Vietnam Journal of Science and Technology ***57*** *(3B) (2019) 144-152*

doi:10.15625/2525-2518/57/3B/14501

OPTIMAL FERMENTATION CONDITIONS FOR ANTIBIOTIC PRODUCTION BY ENDOPHYTIC *Streptomyces cavourensis* YBQ59 ISOLATED FROM *Cinnamomum cassia* Presl

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Received: 15 October 2019; Accepted for publication: 4 November 2019

Abstract. This study determined the optimal nutrient and environmental conditions to produce antimicrobial and cytotoxic agents by *Streptomyces cavourensis* YBQ59 during the fermentation process. The bioactivities of eluted fractions based gradient solvents via chromatography column were also evaluated. *S. cavourensis* YBQ59 exhibited strong antimicrobial activities against methicillin-resistant *Staphylococcus epidermidis* ATCC 35984 (MRSE) and *Salmonella* Typhimurium ATCC 14028 under fermentation conditions as follows: MT6 medium with soluble starch and soybean powder as carbon and nitrogen sources, respectively, temperature 30 ^{o}C, initial pH 7.0, 20 % DO concentration and with 5% initial seed culture. The kinetic of fermentation showed that the antimicrobial activities were highest at between 72 h and 78 h. The fraction **25/1 D-M** (dichloromethane –methanol) exhibited the highest antimicrobial effect against methicillin-resistant *Staphylococcus aureus* ATCC 33591 and MRSE with minimum inhibitory concentrations of 9.4 µg/ml and 6.4 µg/ml, respectively. The fraction **10/1 D-M** had strong cytotoxic effects towards multidrug-resistant A459 and H1299 lung carcinoma cell lines with the cell viability of 11.3 % and 12.4 %, respectively. In conclusion, *S. cavourensis* YBQ59 would be a potential producer of valuable bioactive compounds that may have a board application in pharmaceutical industry and agriculture (biocontrol, livestock, food safety and quality management).

Keywords: antimicrobials, Cinnamomum cassia, endophytic actinomycete, fermentation, food safety, Streptomyces cavourensis.

Classification numbers: 1.2.1, 1.3.2

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1. INTRODUCTION

The World Health Organization (WHO) claims that the antimicrobial resistance (AMR) presents a significant challenge to public health and to the ecosystem. The overuse of antibiotics in these settings has driven the selection of multi-antibiotic resistant (MDR) bacteria, consequently the transmission of antibiotic-resistant strains threats to public health on a global scale [1, 2]. Thus, in order to ensure quality and safety in food industries, it is necessary to control of the AMR emergence in livestock and aquaculture farms by screening new agents having board-spectrum antimicrobial activity from natural sources [1, 2]. In the course of screening for new antibiotics, our research group isolated an endophytic *Streptomyces cavourensis* YBQ59 (GenBank accession number MF950891) from roots of *Cinnamomum cassia* Presl, in Yen Bai, Vietnam [3]. This strain exhibited board-spectrum antimicrobial activity against various human pathogens belonging to Gram-positive and gram-negative bacteria, multidrug-resistant bacteria and yeast. *S. cavourensis* YBQ59 possessed secondary metabolite biosynthetic genes (*pks* and *nrps*) encoding for polyketide synthase and non-ribosomal peptide synthase. In addition, this strain was favorable to produce anthracyclines-like antibiotics [3]. Altogether, it is necessary to isolate and identify bioactive compounds derived from *S. cavourensis* YBQ59. Therefore, the present study aimed to study suitable fermentation conditions for maximal production of antibiotics by *S. cavourensis* YBQ59. The kinetics, antimicrobials and cytotoxic properties were evaluated during fermentation process. Finally, eluted fractions based silica gel column were used for identifying active fractions against multidrug-resistant bacteria and cancer cell lines.

2. MATERIALS AND METHODS

2.1. Materials

Indicator microbes were used for antimicrobial activity testing including methicillin-resistant *Staphylococcus epidermidis* ATCC 35984 (MRSE), methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRSA) and *Salmonella* Typhimurium ATCC 14028. Human lung cancer A549 and H1299 cell lines were kindly prodived by Prof. Jeong-Hyung Lee, Department of Biochemistry, College of Natural Sciences, Kangwon National University, Korea. Ten different antibiotic-producing media (MT1 – MT10) were selected for this study following previous studies [4-6].

2.2. Analytical methods

2.2.1. Antimicrobial activity testing and cytotoxic assay

The antimicrobial activity of *S. cavourensis* YBQ59 against the nine microbes (mentioned above) was performed by using the agar well diffusion method as described previously [3, 7, 8]. The experiments were performed in triplicates. The cytotoxic assay was carried out against human carcinoma cell lines using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as described previously [3, 9].

2.2.2. Selection of fermentation media and conditions

S. cavourensis YBQ59 were incubated in 10 different media (mentioned above). The medium showing the highest antimicrobial activity will be selected as the base medium for the study of fermentation conditions. Effect of carbon and nitrogen sources: the medium MT6 used as the base medium to optimize fermentation conditions for the maximal antibiotic production. The different carbon sources (1 % concentration) were studied including raw molasses, tapioca starch, glucose, saccharose, starch soluble, glycerin, dextrose and mannitol. Similarly, different nitrogen sources were corn extract, soybean power, peanut power, yeast extract, peptone, tryptone, malt extract, meat extract and hydrolyzed casein (2 % concentration). The experiment conditions were performed at 30 °C, shaking at 200 rpm/min. After 72 h, the CFSs were examined for the antimicrobial study. The effect of other important fermentation parameters was also evaluated as follows: temperatures 20, 25, 30, 37 and 40 °C, initial pH ranged between 4.0 and 9.0 (interval step of 1.0); dissolved oxygen (DO) ranged from 5 % to 25 % and rate of seed culture from 1 % to 9 %. The antimicrobial activity was evaluated accordingly.

2.2.3. Kinetic of fermentation process and fractional extraction via silica gel column

The fermentation process was performed in a 5 l Bioflo 110 system (New Brunswick Scientific, USA) with parameters as follows: MT6 medium with starch and soybean as carbon and nitrogen sources, initial pH 7.0; seed culture added 5 % v/v; temperature 30 °C; agitation rate 300 rpm/min; DO concentrations maintained 0.5 l/l/min. The fermentation was carried out for 120 h, and samples were acquired at every 6 h for analysis of dried biomass and antimicrobial activity. After 78 h of the fermentation, 29 l of CFSs was harvested, then were extracted with ethyl acetate (1/1, v/v) under sonication condition at 40 ^{o}C for 30 min (repeated three times) and was concentrated under vacuum. The dried samples (15.0 g) were transferred into the silica gel column of chromatograph system and eluted with gradient solvents (100 % dichloromethane → 100 % methanol) to obtained 9 eluted fractions. The minimum inhibitory concentration (MIC) of eluted fractions against MRSA and MRSE was determined using the micro-broth dilution method as previously described [3]. Azithromycin was used as a positive control. All the experiments were performed in triplicate. The cytotoxic effect of eluted fractions towards A459 and H1299 cell lines was also evaluated [3].

2.2.4. Statistical analysis

The data were expressed as mean ± standard deviation using Excel 2010 and XLSTAT 2016 software for analysis of one-site deviation (ANOVA). The P values ≤ 0.05 expressed statistically significant results.

3. RESULTS AND DISCUSSION

3.1. Effect of fermentation medium

In this study, *S. cavourensis* YBQ59 was able to produce secondary metabolites on all 10 media tested that inhibited the growth of MRSE and *S.* Typhimurium at different levels (Figure 1). Among the media, the MT6 was the most appropriate medium for *S. cavourensis* YBQ59 producing antibiotics. The CFSs obtained from MT6 culture broth showed strong antibacterial activities against MRSE and *S.* Typhimurium (inhibition zones > 26 mm). This result is concordant with previous studies, and therefore the MT6 was selected as the basic medium for

studying nutrient and environmental factors affecting the antibiotic biosynthesis of *S. cavourensis* YBQ59.

3.2. Effect of carbon and nitrogen sources

The effects of different carbon and nitrogen sources are shown in the Figures 2 and 3, respectively. The CFSs exhibited a highest inhibitory effect against MRSE and *S.* Typhimurium with soluble starch as the carbon source (inhibition zones > 26.0 mm). These results could be explained as follows. Firstly, starch soluble is hydrolyzed to glucose slowly in a liquid medium and the absorption rate is slower than glucose, resulting in reduced catabolic pressure due to glucose growth facilitating the growth and antibiotic production [10]. The antimicrobial activity towards MRSE and *S.* Typhimurium was still remarkable with glucose, dextrose, glycerol and mannitol as carbon sources (inhibition zones > 21.0 mm). This suggested that polysaccharides were more suitable than monosaccharide and disaccharide sources for the antibiotic production by *S. cavourensis* YBQ59.

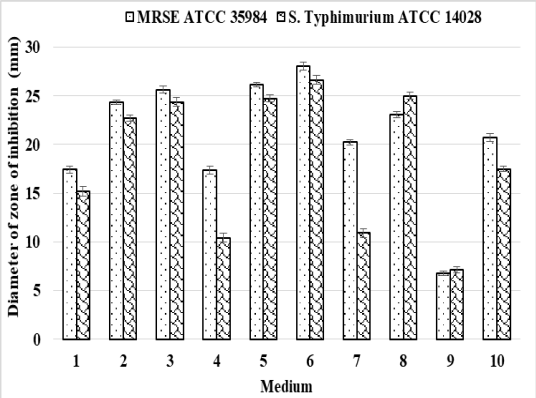

Figure 1. Antimicrobial activity of *S. cavourensis* YBQ59 against MRSE and *S.* Typhimurium (medium 1 – 10: MT1 – MT10).

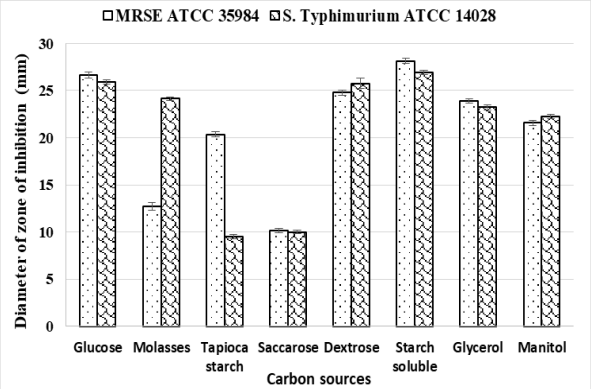

Figure 2. Effect of carbon sources on the antibiotic production of *S. cavourensis* YBQ59.

Simiarly, according to different nitrogen sources, the CFSs of *S. cavourensis* YBQ59 also showed different levels of antimicrobial activity towards *S.* Typhimurium and MRSE. Among the nine nitrogen sources tested, the antimicrobial activity was highest (inhibition zones > 27.0 mm) with soybean powder as the nitrogen source. In fact, soybean powder has a high level of proteins ranging from 36 % to 40 % which contains necessary amino acids for the cell growth such as glutamic acid, aspartic acid, cysteine, vitamins and mineral salts. Therefore, soybean powder has been favored as one of the main ingredients in antibiotic-producing media for many *Streptomyces* species in many studies before [11]. Nevertheless, in agreement with previous studies, the present study showed that other nitrogen sources like yeast extract, tryptone, malt extract, peptone and casein were also appropriate for the antibiotic biosynthesis by *Streptomyces* [12].

3.4. Effect of fermentation conditions

Our study showed that the optimal temperature for the antibiotic production by *S. cavourensis* YBQ59 was around 30 ^{o}C and lower or higher temperatures reduced the production of antimicrobial compounds (Figure 4). This result is consistent with a study of Hassan *et al.*

[13] in which *Streptomyces violatus* produced a high yield of antibiotics at 30 ^{o}C. Similarly, the antimicrobial activity was increased upon increasing the initial pH from 4.0 to 7.0, but any further increase of pH resulted in decreased production of antimicrobial active compounds (Figure 5). According to literature, the initial pH 7.5 was appropriate for the antibiotic biosynthesis of *Streptomyces* sp. KGG32 [14], pH 6.0 for *S. rimosus* MY02 [15], while pH 7.0 was suitable for *Actinomycetes* YJ1 [5]. In addition, our study also showed that the 20 % DO concentration and 5 % seed culture were the appropriate conditions for the antibiotic-producing fermentation of *S. cavourensis* YBQ59 (Figure 6 and 7). In fact, 20 % DO concentration ensured the saturation of oxygen level in the culture and the growth and biosynthesis of secondary metabolites of *Streptomyces* [5]. In concordant with studies of Song *et al.* [5], the optimal seed culture size was 5 % for the antibiotic-producing fermentation. Under these optimal fermentation conditions, *S. cavourensis* YBQ59 exhibited strong antimicrobial activities against MRSE and *S.* Typhimurium (inhibition zones > 25 mm).

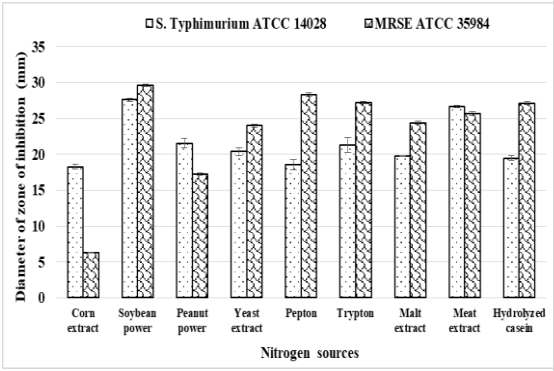

Figure 3. Effect of nitrogen sources on the antibiotic-producing capacity of *S. cavourensis* YBQ59.

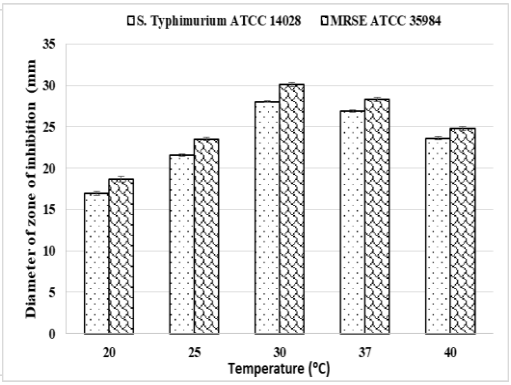

Figure 4. Effect of temperature on the antibiotic production of *S. cavourensis* YBQ59.

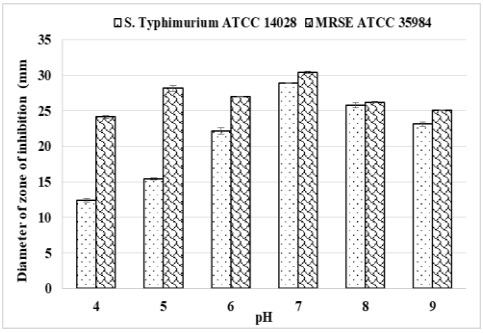

Figure 5. Effect of pH on the antibiotic production of *S. cavourensis* YBQ59.

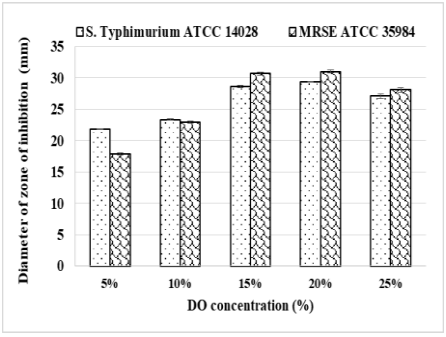

Figure 6. Effect of DO cocentration on the antibiotic production of *S. cavourensis* YBQ59.

3.5. Kinetic of fermentation process

The fermentation process of *S. cavourensis* YBQ59 was carried out for 120 h based the optimal conditions (mentioned above) (Figure 8). The growth rate of this strain rapidly increased the fist 24 h and reached the pick after 72 h with the dried biomass of 11.5 g/l. During this process, the pH was constantly maintained around pH 7,0 (Figure 8). The antibiotic biosynthesis

of *S. cavourensis* YBQ59 was observed after 18 h and the antimicrobial activity against MRSE and *S.* Typhimurium was dramatically increased within 42 h, then slowly increased and reached a peak located between 72 h and 78 h (inhibition zones > 34.0 mm). The antimicrobial activity was stable until 90 h and then slightly decreased after 120 h. Thus, *S. cavourensis* YBQ59 produced a highest yield of antibiotics under the fermentation period between 72 h and 90 h. This result is consistent with previous reports [16, 17] and suggests that the log phase is the best period for harvesting antibiotics produced by *S. cavourensis* YBQ59 under the optimal fermentation conditions.

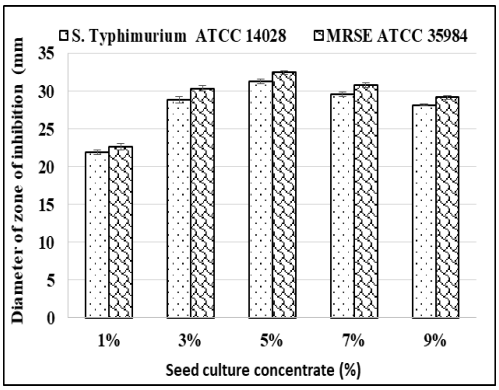

Figure 7. Effect of seed culture on the antibiotic production of *S. cavourensis* YBQ59.

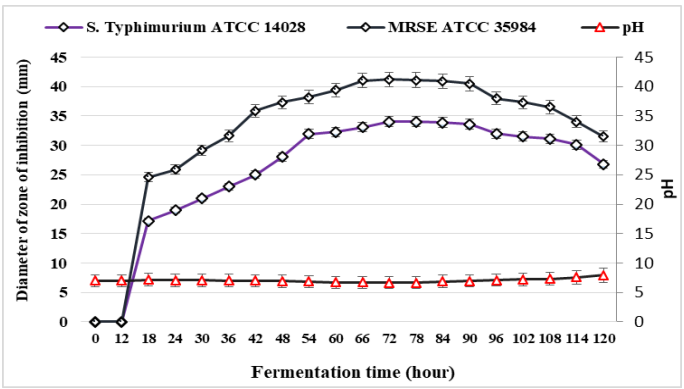

Figure 8. Kinetic of fermentation process for the antibiotic production of *S. cavourensis* YBQ59.

3.6. Antimicrobial and cytotoxic activities of eluted fractions via silica gel column

Table 1. Antimicrobials and cytotoxic effects of eluted fractions.

No	Eluted fractions (%)	MIC (mean±SD, µg/ml)		Cell viability (%)*	
		MRSA	MRSE	A549	H1299
1	**100 D**	28.8 ± 0.12	8.7 ± 0.22	52.95	72.94
2	**50/1 D-M**	24.6 ± 0.18	14.8 ± 0.10	**37.60**	55.52
3	**25/1 D-M**	**9.4 ± 0.09**	**6.4 ± 0.13**	**23.08**	**10.00**
4	**10/1 D-M**	10.7 ± 0.17	13.2 ± 0.25	**11.32**	**12.47**
5	**5/1 D-M**	67.3 ± 0.128	48,2 ± 0.26	54.57	73.26
6	**3/1 D-M**	45.8 ± 0.12	40.5 ± 0.16	**44.64**	75.60
7	**2/1 D-M**	56.7 ± 0.18	34.4 ± 0.14	**43.59**	66.34
8	**1/1 D-M**	46.8 ± 0.12	47.9 ± 0.09	**37.42**	72.81
9	**100 M**	42.8 ± 0.18	32.6 ± 0.14	49.95	82.59
	Azithromycin	11.7 ± 0.21	13. ± 0.12	NA	NA

*: the concentration of eluted fractions tested: 100 µg/ml; NA: not applicable.

The CFSs of *S. cavourensis* YBQ59 was subjected to chemical analysis for the isolation of antimicrobial compounds (Table 1). The bioactive compounds seem to be eluted in the fractions with high concentration of dichloromethane including **50/1 D-M, 25/1D-M** and **10/1 D-M**. Among them, the eluted fraction **25/1 D-M** exhibited highest inhibitory effects towards MRSA and MRSE with the MIC values of 9.4 µg/ml and 6.4 µg/ml, respectively. These results were even better than the antimicrobial activities of azithromycin (Table 1). Similarly, the eluted

fractions **50/1 D-M, 25/1 D-M** and **10/1 D-M** also revealed a strong cytotoxic effect against A549 cells with the reduction of cell viability from approximately 62 % to 88 % (Table 1). The positive inhibitory activity was also found in eluted fractions **3/1 D-M, 2/1 D-M** and **1/1 D-M**. For H1299 cells, only two eluted fractions **25/1 D-M** and **10/1 D-M** exhibited positive inhibitory activities. Taken together, **25/1 D-M** and **10/1 D-M** could be the most important fractions containing valuable bioactive compounds.

In fact, many novel antibiotics and other bioactive compounds have been isolated from endophytic actinomycetes particularly in the *Streptomyces* genus [4]. Moreover, these new antibiotics were active against multidrug resistant bacteria and pathogenic fungi [18]. Many other secondary metabolites active towards different cancer cell lines including multidrug resistant ones have been found in *Taxomyces*, *Streptomyces*, *Micromonospora* and *Kitasatospora* spp. [19]. For example, peptide coronamycin derived from *Streptomyces* sp. MSU-2110 showed similar activity to Taxol and inhibited the growth of HMEC and BT-20 cell lines at a very low concentration (IC_{50} 5-10 µg/ml) [20]. Brartemicin was isolated from *Micromonospora* sp. associated with Brazilian medicinal plants and exhibited strong cytotoxic effects against colon cancer cells with the IC_{50} of 0.39 µmol/l, without any side effect [21]. Our study suggests that *S. cavourensis* YBQ59 would be a potential producer of valuable antibiotics and other bioactive secondary metabolites that have a board application in pharmaceutical – medical industry, bio-control, agriculture and livestock.

4. CONCLUSIONS

The present study determined the optimal fermentation conditions for the antibiotic production by endophytic *S. cavourensis* YBQ59 associated with *Cinnamomum cassia* Presl as follows: MT6 medium with soluble starch as carbon source, soybean powder as nitrogen source, temperature 30 ^{o}C, initial pH 7.0, 20 % DO concentration and with 5 % seed culture supplied. *S. cavourensis* YBQ59 exhibited as a potential producer of strong and board-spectrum antimicrobial and antitumor compounds.

Acknowledgements. This work was funded by Graduate University of Science and Technology, VAST under the grant number GUST.STS.ĐT2017-SH03. We would like to thank the National Key Laboratory of Gene Technology - Institute of Biotechnology, VAST for supporting facilities.

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