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Ferrous ion Enhanced Lipopeptide Antibiotic Iturin A Production from *Bacillus amyloliquefaciens* B128

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Abstract: The effect of different carbon sources, initial pH, and addition of divalent metal cations to the shaker flask cultivation medium was observed with the aim of improving iturin A production from *Bacillus amyloliquefaciens* B128. Among different C-sources tested, soft sugar enhanced iturin A production to 68.78 mg/L, which is 5 fold higher than nutrient broth cultivation concentration. This concentration further enhanced to 71.34 mg/L, after 6 days cultivation by the application of starting cultivation pH at 6.64. Furthermore, when this medium was supplemented with 0.2 mM of iron, iturin A concentration increased to 92.78 mg/L, after 5 days with no pH control cultivation. Interestingly, with the stating pH at 6.64 and 0.2 mM of ferrous sulfate, the highest iturin A production of 121.28 mg/L was obtained. Thus, the present study provides useful information for enhancing iturin A production in cultivation.

Keywords: *Bacillus amyloliquefaciens* B128; Carbon source; Divalent metal addition; Iturin A.

1. Introduction

In the present day, commercially available chemical pesticides and chemical fertilizers in modern agriculture are being removed from the market for their hazardous impact to the natural environment. To cope with these problems, biological control agents, which include effective microorganisms and microbial products, and organic fertilizers have been attracting attention recently as alternatives to chemical agents [1]. *Bacillus amyloliquefaciens* is a Gram-positive, spore forming bacteria, and is closely related to *Bacillus subtilis*. *B. amyloliquefaciens* and other members of the *B. subtilis* group are considered as safe and have "generally recognized as safe" status [2]. Several strains of *B. amyloliquefaciens* have been reported that have potential for biological control of several plant diseases. For example strains 5PVB, B94 and RC-2 against *Botrytis elliptica*, a pathogen of lily grey mould, *Rhizoctonia* seedling disease on soybeans, and *Colletotrichum dematium*, mulberry anthracnose fungus, respectively [3–5]. Importantly, the strain B128 has been reported as a biological control agent against *B. elliptica* which destroys all lilies in field when environmental conditions are conducive to the disease development [6]. Moreover, effective formulation consisting of *B. amyloliquefaciens* B128

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for control lily grey mould in field has been established [7]. Further, *B. amyloliquefaciens* is used industrially for large scale production of proteases, heat-stable amylases [8], bioactive cyclic lipopeptides [9], surfactin [10], the protein toxin barnase [11], iturins [5, 12].

Iturins are constituted by a cyclic heptapeptide like surfactins, but contain a β-amino fatty acid as the lipidic part. The fatty acid chain of lipopeptides varies from 13 to 17 carbon atoms [13]. The iturin group consists of iturin A−E, bacillomycin D, F, and L, and mycosubtilin. All are cyclic peptides with seven α-amino acids (A_1−A_7), and one unique β-amino fatty acid (βAA). They have a general sequence of c[βAA- Asx- Tyr- Asn- A_4- A_5- A_6- A_7] beginning from the first α-amino acid after the β-amino acid as number 1−7 counted from the N-terminus to the C-terminus in the opened-ring peptide [14]. There are isomers for each of the iturins because their fatty acid chain can be in the *n*-, *iso*-, or *anteiso*-form. In nature, iturin A is produced as a mixture of up to eight isomers named as iturin A_1−A_8 [15]. The structure of iturin A is shown below.

R-CHCH2CO→L-*Asn*→D-*Tyr*→D-*Asn*
| ↓
NH←L-*Ser*←D-*Asn*←L-*Pro*←L-*Gln*

The strong efficacy of iturin A against various phytopathogenic fungi is similar to the available chemical pesticides [16]. It has been tested for control of a variety of fungi in pure cultures, and during composting [17–20]. Along with its wide antifungal spectrum, iturin A has low toxicity and low allergic effect on human and animals [18] and stands as a candidate for environmentally safe biological pesticide [21].

Submerged cultivation may provide a more effective system for industrial production of antibiotics like iturin A, which offers the advantages of high homogeneity of heat, mass transfer and easy control of oxygen and temperature, over solid-state fermentation which is limited by practical problems such as difficulties in controlling the above conditions, as well as issues with up-scaling the reactor [22]. In submerged cultivations, the biosynthesis of iturin A antibiotics by spore forming strains of *Bacillus* are not only highly dependent on medium composition but also cultivation conditions as well [23–25]. Further, the regulatory effects of medium composition and trace metals on microbial secondary metabolism have been recorded for a variety of species [26, 27]. However, very little has been published on the effect of cultivation conditions on the production of iturin A from *B. amyloliquefaciens* B128. In our previous study, we have reported the optimized submerged cultivation conditions for the maximal spore production from this strain [28]. The objective of this study is to determine the influence of various carbon sources, initial pH, and metal ions addition on the yield of iturin A by *B. amyloliquefaciens* B128 in shaker flask cultivations.

2. Materials and Methods

2.1. Bacterial strain and culture preparation

The strain *B. amyloliquefaciens* B128 used in this study was isolated from an infected lily plants provided by one of the author Prof. Wen-Shi Wu. The stock culture was maintained on nutrient broth (NB, Difco, Detroit, Mich.) plus 10% glycerol at 4 °C and –70 °C for short term and long term storage, respectively at the Institute of Biotechnology, Chaoyang University of Technology, Wufeng, Taiwan, ROC.

The strain was transferred from culture stock into in a 500 mL Erlenmyer flask containing 200 mL of medium (3% glucose, 0.75% Bacto peptone (Difco, Detroit, Mich.), and incubated for 10 h at 30 °C and 200 rpm.

2.2. Shake-flask studies

The standard spore suspension inoculums 2% (v/v) were used for shaker flask cultures to evaluate the effects of carbon source, starting pH, and divalent metal cations on the production of iturin A from *B. amyloliquefaciens* B128. Cultures were grown at 30 °C and 200 rpm in a rotary shaker incubator (Yih-Der, LM-530R., Taiwan), in 500 mL baffled Erlenmeyer flasks with 200 mL of production media, and no pH control was applied for all cultivations. The complex medium for cultivation of *B. amyloliquefaciens* B128 consisted of Luria-Bertani (LB) medium (Difco Laboratories) and NB medium. NB broth was prepared according to Difco recommendations using an NB preparation containing Bacto beef extract (3 g/L) and Bacto peptone (5 g/L) (Difco Laboratories, Detroit, Mich.). A basal chemically defined medium consisted, 2% of one of the following carbon sources, i.e. fructose, glucose, sucrose (Difco Laboratories), and soft sugar (a sucrose sugar product with a distinctive brown color due to the presence of molasses). The other culture medium components were (%); 1 peptone, 0.1 yeast extract, 0.01 $CaCO_3$, and 0.01 NaCl. For pH studies, the starting pH had been adjusted to 4.0, 5.0, 6.0, 6.64, 7.0, 8.0 or 9.0 by the addition of 2 N HCl or 2 N NaOH. The effect of divalent trace metal on iturin A production was studied by using $FeSO_4.7H_2O$, $MgSO_4$, $CoSO_4.7H_2O$, $MnSO_4.H_2O$, $CuSO_4.5H_2O$ and $ZnSO_4.7H_2O$ (Sigma, St. Louis, Mo., USA).

Submerged cultivations were performed in three replicate experiments, and the analyses were carried out at least in duplicate. The values reported here are mean values with standard deviations being less than 5% in all cases.

2.3. High-performance liquid chromatography (HPLC) analysis

Quantification of iturin A was done by high performance liquid chromatography (HPLC) as described previously (Figure 1) [25]. From the homogenized sample of the cultivation medium 100 µL was transferred into 1.5 mL polypropylene tube and mixed with 900 µL of extraction buffer (acetonitrile:10 mM ammonium acetate (40:60, v/v). The mixture was vortexed at room temperature for 20 min and then centrifuged at 15,000×*g* for 10 min at 4 °C. The supernatant was filtrated through 0.20 µm poly-tetra-fluoro ethylene (PTFE) membrane filter (Advantec, Tokyo, Japan) and 20 µl of the filtrate was injected into HPLC column for iturin A detection. The HPLC system consisted of a Hitachi (Tokyo, Japan) L-7100 pump; a 20 µL fixed loop, and a Model L-7400 variable-wavelength UV-Vis detector. A 7 µm LiChrosorb RP-18 column (250 mm × 10 mm) supplied by Merck KGaA, Darmstadt, (Germany) was used. Isocratic eluent: acetonitrile and 10mM ammonium acetate (40:60, v/v), operated at 30 °C, at a flow rate of 2 mL/min, and the detector monitored absorption at 260 nm for a period of 20 min (Figure 1).

3. Results

In the preliminary experiments using 1–10% (v/v) vegetative inoculum, we found that 2% (v/v) was the optimal level. Therefore, in all cases, inoculum size was 2% (v/v). Furthermore, a series of experiments were carried out to study the effect of temperature and shaker orbital revolution rate on the yield of iturin A production. The results demonstrated that the maximal concentration of iturin A was obtained with the 200 rpm shaker rate and 30 ºC in 6 day cultivation (data not shown).

The cultivations in the complex medium used for screening purposes, when *B. amyloliquefaciens* B128 was grown in the NB medium, the production of the iturin A was relatively poor, and the maximum concentration of about 34.25, and 12.58 mg/L with LB and NB medium, respectively (Figure 2). Using a basal chemically defined medium, various carbon sources (all used in equal concentra-

tions of 2% or 20 g/L) were tested in growth experiments for their ability to support the production of iturin A by *B. amyloliquefaciens* B128. Results for growth after 9 days on these different substrates, in shake flask cultures are given in Figure 2. Compared with complex medium, soft sugar, fructose and glucose supported the production iturin A, which led to a concentration of 68.78, 60.71, and 54.68 mg/L, respectively, after 6 days of cultivation. No pH control was applied for all these cultivations. In addition, the above experiment has shown that soft sugar is the best carbon source for iturin A production (Figure 2). It was thought, therefore, to test whether iturin A production could be improved by varying soft sugar concentration in the cultivation medium. For this purpose soft sugar was applied in different concentrations varied from 1 to 6%. The results showed that production of iturin A reached a maximal value at 2%, above this concentration, the production decreased. As soft sugar concentration increased from 2 to 6% the iturin A production decreased by approximately 35% (data not shown). On the other hand, soft sugar was completely consumed when used at a concentration of 2% or lower. The increase in soft sugar concentration above this level resulted in the accumulation in the cultivation medium, and the remained amount depended on the initial concentration. Therefore, soft sugar in a concentration of 2% was used in the subsequent experiments.

Next, to know the effect of pH on the iturin A production from *B. amyloliquefaciens* B128, the initial pH value varied in the range of 4.0 to 9.0 using soft sugar (2% w/v) as the carbon source. The results in Figure 3 shown that the initial pH of 4.0, 5.0 and 9.0 in the cultivation medium considerably diminished the iturin A production. However, in the cultivation of initial pH as 6.64, the production of iturin A slightly increased about 3.7% (71.34 mg/L) of that of the control (68.78 mg/L) where no pH control was applied. It was noted that during cultivation of *B. amyloliquefaciens* B128, the final pH of the cultivation medium was higher than the initial pH; and that the extent of the increase in pH was proportional to the iturin A production.

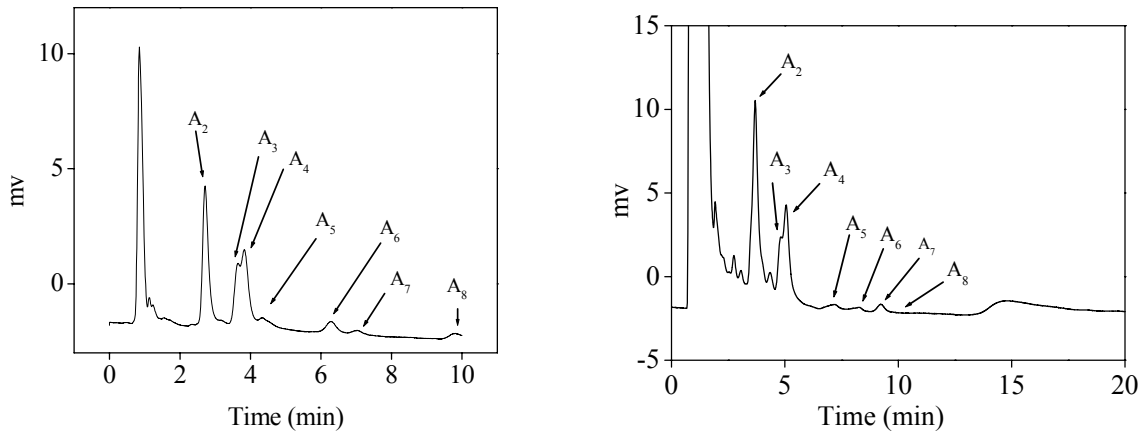

Figure 1. HPLC analyses of iturin A: (A) standard iturin A; (B) Diluted filtrate from *B. amyloliquefaciens* B128 cultivation. Chromatographic conditions are described in materials and methods section.

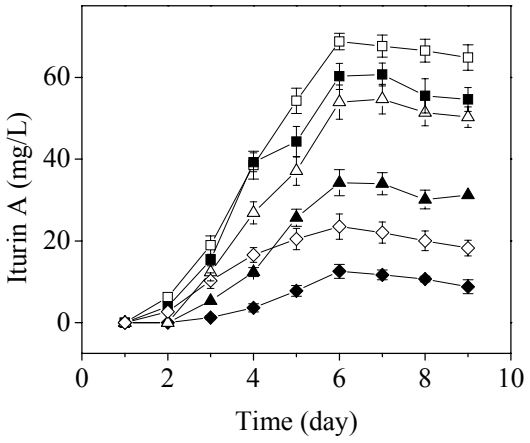

Figure 2. Iturin A production in different medium: Complex medium, LB broth (—▲—), and NB broth (—♦—), and synthetic medium containing carbon source as, soft sugar (—□—); fructose (—■—), glucose (—Δ—), and sucrose (—◊—).

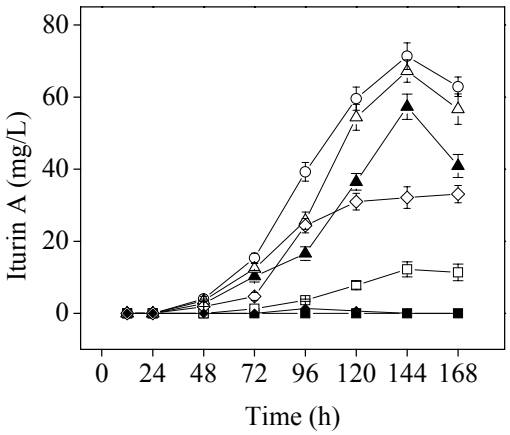

Figure 3. Effect of vario us starting pH on the iturin A production by *B. amyloliquefaciens* B128 in shaker flasks cultures, pH 4.0 (—■—), 5.0 (—□—), 6.0 (—▲—), 6.64 (—○—), 7.0 (—Δ—), 8.0 (—◊—), and 9.0 (—♦—).

Next cultivations were carried out to know the effect of metal cations on iturin A production. To 500-mL shaker flasks were added 200 mL of the basic media containing soft sugar as carbon source, and about 2 mM of various metal salts. All metals were added aseptically as sulfate salts to autoclaved growth medium, with an untreated culture serving as the control. The flasks were incubated at 30 °C for 8 days and compared to control flasks. Only one salt, $FeSO_4$ caused significant enhancement of iturin A, where the maximum concentration of 74.46 mg/L was obtained after 6 days cultivation (Figure 4). Further, the salt $MgSO_4$ at 2 mM concentration had no noticeable effect on iturin A production. In contrast, the salts $ZnSO_4$, suppressed growth of *B. amyloliquefaciens* B128, while $CuSO_4$, $MnSO_4$ and $CoSO_4$ completely inhibited growth.

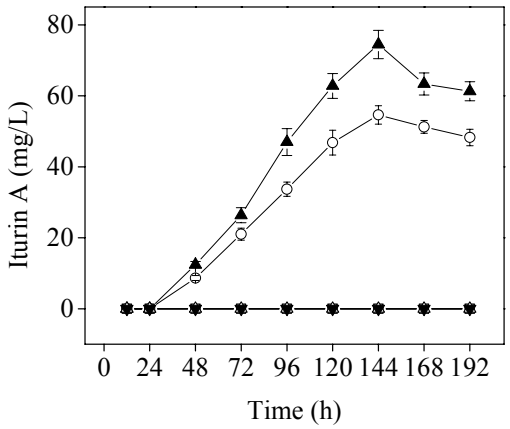

Figure 4. Effect of various divalent cations on iturin A production by *B. amyloliquefaciens* B128 in shaker flasks cultures; Co^{2+} (—■—), Cu^{2+} (—□—), Fe^{2+} (—▲—), Mg^{2+} (—○—), Mn^{2+} (—Δ—), and Zn^{2+} (—▼—).

Accordingly, the effects of iron and magnesium on growth and iturin A production were further assessed using glucose, fructose, and soft sugar as carbon source. A control flask with no added iron or manganese had almost normal growth and produced only 68.78 mg/L of iturun A, after 6 days of cultivation. In several sets of experiments, the results show that iron dramatically affected the production

of antibiotic synthesized (Figure 5). Importantly, in the cultivation of soft sugar as carbon source and iron addition in the concentration of 0.2 mM increased the antibiotic production to 92.78 mg/L, after 5 days of cultivation. Magnesium at a similar concentration to that of iron displayed trend that was similar but not as marked (Figure 5). For example, at 0.2 mM of magnesium, iturin A production (71.25 mg/L) was still considerable, being only slightly increased to 3.6% of that of the control (68.78 mg/L). To find the optimal iron and magnesium concentration on maximal production of iturin A, we added both metals at varying concentrations (0.2, 2 and 10 mM) to the cultivation medium before inoculation with bacterial suspension. Metal ions at low concentrations caused an increase in iturin A productivity, whereas the same ions at higher level prompted a decrease in concentration. It was found that the maximum iturin A was measured in the presence of 0.2 mM of both metals. Increased concentrations of both the metals (from 0.2 mM to 10 mM) repressed iturin A production (up to 90% at 10 mM). Furthermore, the effect of mixtures of iron and magnesium metals on iturin A production was also examined. The results in Figure 6, shown that when iron was present, manganese did not affect iturin A production at various concentrations tested. Consequently, 0.2 mM of iron was chosen for further cultivations.

Finally, three additional cultivations were performed for each condition. In case of soft sugar as carbon source, the three replicate experiments produced an average of 68.78 and 71.34 mg/L, in no pH control, and controlled pH at 6.64, respectively, after 6 days of cultivation (Figure 7). However, by the addition of 0.2 mM, iron to the cultivation medium, the concentration further enhanced to 92.28 and 121.36mg/L, in no pH control, and controlled pH at 6.64, respectively, after 5 days of cultivation (Figure 7).

4. Discussion

The biosynthesis of secondary metabolites by *Bacillus* is controlled by several external (cultivation conditions) and internal factors (medium composition) involved a highly complex regulation [24]. Carbon source studies on iturin A production by *B. amyloliquefaciens* B128 revealed that, among different carbohydrates tested, soft sugar was the best source to support the antibiotic production. The optimal concentration of soft sugar in the cultivation medium was 2% (w/v), above which a significant decrease in iturin A production observed. The negative effect of high soft sugar concentrations may be explained on the basis of carbon catabolite regulation mechanism. These results are in agreement with previous reports that the carbon source played an important role in the antibiotic rapamycin and simocyclinones production from *Streptomyces hygroscopicus* and *S. antibioticus*, respectively [29, 30].

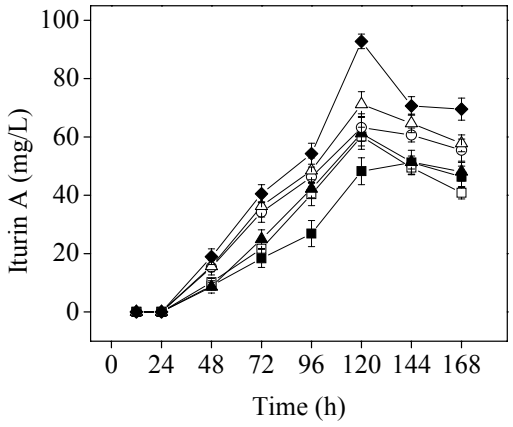

Figure 5. Effect of 0.2 mM individual divalent cations Mg^{2+} and Fe^{2} addition, and carbon source (glucose, fructose and soft sugar) in the cultivation medium on iturin A production by *B. amyloliquefaciens* B128 in shaker flasks cultures; glucose and Mg^{2+} (—■—), glucose and Fe^{2+} (—□—), fructose and Mg^{2+} (—▲—), fructose and Fe^{2+} (—○—), soft sugar and Mg^{2+} (—Δ—), soft sugar and Fe^{2+} (—♦—).

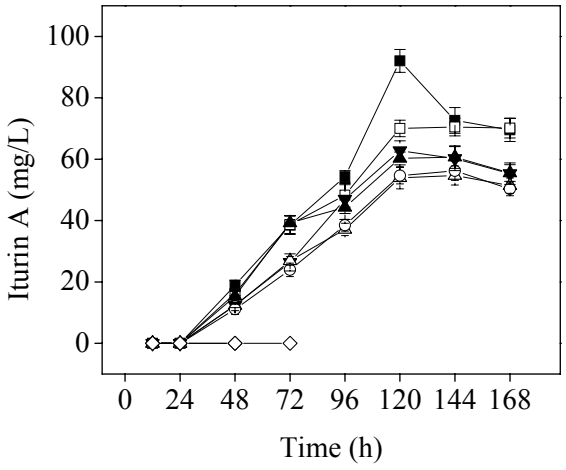

Figure 6. Combined effect of Mg^{2+} with Fe^{2+} on iturin A production by *B. amyloliquefaciens* B128 in shaker flasks culture (Fe^{2+} and Mg^{2+} in mM); 0.2 and 0.2 (—■—), 0.2 and 2.0 (—□—), 0.2 and 10.0 (—▲—), 2.0 and 0.2 (—▼—), 2.0 and 2.0 (—Δ—), 2.0 and 10.0 (—○—), 10.0 and 0.2 (—●—), 10.0 and 2.0 (—♦—), 10.0 and 10.0 (—◊—), respectively. Soft sugar was used as carbon source.

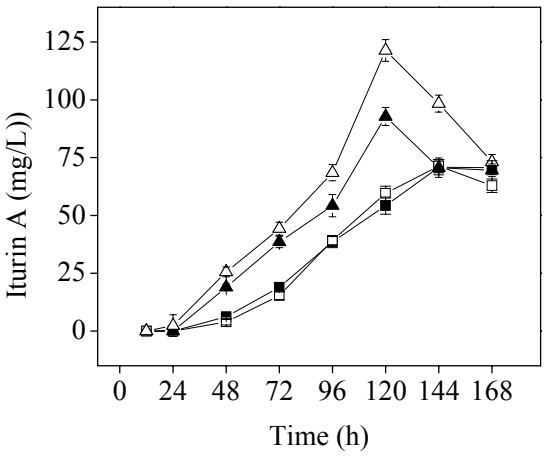

Figure 7. Optimal conditions for the maximal production of iturin A by *B. amyloliquefaciens* B128 in shaker flasks; only soft sugar and no pH control (—■—), soft sugar and controlled pH at 6.64 (—□—), soft sugar together with 0.2 mM Fe^{2+} at no pH control (—▲—), and soft sugar together with 0.2 mM Fe^{2+} and controlled pH at 6.64 (—Δ—).

Because of culture pH is an important bioprocess parameter which affects a series of regulatory processes in bacteria, and has been reported to affect secondary metabolite production [27]. As can be seen from Figure 3, in shaker flask cultivations, the iturin A production was affected to different extent by application of pH control between 4.0 and 9.0. It seems that the pH value affected the growth of *B. amyloliquefaciens* B128 as well as the release of iturin A into the medium. The optimal value of the pH of the media for iturin A production from *B. amyloliquefaciens* B128 was found to be 6.64.

It is known that metals are necessary for the normal metabolism of microorganisms as microelements, as well as for the production of secondary metabolites [31]. In shaker flask experiments, when *B. amyloliquefaciens* B128 was grown on a nutrient broth medium, the concentration of iturin A was about 12.58 mg/L (Figure 2). Upon studying the effect of medium supplementation with divalent metal cations, results revealed that the addition of metal salts to the cultivation medium was found either enhanced, or inhibited iturin A production. Only iron and magnesium salts caused significant enhancement of iturin A production. However, iron sulfate caused a much larger increase in iturin A production than the magnesium salt. Furthermore, higher iron concentrations, above 0.2 mM, decreased the production of antibiotic iturin A. The results of ferrous ion was compared with the reported that ferric ion (trivalent iron at 0.25–1.0 mM) significantly enhanced the antibiotic zwittermicin A production from the cultivation of *B. cereus* UW85 [31]. These results are in agreement with previous reports that the iron supplement strategies enhanced

surfactin production from *B. subtilis* [32]. Although, the maximal concentration of 121.36 mg/L obtained in this study is comparable with previous reports from *B. subtilis* [22, 23, 25, 33], however, much higher than those reported until now from the cultivation of *B. amyloliquefaciens* strains [5, 12]. In addition, effect of carbon source, pH and metal ion addition strategy provides useful information for enhancing iturin A production in cultivation.

5. Conclusions

Iturin A is an industrially important antibiotic and its demand is increasing in line with the growing global markets for processed food. The present study contributed toward the optimization of nutritional parameters and cultural conditions. Important findings included selection of carbon source, optimization of cultivation pH, and use of metal ions.

Acknowledgments

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