): 679–682