Production of an antibiotic enterocin from a marine actinobacteria strain H1003 by metal-stress technique with enhanced enrichment using response surface methodology

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Abstract: Elicitation by chemical means including heavy metals is one of a new technique for drug discoveries. In this research, the effect of heavy metals on marine actinobacteria *Streptomyces* sp. H-1003 for the production of enterocin, with a strong broad spectrum activity, along optimized fermented medium was firstly investigated. The optimum metal stress conditions consisted of culturing marine actinobacteria strain H-1003 with addition of cobalt ions at 2mM in optimized Gause's medium having starch at 20mg/L for 10 days at 180 revolution/min. Under these conditions, enterocin production was enhanced with a value of 5.33mg/L, which was totally absent at the normal culture of strain H-1003 and much higher than other tested metal-stress conditions. This work triumphantly announced a prodigious effect of heavy metals on marine actinobacteria with fringe benefits as a key tool of enterocin production.

Keywords: Metal-stress, drug discovery, enterocin, optimization, marine actinobacteria.

INTRODUCTION

Marine microorganisms adapt and respond briskly to changes in the availability and concentrations of metals within their harsh and dynamic environment (Holden and Adam, 2003, Ding *et al.,* 2016). Marine microorganisms, living in a stressful environment, are of great interest as new promising sources of therapeutically active products (Pan *et al.,* 2016). Contrary to insight, that metals hinder secondary metabolite production, recent studies have revealed that metals can induce or enhance the activity of possibly potent medically and nutraceutically relevant metabolites (Ding *et al.,* 2016).

Natural products have been an important part of pharmaceutical and nutraceutical throughout human history. In recent years, the use of naturally derived drugs has been tremendously elevated because of their efficacy and safety as compared to synthetic drugs. Although the potential of marine microorganisms to produces novel secondary metabolites seems limitless, because of few major obstacles that hinders the transformation of biomolecules into medicines. One of the major obstacle in identification of secondary metabolites of these medicinal important drugs are genes clusters or non-activated biosynthesis pathways. Such gene clusters become entitled as sleeping gene clusters (Ding *et al.,* 2016). Activation of sleeping genes or producing structures with stereo chemical affection which facilitate metal complexation and their transportation in biological systems may account for the mechanisms behind the metal-induced metabolite phenomenon (Ding *et al.,* 2016).

In this study, the capability of marine actinobacteria *Streptomyces* sp. H-1003 strain to produce secondary metabolites in the presence of metal ions in the culture medium by a metal-stress technique was investigated. Furthermore, the production of metal-elicited compound was enhanced by using response surface methodology. This is the first ever work reported on enhanced enrichment of stress-driven compound by using response surface methodology.

MATERIALS AND METHODS

Instruments

High-performance liquid chromatography (HPLC) system composed of a Waters 717 plus Autosampler, a Waters 996 Photodiode Array Detector, the Waters 600 Controller and a Waters Millog workstation (Waters, Shinagawaku, Tokyo, Japan). ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured at 25°C on a Bruker AVANCE DMX 500 NMR spectrometer with TMS as internal standard. Preparative HPLC was operated on an Agilent-1100 system.

Soil sample collection

Soil sediments samples were collected at 50meter intervals from the Zhoushan, Zhejiang East China sea area in a 900meter flow path after merging Zhoushan Island. The Sediments were taken from unpolluted sites

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within the coastal limits of Zhoushan Island, China. Mixed soil samples, pure soil samples and sand samples were collected from different zones with at least difference of 3-4 kilometer. Soil for actinobacteria analyses were collected from the different sites in sterile plastic bags. The samples were immediately brought to the laboratory for fresh soil isolation and stored in the refrigerator at 4ºC.

Isolation and storage of H-1003 strain

The fresh soil sample approximately 1-2g was directly inoculated into the pre-sterilized glass tubes and diluted with artificial sea water to achieve its 10^{-1} dilution. The mixture was subjected to sonication for 1 minute to release microorganisms bound to the soil particles and shaken at room temperature for 15 minutes. Subsequently, a series of dilutions (10^{-2}, 10^{-3} and 10^{-4}) were prepared. Three isolation media Gause's synthetic agar (GS), ISP2 and starch-yeast extract-peptone agar (M1) were prepared and supplemented with Nystatin (0.05g/L) to prevent fungal contamination. After preparing dilutions, aliquots of 100 µl of each dilution were inoculated on each media and spread using a pre-sterilized spreader. All the sample aliquots were analyzed in triplicate. The plates were incubated at 28ºC for 15-20 days.

After 5 days of incubation growth on media was observed and purified colonies of actinobacteria were obtained and preserved on solid agar media slants containing Nystatin (0.5g/L) and stored at 4ºC. The actinobacteria were distinguished based on their morphology and their colors. The H-1003 strain was identified as a *Streptomyces* sp on the basis of ITS 16S fragment.

Normal and metal stress cultivation

The normal and metal stress culture of strain H-1003 were carried out parallel in 500mL flasks containing 200mL liquid Gause's, ISP2 and M1 medium in a rotatory shaker at 180 rpm for 10 days at 28ºC. The normal broth culture of strain H-1003 was initially carried out in 2 flasks as a control. The stressed culture media for H-1003 consisted of additional 0.5mM, 1mM, 2mM and 4mM Co^{2+}, Ni^{2+} and Zn^{2+} . The mycelium was removed and culture broth was extracted with an equal volume of EtOAc twice.

Chemical screening by HPLC analysis and identification of stress metabolite

For detection of chemical compounds of different groups, an analytical reversed-phase HPLC-UV experiments were performed using a C18 column (Sepax Amethyst C18-H, 100mm × 3.00mm) applying an H_2O/MeOH gradient from 20%~100% for 30 min, 100% MeOH from 30-50 min, flow rate 0.8ml/min with column temperature 25°C, using 210 wave length, on a LC20A system (Shimadzu, Kyoto, Japan) equipped with a Prominence CBM-20A/20 Alite controller, SPD-20A UV detector and Prominence CTO-20A column oven. The stress-induced compound was isolated by orthogonal purification technique (preparative HPLC).Based on the results on analytical HPLC analysis, a constant mobile phase of 65% MeOH for 30 min and a flow rate of 10 ml/min were employed for isolation and purification of stressed compound.

Extraction and isolation

The 13 L of fermented actinobacterial strain H1003 broth were extracted with EtOAc (2×200ml). After evaporation of the solvent, the crude extract was dissolved in methanol and subjected for centrifugation 1, 20,000rpm for 10 minutes prior to subjection for analytical HPLC. The metal induced substance was purified by preparative HPLC. enterocin (9.1mg, tR=18 min) was obtained using MeOH: H_2O (65:35) as an eluent.

Optimization of medium composition by response surface methodology

Box-Behnken design (BBD) was applied to accurately optimize the concentration of culture time (A), metal ions (B) and starch (C). This design was based on a 3^3 factorial design, 3 replicates of the central run, leading to 17 set of experiments, allowing each experimental response to be optimized. The responses were examined using a Box-Behnken statistical experimental design. The process of optimization involves evaluating the response of the statistically designed combinations, predicting the response of the fitted model, assessing the coefficients by fitting the experimental data to the response function, and accounting the adequacy of the model. Before taking start on optimization experiments, it is crucial to identify the important factors affecting the quality of the derived outcomes. The stages for the variables were selected on the basis of their minimum and maximum effect on yield and also each stage has been tested at lower and higher end. The stages of the three factors evaluated in this design are listed in (table 1). A three factor, three-Level Box-Behnken design was practiced for the optimization process, using the software Design Expert V 8.0.5. All other factors, for example temperature, volume and detection wavelength were maintained constant.

Bioassay

Extracts of normal and metal stress cultures were tested for its antibacterial activity by agar diffusion method. *Staphylococcus aureus* [CMCC (B) 26003], *Escherichia coli* [CMCC (B) 44102], and *Bacillus subtilis* [CMCC (B) 63501] were selected as test organisms. Isolates were inoculated onto Nutrient agar and PDA slants for 24 hours. Bacterial suspension was prepared by using PBS and matched with 0.5 McFarland's Index i.e. 10^8cfu/ml. After that 20 ml of MHA at 70ºC were inoculated with 20 µl from 10^8 of culture suspension in order to achieved 10^6 cfu/ml and immediately poured plate and allowed it to set for at least 30 min inside the safety cabinet. Ten micro liter of each crude extract testing sample was spotted onto plates (Anjum *et al*., 2015). Plates were incubated at 37ºC for at least 24 hours.

RESULTS

Bioactive-guided screening of metal-stressed cultivations:

For determining the therapeutic efficacy of actinobacteria strain among 11 isolated actinobacteria strains in metal culture, primary bioassay was performed. The crude extracts from metal spiked culture of H-1003 displayed very strong biological activity towards several medically relevant test organisms containing gram-positive *Staphylococcus aureus* [CMCC (B) 26003], *Bacillus subtilis* [CMCC (B) 63501] and gram-negative *Escherichia coli* [CMCC (B) 44102]. The net weight of metal and non-metal crude extracts of 5 mg/ml dissolved in DMSO was initially used to check the bioactivity potential of H-1003 strain. Strain H-1003 displayed bio-activity only with extracts of the 2mM/L $CoCl_2$ metal grown culture (fig. S1). This suggests a molecular mechanism of induction due to metal supply. It can be hypothesized, the metabolites produced in these cultures are synthesized only in the presence of metals by activation of sleeping genes clusters or nonsynthetic bio synthesis pathways. From this initial limelight response the H-1003 strain with 2mM/L $CoCl_2$ attracted our interest to explore more and to know the hidden treasure responsible for this bioactivity.

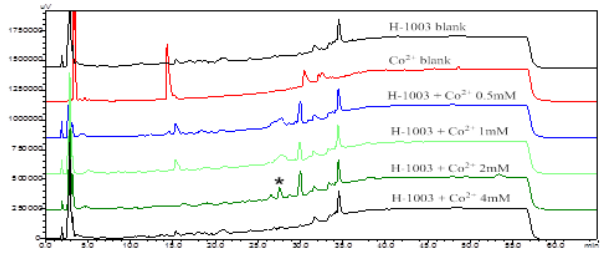

Fig. 1: HPLC profile of metal treated and untreated H-1003 strain with four different cobalt metal ions. The * indicates the new peak in HPLC chromatogram.

Chemical investigations of metal treated and untreated extracts by HPLC

To define the optimal HPLC condition for separation the stressed metabolite of marine soil derived actinobacteria, the effects of eluted mode, mobile phase, flow rate, detection wavelength and column temperature were investigated. Three different kind of metals Co^{2+}, Zn^{2+} and Ni^{2+} were selected as initial elicitors on the base of their previous secondary metabolites production pattern with microorganisms (Ye *et al., 2014,* Ding *et al., 2016*). The cultivation experiment of strain H1 were carried out in Gause's medium having Co^{2+}, Zn^{2+} and Ni^{2+} ions with four different initial concentrations in a rotatory shaker at 180 rpm for 10 days at 28ºC. The two metals Zn^{2+} and Ni^{2+} didn't elicit any compound at any concentration (fig. S5, S6) but a new peak was elicited in Co 2mM (fig. 1). The change in the metabolic pattern after metal ion induction was verified by setting up one medium without metal ions as a Blank, one medium without actinobacteria as a metal control and four sets of media with different ionic concentrations (0.5mM to 4mM). Mycelium and culture broth were separated by a gauze filter and the culture broth was extracted with EtOAc (2 × 200mL). Results revealed from the HPLC chromatogram that one target stress induced compound in the marine actinobacteria *Streptomyces* sp(27.3min for enterocin) was observed in metal treated culture which was almost invisible in non-metal culture (fig. 1). Among different metal ion concentrations the 2mM Cobalt was observed as most effective. While, the strain H-1003 didn't show any kind of metabolites under normal culture conditions (fig.1). When marine actinobacteria strain H-1003 cultured solely in the usual fermentation media, no peak at 27.3 min was observed (fig. 1). A new peak was shown in the 2mM/L Cobalt stressed fermentation, indicating a stress metabolite elicited by Cobalt stress.

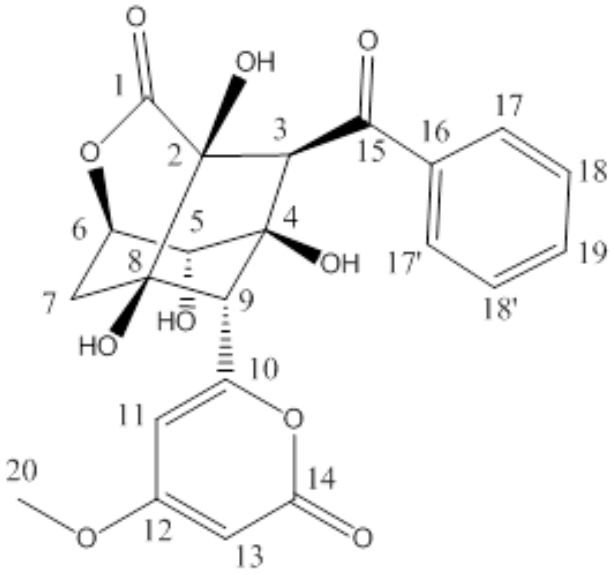

Fig. 2:Chemical structure of enterocin.

Identification and structure determination of metal-induced secondary metabolite

Stress metabolite 1 was isolated as a pale yellowish powder from the marine actinobacteria *Streptomyces* sp. H-1003. The 1H, 13C NMR and DEPT data for the stress metabolite 1 are shown in (fig. S2, S3, S4). From NMR data, stress metabolite 1 was identified as the known compound enterocin as shown in (table 2) (fig. 2) (Saurav *et al.*, 2014). It is also known as vulgamycin. The ^{13}C NMR spectrum in enterocin showed the presence of 22 signals including a monosubstituted benzene ring and a methoxy-substituted α-pyrone ring. enterocin 1 was reported to possess strong bactericidal activities (Gálvez *et al.*, 2007, Ghairi and Hani, 2015). Enterocin (1) was reported to be bacteriostatic against Gram-positive and Gram-negative bacteria, including *Escherichiu coli* and species of *Proteus*, *Sarcina*, *Staphylococcus* sp., and *Corynebacterium*, at the high concentration of 4mg/mL

Table 1: Levels of tested parameter for Box–Behnken design.

Independent factors	Unit	Symbol	Levels	
			Low	High
Time	Days	A	8	12
Metal ions	mM	B	1.5	2.5
Starch	gm	C	15	20

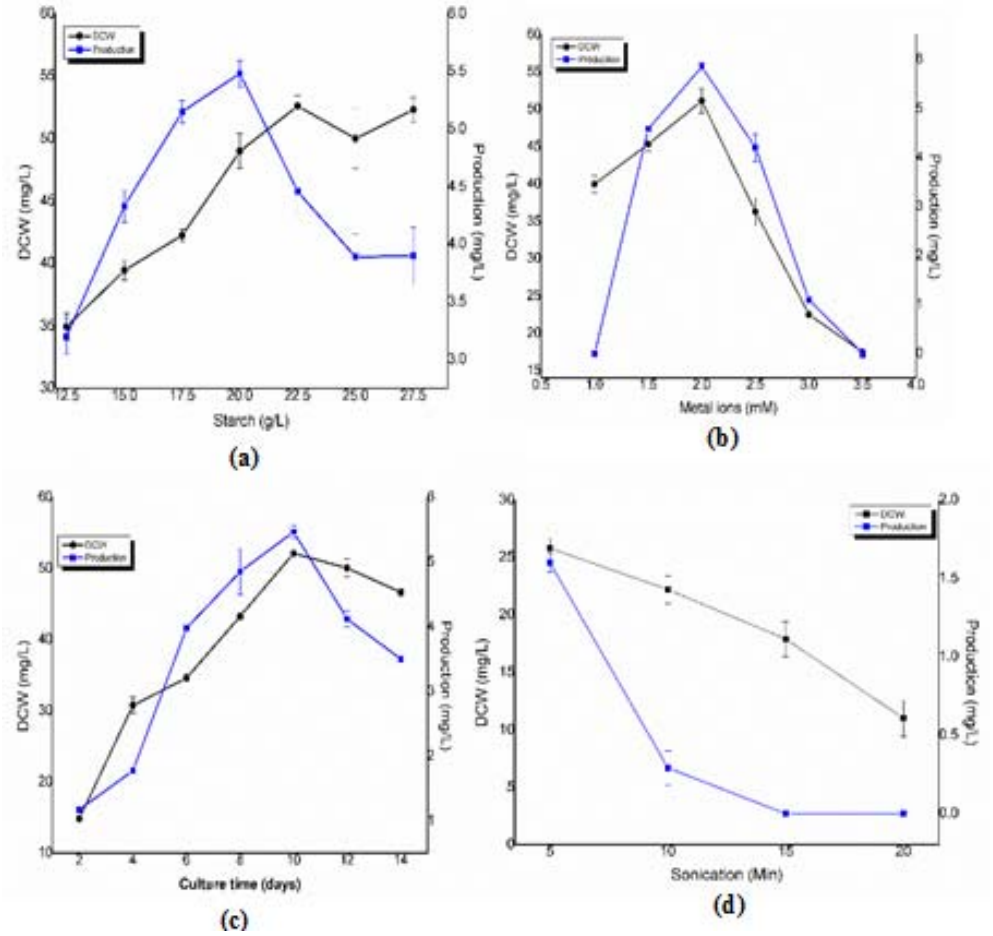

Fig. 3: The enterocin production and DCW of H-1003 strain cultured in Gause's optimized medium. (a) Culture conditions of H-1003 strain with different starch level and constant culture time and Co^{2+} 2mM ions. (b) Culture conditions of H-1003 strain with different metal ion concentrations and constant culture time 10days and starch 20mg. (c) Culture conditions of H-1003 strain with different culture time and constant metal ions concentration Co^{2+} 2mM and starch 20mg. (d) Sonication of H-1003 strain with different passage of time.

(Gálvez *et al.,* 2007, Ghairi and Hani, 2015). This is the first example in which enterocin was induced by metal elicitation. In this case, we have also calculated the simulated peak to demonstrate the dose-dependent effect of elicitation.

Antimicrobial activity of enterocin

The second phase of bioassay was performed for the pure compound enterocin. The pure compound enterocin displayed a potent strong broad spectrum bactericidal activity against *Staphylococcus aureus* [CMCC (B) 26003] with MIC value of 62.5µg/ml, *Escherichia coli* [CMCC (B) 44102] with MIC value of 31.25 and *Bacillus subtilis* [CMCC (B) 63501] with MIC value of 31.25 µg/ml respectively. The pure compound also displayed a marked zone of inhibition against *Staphylococcus aureus* [CMCC (B) 26003] (fig. S7). Ampicillin was used as a positive control.

Factors affecting the enhancement of enterocin production

In order to properly enhancement of enterocin production, it is necessary to up-regulate the factors affecting the elicitation of enterocin biosynthesis. As reported previously, the enterocin enhancement can be stimulated by nutrient composition (starch) and other external factors

such as culture time, physical stimulation (temperature and ultrasonication) and inducer supplementation (metal ions) (Malheiros *et al.,* 2015). Therefore, nutrient supplementation (starch), culture time (days) and inducer supplementation with metal ions were elected to enhance the enterocin production. The supplementation concentration of each factor on enterocin production along with culture time was optimized as single factor base experiments. The enterocin production may be regulated by the amount of starch in the medium. As, starch is one of the basic nutrient supplement in the medium. Therefore, when culturing H1 actinobacteria in Gause medium with different amount of starch level, the enterocin production was altered with different level and the production rate was sky rocketed when starch level reached to the amount of 20gm/L. The starch addition condition and its influence on enterocin production and DCW (Dry cell weight) were summarized in (table S1) (fig. 3a).

Table 2: ^{1}H NMR data (500 MHz, δ in ppm, *J* in Hz) and ^{13}C NMR data (125 MHz, δ in ppm) for compound 1 in CD_3OD-d_4

position	1	
	δ_C, type	δ_H (*J* in Hz)
1	175.8, C	
2	80.1, C	
3	56.7, CH	4.66, d (4.5)
4	77.5, C	
5	80.9, CH	4.81, m
6	71.3, CH	4.74, d (2.7)
7	36.8, CH_2	2.65, dd (14.6, 3.1) 1.88, dt (14.6, 2.8)
8	77.7, C	
9	54.7, CH	4.70, s
10	162.4, C	
11	107.4, CH	6.41, d (2.0)
12	173.6, C	
13	89.1, CH	5.66, d (2.2)
14	167.2, C	
15	197.9, C	
16	140.9, C	
17/17’	129.6, CH	7.94, dd (7.3, 1.3)
18/18’	129.7, CH	7.49, t (7.7)
19	134.3, CH	7.59, t (7.4)
20	57.1, CH_3	3.90, s

The impact of metal ions in the culture medium can induce or enhance the synthesis of secondary metabolism. Metals may alter the production of bioactive compounds by changing aspects of secondary metabolism (Verpoorte *et al.,* 2002). Metals including Cu, Co, Ni, Zn, Ag and Fe have been shown to elicit the production of secondary metabolites in a variety of microorganisms (Ding *et al*., 2016). The enterocin production was altered by different concentration of Co^{+} ions. The enhancement reached to its peak when the metal concentration was 2mM in the fermentation medium. The decreased amount of enterocin production was observed in initial and high concentrations of metal ions but the DCW amount was decreased in high concentration of metal ions (table S1) (fig. 3b). These results suggest that heavy metals has been proven to be an effective mean to stimulate their cryptic secondary metabolism.

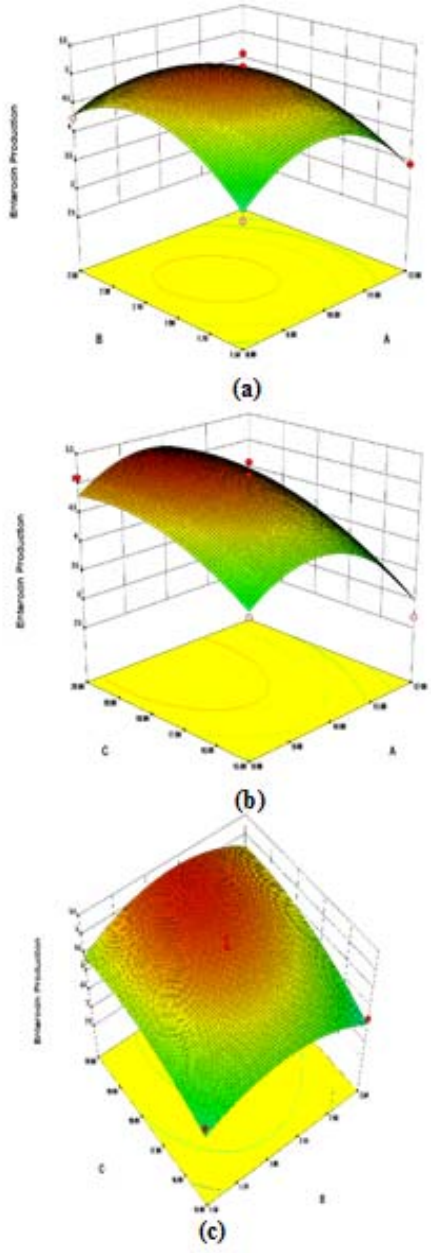

Fig. 4: Response surface graphs representing the effect of Time (a), Metal ions (b) and starch (c) on the responses. (a) Relative effect of Time and metal ions on enterocin production at constant starch level (b) Relative effect of Time and starch level on enterocin production at constant metal ions (c) Relative effect of metal ions and starch level on enterocin production while keeping time as a constant factor.

The bacteria have 4 different stages in their life cycle during which they first start their growth by maturing them self and entering second phase of life where they start cell-doubling, if the essential nutrients didn't provide enough then bacteria move towards stationary phase where they stop their growth due to growth-limiting factor and at last bacteria cell starts dying if the nutrients still didn't provide for long period (*Fankhauser and David, 2004)*. The enterocin production was highly influenced by culture period. The production started to grow from 2nd day and reached to its peak on 10th day with highest amount of DCW and enterocin production and started decline from 12 day onwards (table S1) (fig. 3c). Contrary to this, activity base guidance also re-proved the elevated level of enterocin production on 10th day by doing anti-bacterial assay against three microorganisms including *Staphylococcus aureus* [CMCC (B) 26003], *Escherichia coli* [CMCC (B) 44102] and *Bacillus subtilis* [CMCC (B) 63501]. The 10th day crude extract exhibited a high level of antibacterial activity hence proving elevated level of enterocin production at that time (fig. S8). Higher amount of enterocin were produced when the incubation temperature was between 25°C to 30°C. At temperature above or below these scales negotiate the enterocin production (Malheiros *et al.,* 2015). Therefore, understanding the important role of temperature in enterocin production we selected the constant temperature 28°C during our total optimization experiments. The physical stimulation ultra-sonication didn't enhance the enterocin production but imposed the negative effect on enterocin production and DCW. After culturing H-1003 strain for 10 days in Starch 20 mg with Co^{2+} 2mM ions, we subjected the matured culture for ultra-sonication setting initial time from 5 minutes till 30 minutes with a gap of 5 minutes between each batch, but after initial ultra-sonication result we observed a specific decline in enterocin production and DCW, and at a level of 3rd batch on 15 minutes ultra-sonication the enterocin production was totally diminished (fig. 3d).

The time profile of dry cell weight (DCW) and enterocin production of H1 strain effected by individual factors time (A), metal ions (B) and starch (C) summarized in (table 3) are an evident from the data that all these three variables have a direct effect on enterocin production.

As shown in (fig. 1, fig. 3 and fig. 4) the actinobacteria growth and enterocin production in Gause medium having starch 20 mg, with Co^{2+} 2mM, sky rocketed on 10th day with highest production value of 5.33mg/L. These results demonstrated that Gause medium having starch 20 mg with 2 mM Cobalt ions, cultured for 10 days was the most favored condition for the actinobacteria growth and enhanced enterocin production among these three tested factors. The difference of cell growth and enterocin production may be related to the different concentrations of metal, medium ingredients and culture time, as the actinobacteria growth and secondary metabolism of actinobacteria can be greatly influenced by media ingredients and metal ions in their surroundings (Ding *et al.*, 2016). However, mechanisms of various metal ions with different media ingredients coping with actinobacteria should be further investigated for the best fermentation achievement.

Response surface optimization for enterocin

The key parameters most influencing on the metal stress technique for the elicitation of compound from marine actinobacteria for enhancement of enterocin, were time (A), metal ions (B) and starch (C) studied for this study. The response was measured in terms of actual factors of production. The results of experimental runs are summarized in (table 3). Data collected from experimental runs were analyzed by using the Design Expert software, version V 8.0.5.

To analyze and optimize the influence of three independent parameters for the extraction, namely: metal ions concentration, starch quantity and culture time (days) on the enterocin production, response surface methodology (RSM) with a 3 factorial experiments was designed. The mean values of production (Y, dependent parameter) obtained from the triplicate experiments were fitted to a quadratic polynomial model which reads as follows (Pan *et al.,* 2015)

$$Y=\beta_0 + \Sigma\beta_i X_i + \Sigma\beta_i X^2_{ii} + \Sigma\beta_{ij} X_i X_j$$

Where Y is the predicted response (the yield of extractive substances in mg), b_0, b_j, b_{jj} and b_{ij} are the regression coefficients for intercept, linear, quadratic and interaction terms, respectively, while X_i and X_j are the independent variables. The statistical software Design Expert (Trial version 8.0.5) was used to calculate the experimental data and to find the contour plots and response surfaces of the response model. The significance of the independent parameters and their interactions were calculated by the ANOVA. The suitability of the model was expressed by the coefficient of determination (R^2) and adjusted R^2, while the F-test and p-value were used to check the significance of the regression coefficient. Finally, in order to determine the suitability of the fitted model, the experimental and predicted values were compared (table S2).

This multiple nonlinear model resulted in response surface graphs for enterocin production (fig. 4a, 4b and 4c). Point prediction tool of the software was used to calculate maximum production. Finally the optimum values for enterocin production, time 9.5 days, 2mM cobalt ions and starch 19.28gm resulted in production of 5.35 mg/L. The optimized values of these parameters were validated under similar conditions. An average 5.33 mg/L of production was produced under optimized conditions with 99.6% validity. The above results obtained by use of these conditions suggested that the

response surface predictions were in good agreement with the experimental results. Therefore, Response surface methodology is a powerful and reliable tool for optimizing biotechnological processes and Box–Behnken statistical design was reliable and effective in determining the optimum conditions (Malheiros *et al.,* 2015).

DISCUSSION

This work demonstrated by the two procedures of biological and chemical screening that the secondary metabolite patterns of selected strain can vary under the influence of heavy metals added to the fermentation medium. This work established a very easy, facile and fast method for enhanced enrichment of enterocin by optimized conditions. Under the optimal condition, enterocin production was enhanced with a value of 5.33mg/L, which was absent at the normal culture. Strains isolated from marine soil sediments, display an altered secondary metabolite pattern visualized by HPLC and varied antibiotic activity in bioassays after growth in metal containing medium. It certainly would be worthwhile to re-screen already established microbial strain collections for novel secondary metabolites produced under the influence of heavy metals. The results of this study showed that stress-driven discovery of drug candidate from the sea is an effective strategy to unveil the untapped reservoir of small molecules from marine species.

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Supplementary data

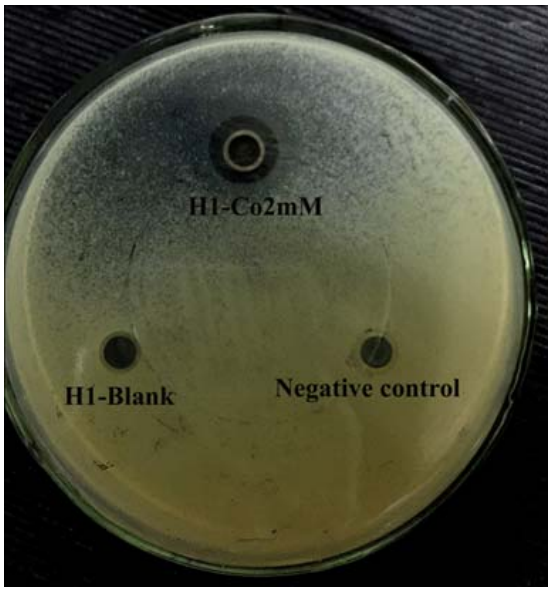

Fig. S1: Antibacterial activity of H1 strain with and without metal ions against *S. aureus* [CMCC (B) 26003]. The negative control shows the 50%DMSO as H1+Co-2mM was dissolved in 50%DMSO.

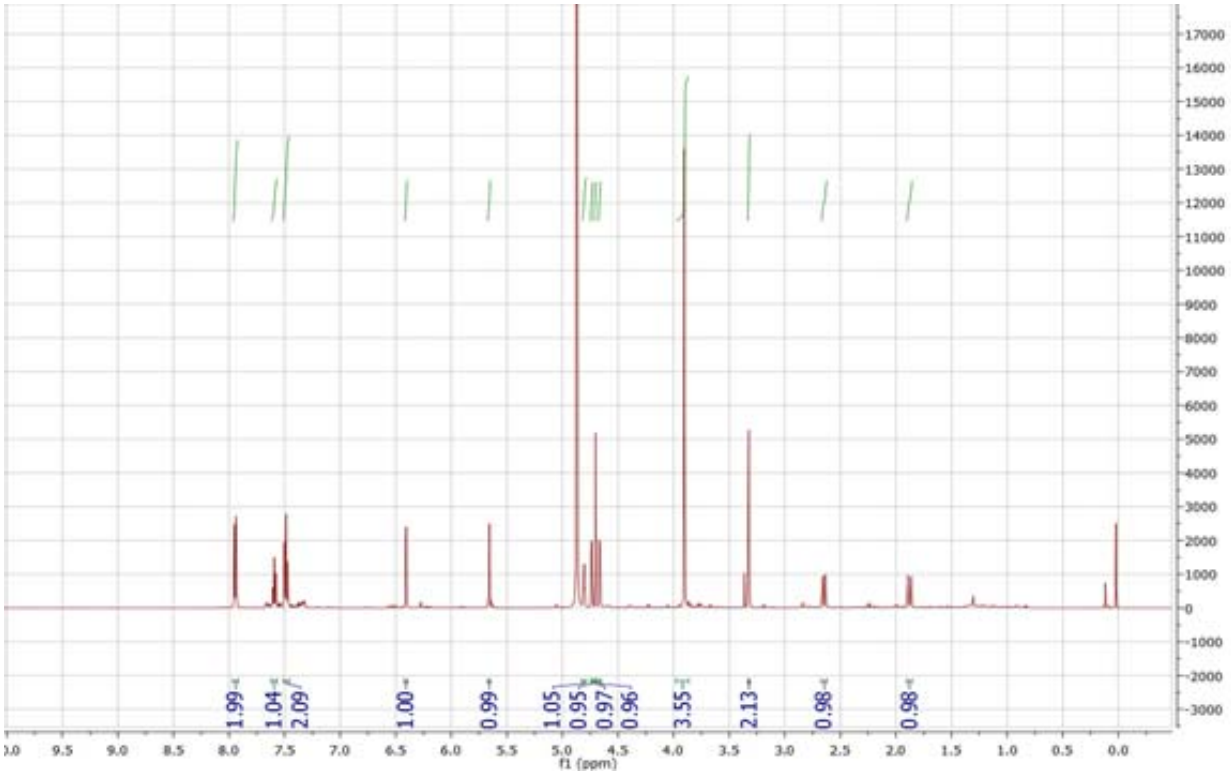

Fig. S2: ^{1}H NMR in CD_3OD for Enterocin

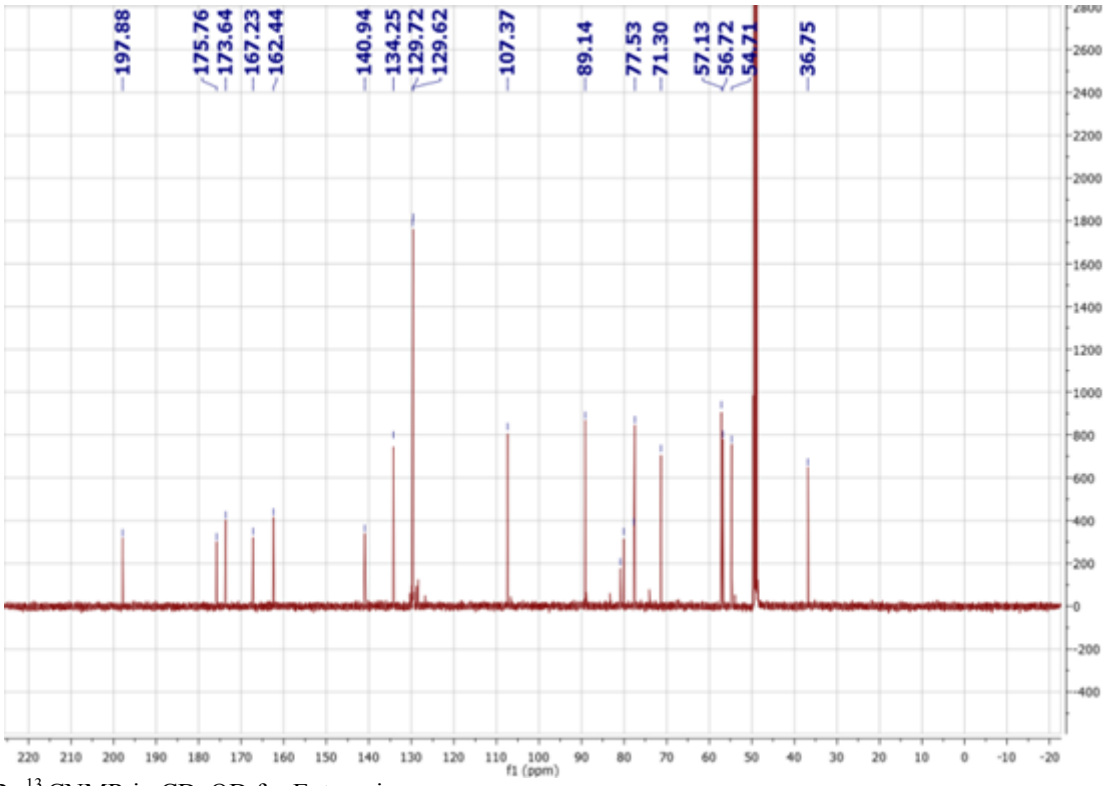

Fig. S3: ^{13} CNMR in CD_3OD for Enterocin

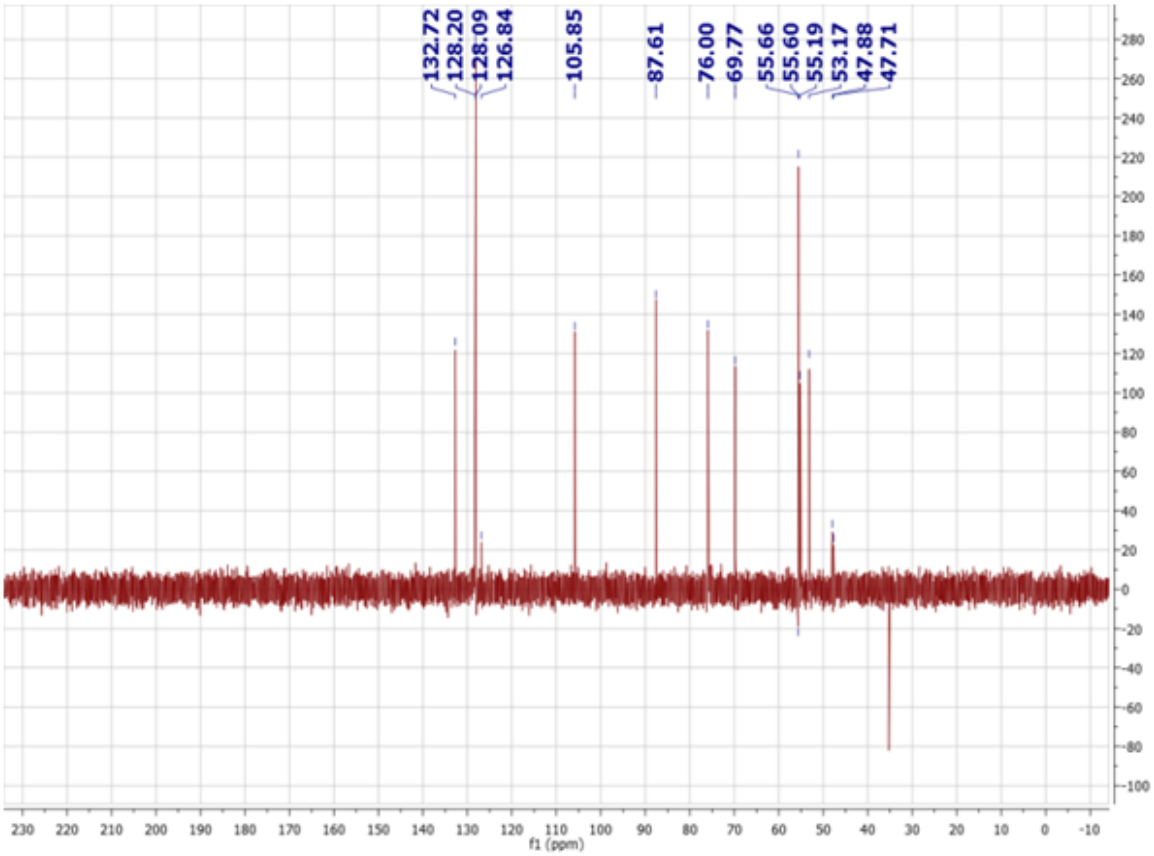

Fig. S4: DEPT in CD_3OD for Enterocin

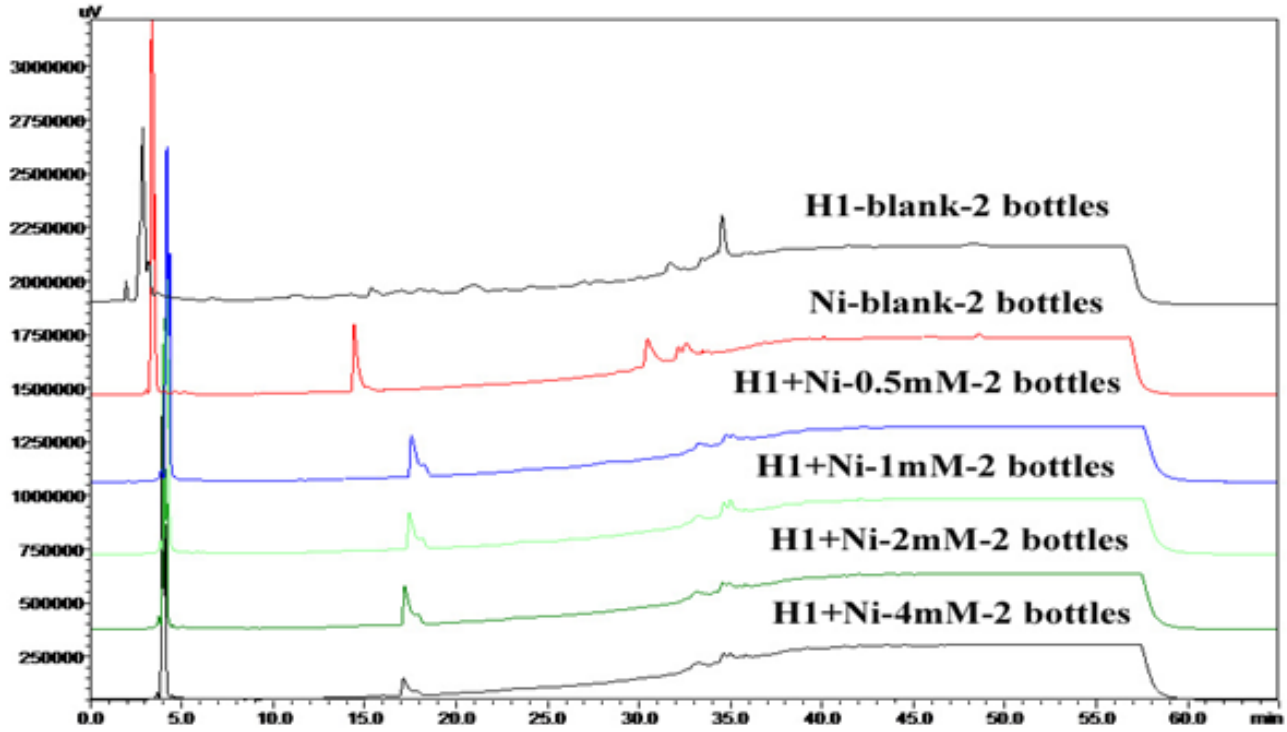

Fig. S5: HPLC profile of metal treated and untreated H1 strain with four different concentrations of Nickel ions.

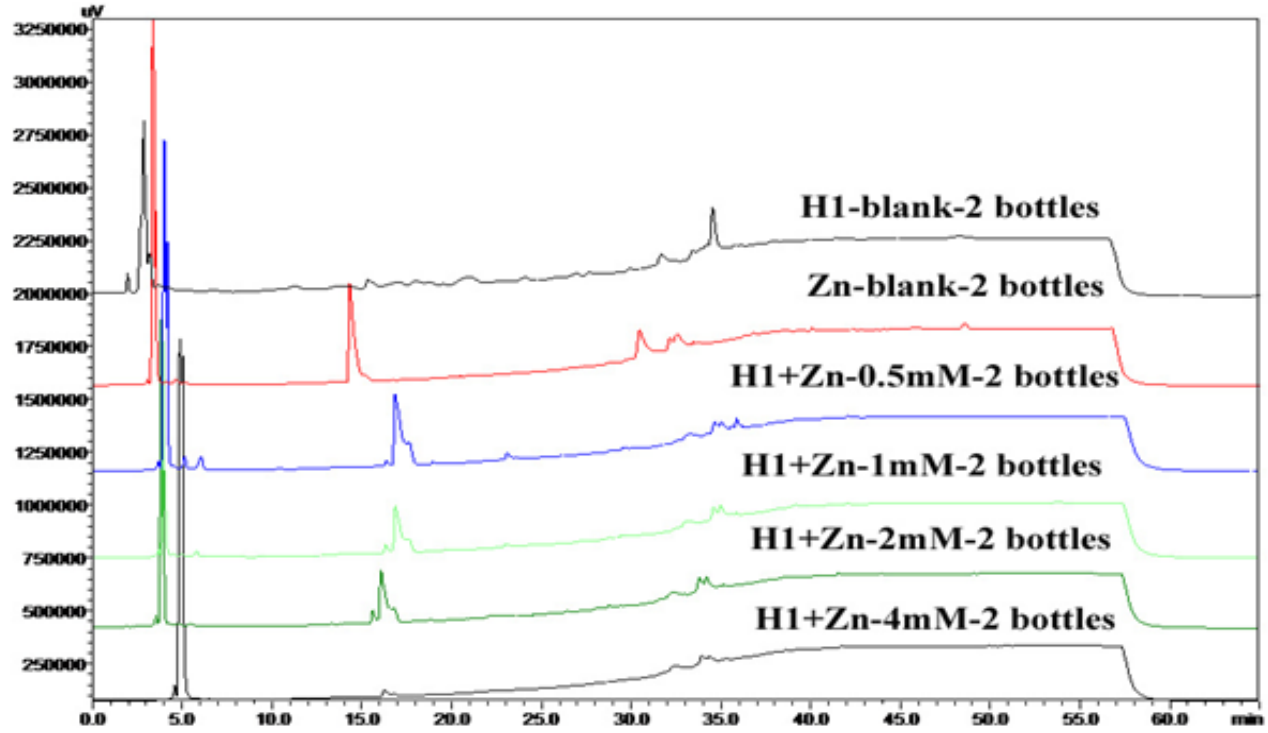

Fig. S6: HPLC profile of metal treated and untreated H1 strain with four different concentrations of Zinc ions.

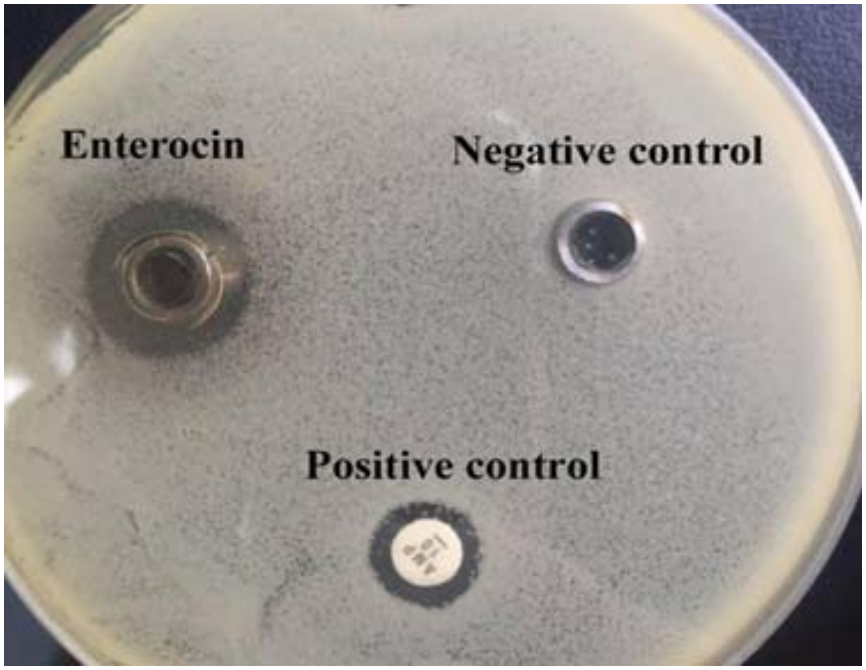

Fig. S7: Antibacterial activity of Enterocin against *S. aureus* [CMCC (B) 26003]. The negative control shows the 50%DMSO as Enterocin was dissolved in 50%DMSO. Ampicillin was used as a positive control.

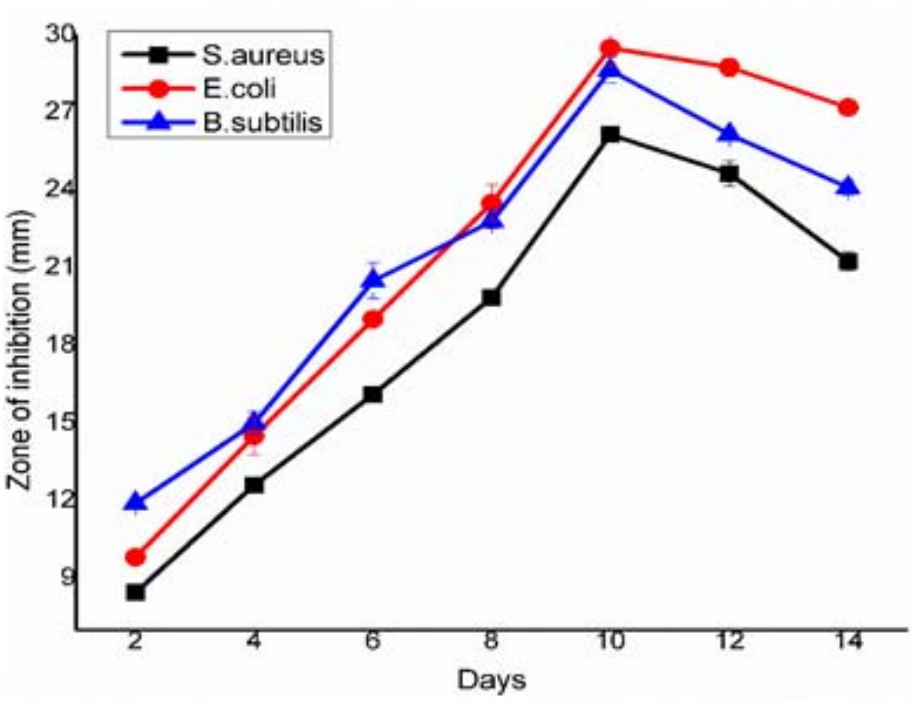

Fig. S8: Antibacterial activity of different culture days of enterocin production.

Table S1: Experimental design and results of DCW and enterocin production by three variables

Runs	Factors			Production (mg/L)	DCW (mg/L)
	Days	Metal ions (mM)	Starch (gm/L)		
1	10.00	2.00	12.50	3.3 ± 0.15	34.95±1.06
2	10.00	2.00	15.00	4.33 ± 0.14	39.45 ± 0.77
3	10.00	2.00	17.50	5.15 ± 0.09	42.25 ± 0.49
4	10.00	2.00	20.00	5.48 ± 0.11	50.2 ± 0.41
5	10.00	2.00	22.50	4.46 ± 0.18	52.6 ±0.84
6	10.00	2.00	25.00	3.89 ± 0.19	50 ± 0.98
7	10.00	2.00	27.50	3.9 ± 0.24	52.3 ± 0.92
8	10.00	1.00	20.00	0 ± 0	40 ± 1.13
9	10.00	1.50	20.00	4.58 ± 0.23	45.3 ± 0.91
10	10.00	2.00	20.00	5.41 ± 0.07	51.1 ± 0.72
11	10.00	2.50	20.00	4.2 ± 0.28	36.3 ± 1.38
12	10.00	3.00	20.00	1.09 ±0.02	22.2 ± 0.70
13	10.00	3.50	20.00	0 ± 0	17.5 ± 0.70
14	2.00	2.00	20.00	1.17 ± 0.004	14.85 ± 0.21
15	4.00	2.00	20.00	1.78 ± 0.042	30.8 ± 1.13
16	6.00	2.00	20.00	3.97 ± 0.005	34.6 ± 0.56
17	8.00	2.00	20.00	4.85 ± 0.35	43.3 ± 0.42
18	10.00	2.00	20.00	5.47 ± 0.09	52.1 ± 0.21
19	12.00	2.00	20.00	4.12 ± 0.12	50.1 ± 1.27
20	14.00	2.00	20.00	3.5 ± 0.02	46.6 ± 0.49

DCW (dry cell weight). Data are expressed as the M ± S.D., while M means to Mean and S.D means Standard deviation. N=3

Table S2: Table of ANOVA analysis.

Source	Sum of Squares	df	Mean square	F value	P-value Prob > F	
Model	9.71	9	1.08	15.42	0.0008	significant
A	1.51	1	1.51	21.65	0.0023	
B	$4.512*10^{-3}$	1	$4.512*10^{-3}$	0.065	0.8068	
C	1.80	1	1.80	25.68	0.0015	
AB	0.28	1	0.28	3.94	0.0875	
AC	$4.225*10^{-3}$	1	$4.225*10^{-3}$	0.060	0.8129	
BC	0.058	1	0.058	0.82	0.3943	
$A^2$3.12	1	3.12	44.58	0.0003		
$B^2$1.91	1	1.91	27.27	0.0012		
$C^2$0.48	1	0.48	6.88	0.0343		
Residual	0.49	7	0.070			
Lack of fit	0.41	3	0.14	6.61	0.0497	significant
Pure Error	0.082	4	0.021			
Cor total	10.20	16				