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OPTIMIZATION OF CULTURE CONDITIONS OF *STREPTOMYCES ANTIBIOTICUS* STRAIN 1083 TO IMPROVE THE ANTIMICROBIAL ACTIVITY AGAINST *AEROMONAS HYDROPHILA*

Nguyen Xuan Canh[1,✉], Tran Thi Thu Hien[1], Nguyen Thanh Huyen[1], Pham Le Anh Minh[1], Tran Bao Tram[2], Nguyen Thi Thanh Mai[2]

[1]*Vietnam National University of Agriculture*
[2]*Center for Experimental Biology, National Centre for Technological Progress*

✉ To whom correspondence should be addressed. E-mail: nxcanh@vnua.edu.vn

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SUMMARY

Fish is a healthy, high protein and low fat food that encourages the health and growth of people, especially children. However, in fact fish is very sensitive to many diseases which affects the productivity and quality of fish. Therefore, identifying the cause of the diseases and finding preventive measures become an urgent task today. In the previous study, we isolated *Streptomyces antibioticus* strain 1083 that has the ability to antagonize *Aeromonas hydrophila* - a pathogenic bacterium in fish. Based on the obtained results, we continue to perform this study to determine optimal conditions for the culture of *S. antibioticus* strain 1083 in order to produce antimicrobial compounds against *A. hydrophila.* The production of antagonists by the strain 1083 was optimized by controlling the condition of different inoculations such as media, pH, temperature and incubation period. The results indicated that International Streptomyces Project 2 (ISP2) was the best medium for *S. antibioticus* strain 1083 to produce the highest antimicrobial activity against *A. hydrophila* with 32 mm in diameter of inhibited zone. The actinomycete strain 1083 could express the maximum antimicrobial activity when they were incubated in shaker incubator (200rpm) at 40^{o}C with pH8 in 8 days. The ability of the actinomycete strain in antagonism against *A. hydrophila* was evaluated by adding different culture medium volume of *S. antibioticus* strain 1083. With adding 10% cultured solution volume of *S. antibioticus* strain 1083 into the culture medium of *A. hydrophila*, after 1 day of inoculation the number of pathogenic bacteria cells were completely eliminated.

Keywords: *Aeromonas hydrophila, Antimicrobial activity, Culture condition, Optimization, Streptomyces antibioticus*

INTRODUCTION

Fish is an important food source of protein for humankind. Fish not only is a high-protein food, but also provides essential nutrients and micronutrients for the development of human (FAO, 2014). However, fish is susceptible to a wide range of diseases, which are very difficult to control. The diseases cause the decline of fish productivity, which leads to economic losses of fish farmers. In 1997, World Bank estimated that financial loss caused by the diseases to aquaculture was about US$ 3 billion per annum (Subasinghe, Phillips, 2002). According to Faruk *et al.,* (2004), the fish diseases have a huge impact on Bangladesh's economy with approximately 15% of annual average fish production lost (Faruk *et al.*, 2004). Among the causes of fish diseases, bacterial pathogens are responsible for serious diseases in fish. Mohan (2007) reported that a lot of bacterial pathogens causing the mortality of fish seed in hatcheries which include *Aeromonas, Vibrio* and *Pseudomonas* (Mohan, 2007). One of the major bacterial pathogens is *Aeromonas* spp. In particular, *A. hydrophila* causes surface ulcerative disease in fish known as "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia", or "Ulcer Disease". The disease is characterized by swollen abdomen, red mouth, hemorrhage in external surface and surrounding of the anus (Alain, 2009). In Vietnam,

A. hydrophila also causes serious damage to fish farmers in the Mekong Delta because the fish disease appears throughout the year. In acute cases, infected fish can be fatal from 80% to 90% (Lien, 1998).

Streptomyces are economically and biotechnologically valuable prokaryotes that they are responsible for production of bioactive secondary metabolites, notably antibiotics, antitumor agents, immunosuppressive agents and enzymes. Among biological factors to inhibit pathogenic bacteria, *Streptomyces* is the most potential group because they produce a large amount of antibiotics (Selvakumar *et al*., 2010). Up to now, approximately 80% of 8000 antibiotics have been produced from *Streptomyces* (Dhanasekaran *et al*., 2012). In the previous study "Characterization and identification of a *Streptomyces* strain with biocontrol activity against *A. hydrophila* causing haemorrhage disease in fish", *S. antibioticus* strain 1083 expressed strong antagonism against *A. hydrophila*. Thus, in this study, experiments were conducted to test effects of different culture conditions of *S. antibioticus* strain 1083 on their antagonism against *A. hydrophila* and identify the optimal conditions which lead the *Streptomyces* strain to produce the most bio-products inhibiting *Aeromonas* infection in fish.

MATERIALS AND METHODS

Materials

S. antibioticus strain 1083 used in this study was isolated, identified and stored at Laboratory of Microbial Biotechnology Department, Biotechnology Faculty, Vietnam National University of Agriculture (Canh *et al*., 2018). *A. hydrophila* causing haemorrhage disease was received from Aquaculture Faculty, Vietnam National University of Agriculture.

Selection of culture media

Four liquid media SCA (soluble starch 10 g, NaCl 3 g, KH_2PO_4 0.5 g, casein 10 g, MgSO4 0.5 g, distilled water 1000 mL, pH 7.5-7.8); GAUSE-1 (Soluble starch 20 g, KNO_3 1 g, NaCl 0.5 g, $K_2HPO_3.3H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, distilled water 1000 mL, pH 7.2-7.4); ISP2 (yeast extract 4 g, malt extract 10 g, glucose 4 g, distilled water 1000 mL, pH 7.3); M1ASW (soluble starch 15 g, glucose 5 g, peptone 5 g, distilled water 1000 mL, pH 7.5-7.8) (Trang PT et al., 2014) were used in this study. The inoculated tubes were incubated at 30^{o}C to choose the optimum medium for the production of antagonist.

Effect of pH and temperature

After the selection of the medium, the initial pH of media was adjusted from 6 to 9 (6, 7, 8 and 9) by using 0.1 M HCl and 0.1 M NaOH to define the best pH for the highest antibiotic production.

The optimal temperature for the maximum antibiotic production was tested by the ISP2 medium at different temperatures such as 25, 30, 35, 40 and 45^{o}C.

Effect of incubation period

S. antibioticus strain 1083 was inoculated in the optimal medium, pH and temperature in shaker incubator at 200rpm within 9 days. The broth culture centrifuged and the supernatant were extracted to determine the antibacterial activity by agar well diffusion method against *A. hydrophila* every day.

Effect of the cultured solution of *S. antibioticus* 1083

The strain 1083 was fermented in ISP2 medium with shaking at 200rpm. After 8 days, fermented solution was centrifuged at 10.000rpm for five minutes to remove the cells. The supernatant was transferred with different volumes of 0, 20, 50, 100, 200, 500 and 1000 µl into 10 mL of the selected medium. After incubation for 1 day, the antimicrobial activity was evaluated by checking the number of bacteria colonies.

Determination of antimicrobial activity

Antimicrobial activity of the isolate was determined by agar well diffusion method. Tubes were incubated in a shaker incubator at 200rpm for 8 days, the cultured solutions were centrifuged at 10.000 rpm for 5 min at 4^{o}C, the supernatant was then transferred into wells of the plate which had been spread with *A. hydrophila*. Plates were incubated in the incubator at 30^{o}C for 1 day to pick out the optimal conditions based on inhibited zone.

RESULTS AND DISCUSSION

Effect of culture media on antimicrobial activity of *S. antibioticus* strain 1083

Actinomycetes are an important group of filamentous, gram-positive bacteria producing secondary metabolites of agricultural and medicinal

importance. *Streptomyces* spp. covers around two-third of the clinically important antibiotics. Production of secondary metabolites by *Streptomyces* is not promising nature but can be increased or completely decreased under various nutritional conditions. Changes in the nature and type of carbon, nitrogen or phosphate sources and trace element have been reported to affect antibiotics biosynthesis in *Streptomyces* (Sarad *et al.*, 2015). Optimization of media is an important task for maximum secondary metabolites production, thus we used different types of media to select the best medium for the efficient production of antagonists (Fig. 1).

The results of Tab.1 show that the ISP2 medium in which the *S. antibioticus* strain 1083 showed maximum production of antimicrobial activity, expressed in terms of zone of inhibition (reached to 32 mm). This optimized medium was used for further study.

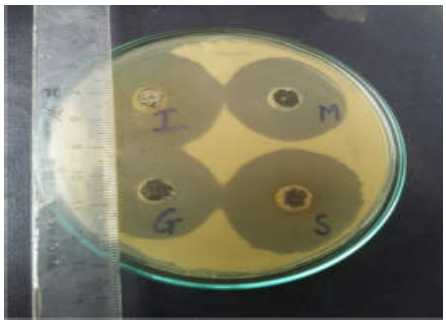

Figure 1. Effect of culture media on antimicrobial activity of *S. antibioticus* strain 1083 against *A. hydrophila*. *Note: S: SCA; M: M1ASW; I: ISP2; G: Gause-1.*

Table 1. The size of inhibition zone of *S. antibioticus* strain 1083 cultured in 4 different media.

Culture media	SCA	M1ASW	ISP2	GAUSE-1
Diameter of inhibition zone (mm)	30	24	32	22

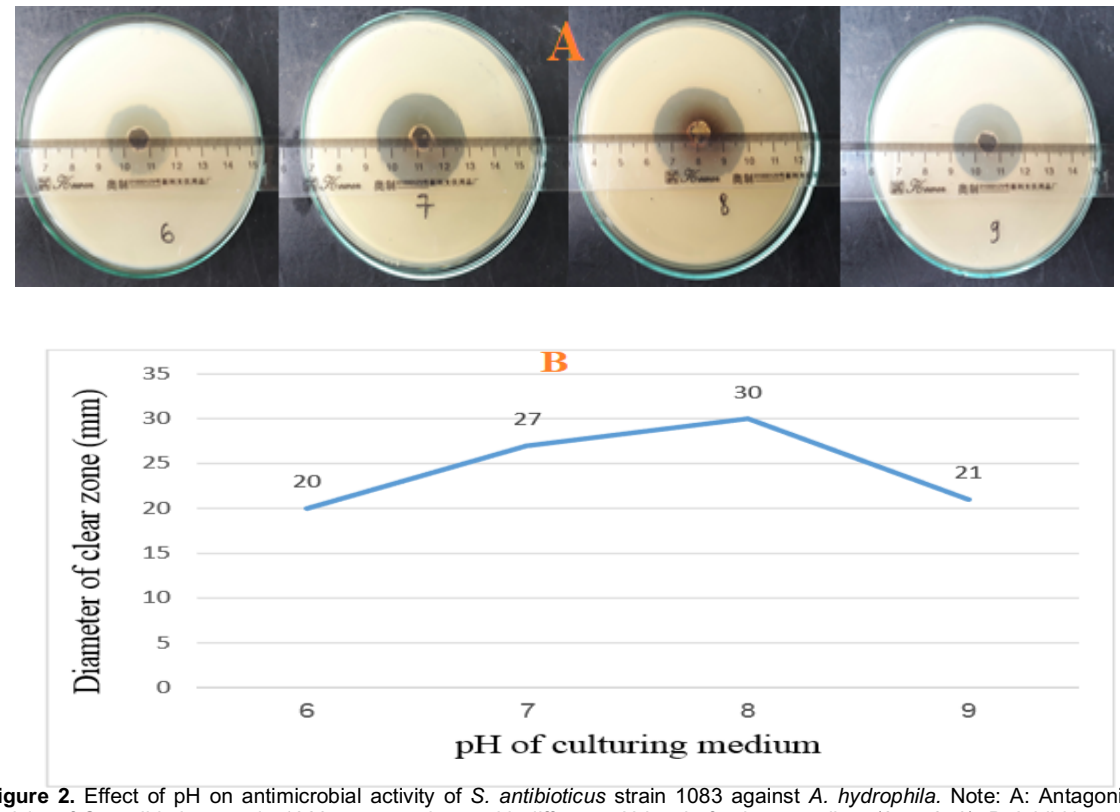

Figure 2. Effect of pH on antimicrobial activity of *S. antibioticus* strain 1083 against *A. hydrophila.* Note: A: Antagonistic activity of *S. antibioticus* strain 1083 on agar plates with different pH-level of culture medium (6, 7, 8, 9). B: Inhibition zone diameter chart.

Effect of pH on antimicrobial activity of *S. antibioticus* strain 1083

The value of pH has a significant impact on growth kinetics of microorganisms as enzyme and antimicrobial activities in producing strains are strongly sensitive to its changes (Elmahdi *et al, 2003*). Most of bacterial strains have their optimum growth on neutral environments. Thus, pH is also an important factor related to antimicrobial production of the strain 1083.

The cultured solution of the strain 1083 in ISP2 media with different pH was used to check antagonistic activity against *A. hydrophila* (Fig. 2).

The results show that pH 8 was the most suitable for the strain 1083 to create antimicrobial compound with the inhibit zone 30 mm. Similarly, the highest biomass of *Streptomyces* spp. yield was also observed at pH 8.0 (Palanichamy *et al.*, 2011) while the maximum production of antimicrobial compound from *S. albidoflavus* was found at pH 7.0 (Sarad *et al*., 2015) or *S. albovinaceus* was at pH 7.2 (Abdelghani *et al*., 2011). With the achieved result, pH 8 was used for culturing *S. antibioticus* strain 1083 in the next experiments.

Effect of temperature on antimicrobial activity of *S. antibioticus* strain 1083

The results in Fig. 3 were shown that the strain 1083 cultured in a range of temperature from 25°C to 40°C had obtained the good antagonistic activity against *A. hydrophila*, but the antagonistic activity of the isolate incubated at 45°C declined significantly.

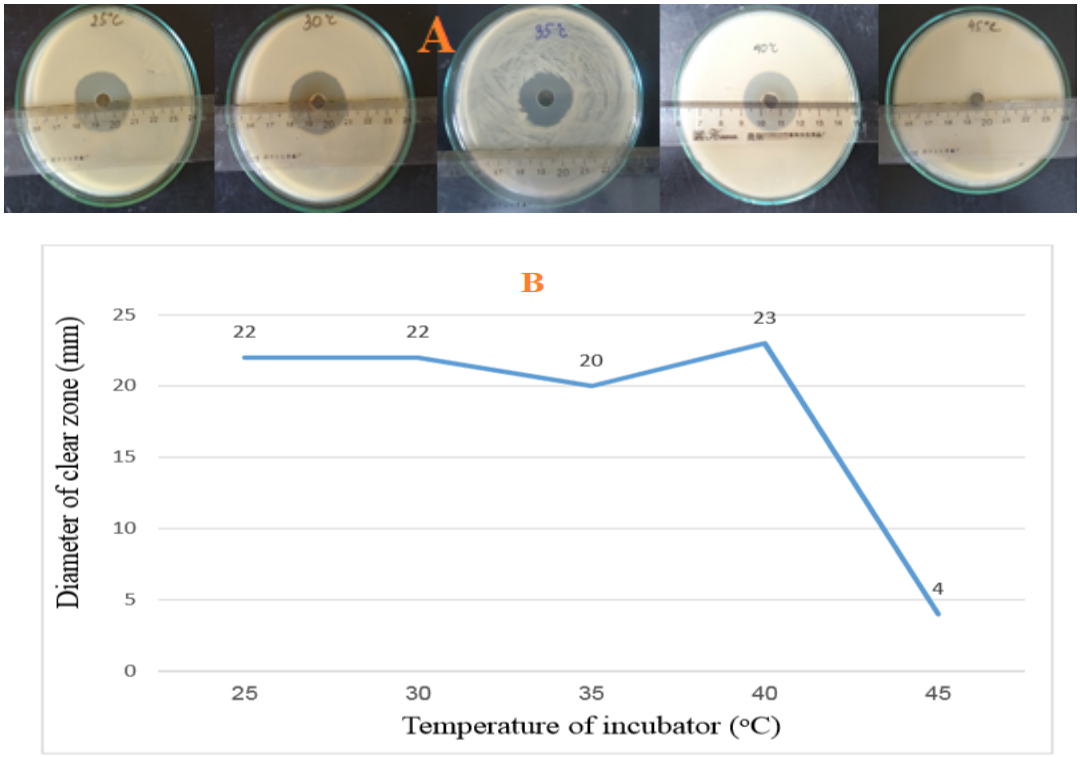

Figure 3. Effect of temperature on antimicrobial activity of *S. antibioticus* strain 1083 against *A.hydrophila*. Note: A: Antagonistic activity of *S. antibioticus* strain 1083 on agar plates with different culture temperature (25, 30, 35, 40, 45°C). B: Inhibition zone diameter chart.

In previous studies, the optimum temperature for growth of most *Streptomycetes* is close to 30°C. The optimum temperature for growth and antibiotic production in *S. aureofaciens* MY18 and *S. roseviolaceus* MR13 was 30°C (Tawfik *et al.,* 1991). Monamycin and erythromycin production at 26°C and 33°C were highest and the optimum temperature for antifungal antibiotics production by *S. rimosus* is 28°C. Nevertheless, antibiotic production might happen on higher temperatures in specific

Streptomycetes (James *et al.*, 1989). Thus, the optimal temperature (40°C) for antimicrobial production of *S. antibioticus* strain 1083 in this study was consistent with previous studies.

Effect of culture period on antimicrobial activity of *S. antibioticus* strain 1083

The strain 1083 was cultured in ISP2 medium, pH 8 at 40°C with shaking at 200 rpm. After 5, 6, 7, 8 and 9 days of incubation, the cultured solutions were used to evaluate antagonistic activity against *A. hydrophila* as Fig. 4.

Antagonistic activity of *S. antibioticus* strain 1083 was expressed from 5th day and reached a maximum after 8 days; however, the activity was decreased in the next days. The production of antimicrobial compound by *S. antibioticus* AZ-Z710, *S. kanamyceticus* M27 and *S. zaomyceticus* RC2073 was also reported maximum after 5 days of incubation (Sarad *et al.*, 2015). Thus, the optimal time (8 days) for incubation of the strain 1083 was consistent was consistent with previous studies and the information was used in the next experiments.

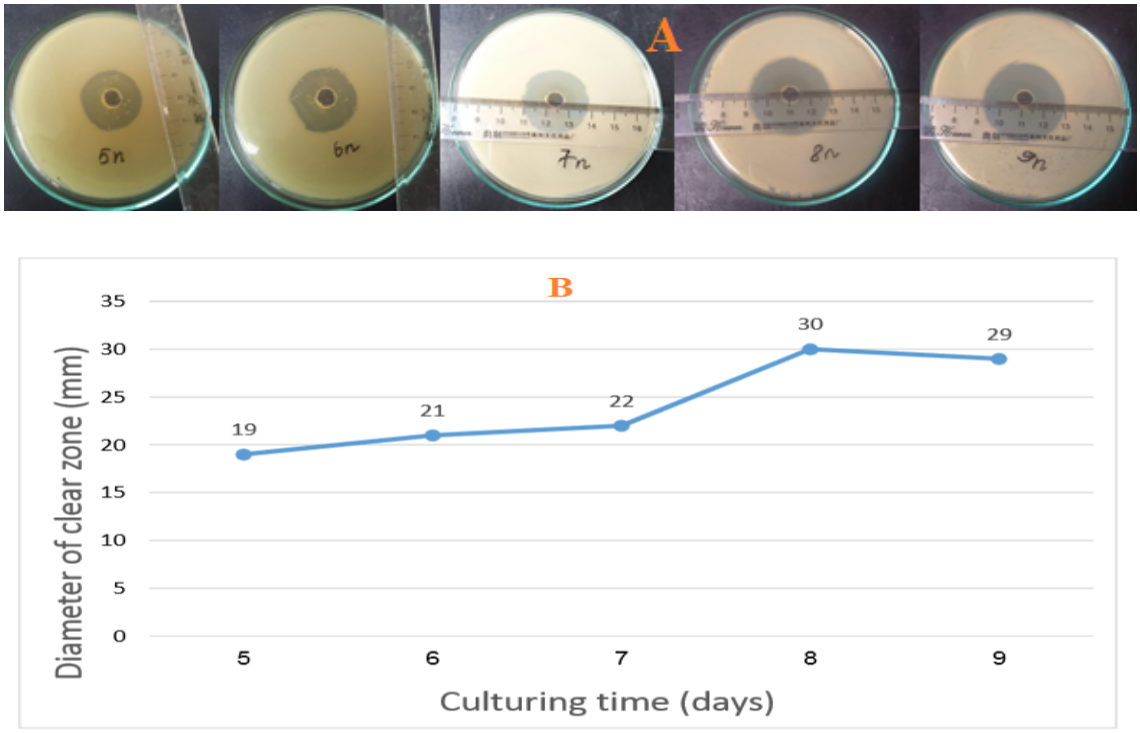

Figure 4. Effect of incubation period on antimicrobial activity of *S. antibioticus* strain 1083 against *A. hydrophila*. Note: A: Antagonistic activity of *S. antibioticus* strain 1083 on agar plates with different culture period (5, 6, 7, 8, 9 culture days). B: Inhibition zone diameter chart.

Effect of the concentration of *S. antibioticus* strain 1083 on their antimicrobial activity

Different initial concentrations of the strain 1083 were added into ISP2 medium to determine the minimum inoculum for the maximum expression of antimicrobial effectiveness. The results were shown in the Fig. 5.

It was observed that adding of 0.2% and 1% cultured solution volume of the strain 1083 made number *of A. hydrophila* cells go down to 210 cells/ml and 150 cells/ml respectively, compared to that in the control sample (580 cells/ml). The bacteria were eliminated completely when 10% volume of supernatant of the strain 1083 were added.

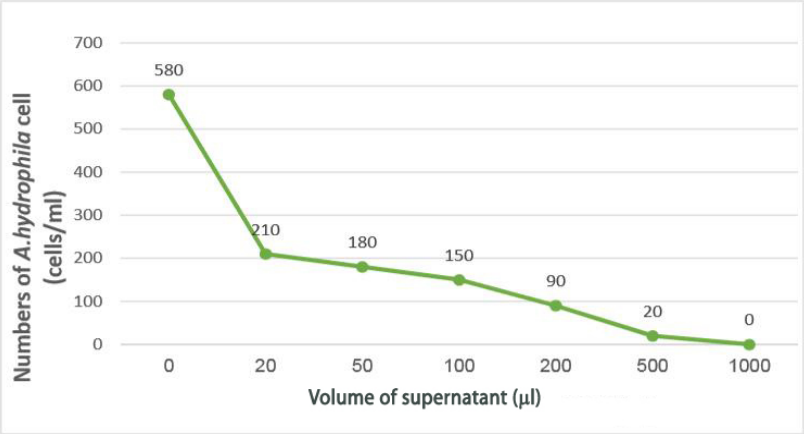

Figure 5. Effect of the cultured solution of the strain 1083 on the growth of *A. hydrophila*.

CONCLUSIONS

The optimum medium for *S. antibioticus* strain 1083 expressing antimicrobial activity against *A. hydrophila* is ISP2 at pH 8. After 5 days of incubation, the strain 1083 secretes antimicrobial compound but it shows the strongest antagonism after 8 culture days at 40^{o}C with shaking at 200rpm. The optimal cultured solution of *S. antibioticus* strain 1083 added into the medium in order to inhibit *A. hydrophila* are from 5-10%.

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NGHIÊN CỨU XÁC ĐỊNH ĐIỀU KIỆN NUÔI CẤY CHỦNG *STREPTOMYCES ANTIBIOTICUS* 1083 NHẰM TĂNG CƯỜNG KHẢ NĂNG ĐỐI KHÁNG VỚI CHỦNG *AEROMONAS HYDROPHILA* GÂY BỆNH TRÊN CÁ

Nguyễn Xuân Cảnh[1], Trần Thị Thu Hiền[1], Nguyễn Thanh Huyền[1], Phạm Lê Anh Minh[1], Trần Bảo Trâm[2], Nguyễn Thị Thanh Mai[2]

[1]Học viện Nông nghiệp Việt Nam
[2]Trung tâm Sinh học thực nghiệm, Viện Ứng dụng công nghệ

TÓM TẮT

Cá là một loại thực phẩm lành mạnh và cung cấp nhiều chất dinh dưỡng có lợi cho sức khỏe con người. Tuy nhiên, cá là đối tượng rất nhạy cảm với nhiều loại bệnh. Vì vậy, xác định được đối tượng gây bệnh và tìm ra biện pháp phòng trừ là một nhiệm vụ cấp bách hiện nay. Ở nghiên cứu trước, chúng tôi đã tìm ra được chủng *S. antibioticus* 1083 có khả năng đối kháng với vi khuẩn *A. hydrophila* gây bệnh ở cá. Dựa trên những kết quả đã đạt được, chúng tôi tiếp tục thực hiện nghiên cứu một số yếu tố ảnh hưởng tới khả năng sinh trưởng và hoạt tính kháng vi khuẩn *A. hydrophila* của chủng *S. antibioticus* 1083 nhằm xác định được điều kiện tối ưu cho việc nuôi cấy chủng *S. antibioticus* 1083, từ đó chúng tôi có thể kiểm soát bệnh do vi khuẩn *A. hydrophila* gây ra hiệu quả nhất. Kết quả của nghiên cứu này đã cho thấy, chủng *S. antibioticus* 1083 sinh trưởng tốt nhất trên môi trường ISP2, ở 40^{o}C, pH 8 và sau 8 ngày nuôi cấy trong điều kiện nuôi lắc 200 vòng/ phút. Nghiên cứu cũng đã đánh giá khả năng kháng khuẩn của *S. antibioticus* 1083 trong điều kiện môi trường giả định, kết quả cho thấy khi bổ sung 10% thể tích dịch nuôi cấy *S. antibioticus* vào môi trường nuôi vi khuẩn *A. hydrophila* các tế bào vi khuẩn *A. hydophila* bị loại bỏ hoàn toàn sau một ngày.

Từ khoá: *Aeromonas hydrophila, Hoạt tính kháng khuẩn, Điều kiện nuôi cấy, Tối ưu hóa, Streptomyces antibioticus*