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CONTENTS

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Improvement of production of lipopeptide antibiotic iturin A using fish protein

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Abstract

To enhance the production of lipopeptide antibiotic iturin A, nutrient contents of the culture mediums were investigated in both submerged and biofilm fermentations. As a carbon source maltose and as nitrogen source, fish protein was used. In submerged fermentation maltose uptake was found lower (12%) compared to biofilm fermentation (15%) that was associated with higher cellular growth in biofilm. However, requirement of nitrogen (fish protein) concentration was found similar in both submerged and biofilm fermentations. Production of iturin A in submerged fermentation with 12% maltose and 5% fish protein was 4450 mg/L, and in biofilm fermentation it was 5050 mg/L when 15% maltose and 5% fish protein was used.

Key words: *Bacillus subtilis*; iturin A; lipopeptide antibiotic; biofilm fermentation

Introduction

Iturin A is one of the lipopeptide antibiotics produced by *B. subtilis* and its several strains as a secondary metabolite. Along with its wide antifungal spectrum, iturin A has low toxicity and low allergic effect on human and animals and thereby stands as a candidate for environmentally safe biological pesticide. Iturin A is a cyclic lipopeptide (Peypoux et al., 1978), and has been tested to control a variety of fungi in pure cultures and during composting (Phae and Shoda, 1990). At present commercially available chemical agents against plant diseases are being removed from the market for their hazardous impact to the nature, creating the demand for the safer products as fungicide like iturin A. The strong efficacy of iturin A against various phytopathogenic fungi is similar to the available chemical pesticides. As a potential biocontrol agent the production of iturin A and its improvement is being researched elsewhere including our laboratory. During the cultivation of microorganisms, the nitrogen source often represents the major part of the production cost in the fermentation industry (Broise et al. 1998). Commercially available peptones, like Polypepton and Polypepton S, which are derived from soybean and milk casein respectively, are being used as standard peptones for microbial growth. Previous studies revealed that marine resources like tuna red meat hydrolysate (Raghunath 1993), cod viscera (Dufosse et al. 2001), viscera from squid, yellow fin tuna, swordfish and rainbow trout (Vazquez et al. 2004) had been used as alternative nitrogen sources.

Fish Protein Powder is a new source of protein to replace overused soy and other protein powders. Fish is harvested from the ocean and clean waters and processed using low temperature technology in order to preserve the integrity of the delicate components of the protein. The hydrolysate is processed to stringent quality control standards. The powder can be added to a variety of food products to provide a healthy source of easily digestible protein. Bakery and pasta products are particularly amenable. Recently fish protein powder is being used as an excellent dietary supplement for human that can be added to a diverse range of products to provide a healthy source of easily digested protein. Fish protein powder can be added to weight loss and protein supplements and can be used as powdered capsule filler. Fish protein consumption can benefit the heart by lowering blood pressure and plasma total cholesterol. In any area of the food chain where protein is a requirement for health and nutrition, as well as for all animal feed products, fish protein powder can be used as the protein base.

From this perspective, it was tried to use powdered

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fish protein from Japanese cod as a marine resource for laboratory experiments. As basic information the major components and the amino acid composition of fish protein are shown in **Table 1**. Nowadays studies of microbial productions almost entirely are based on the submerged culture system where the microorganisms are offered with higher oxygen transfer rate by the continuous movement or transfer of the medium. Cells remain planktonic in this culture and the entire fermentation system is based on the sum performance of the each of the individual cells. On the other hand, biofilm development of *B. subtilis* has become a hot topic in recent microbiology. *B. subtilis* cells show significantly independent genetic and morphological development during biofilm formation compared to planktonic culture (Pratt and Kolter, 1999). When the culture is incubated on a liquid medium at a static condition, wild type strain of *B. subtilis* RB14 produces thick and stable biofilm in the liquid-air surface of the culture medium and surprisingly iturin A is also found to be produced from the culture (Rahman et al., 2007, Zohora et al., 2009). In this study production of iturin A and its improvement in both submerged and biofilm fermentation using FP medium is described. In order to improve the production of iturin A, optimization of carbon and nitrogen concentration was attempted in both submerged and biofilm fermentation.

1 Materials and methods

1.1 Culture medium composition

Pre-culture medium composition and the details of conducting pre-culture were similar as described in Zohora et al., (2009). Iturin A production was carried out initially in medium containing 80 g/L fish protein (Suzuhiro Co., Ltd., Odawara, Japan), 67 g/L of maltose, 5 g/L of KH_2PO_4, 0.5 g/L of $MgSO_4{\cdot}7H_2O$, 25 mg/L $FeSO_4{\cdot}7H_2O$, 22 mg/L $MnSO_4{\cdot}7H_2O$ and 184 mg/L $CaCl_2$ (pH 7), was used as a base medium for both submerged and biofilm culture. In order to improve the production of iturin A, as carbon source, maltose concentration was gradually increased from 6% up to 15%. And as nitrogen source, fish protein concentration was investigated within 3% to 8% range. L-agar plate containing LB medium with additional 1.5% agar (Shimizu Shokhin Co., Ltd., Japan) was used for the determination of colony-forming units (cfu).

Table 1 Amino acid composition of fish protein

Amino acid	Amount per 100 gm
Alanine	5.62 g
Arginine	6.44 g
Asparagine	10.2 g
Cysteine	0.92 g
Glutamine	16.4 g
Glycine	3.57 g
Histidine	2.13 g
Isoleucine	4.45 g
Leucine	7.93 g
Lysine	9.56 g
Methionine	2.99 g
Phenylalanine	3.45 g
Proline	3.17 g
Serine	4.17 g
Threonine	4.46 g
Tryptophan	1.07 g
Tyrosine	3.66 g
Valine	4.87 g

1.2 Cultivation of *B. subtilis* RB14

A 400-μL of *B. subtilis* RB 14 preculture was inoculated into 40 mL of iturin A production medium in 200 ml Erlenmeyer Flasks. The flasks were then incubated at 30°C at 120 strokes per minute in submerged condition and 25°C at static condition to allow biofilm formation. Both the fermentation techniques were designed for 8 days. Cellular growth was estimated by counting vegetative cells and spores as described in literature (Zohora et al., 2009). Spore numbers was detected using the samples from the culture that was heated at 80°C for 10 min in water bath and spread on agar plate. During the sample preparation in corresponding time course of cellular and spore numbers, pH was also recorded at regular intervals.

1.3 Estimation of cellular growth

At specific time intervals samples were collected from the culture flasks of submerged fermentation to measure bacterial growth by counting CFU (colony forming units). On the contrary entire biofilm produced during fermentation and culture medium in which the biofilm was produced were collected and homogenized by using Physcotron homogenizer (NS-310E, NITI-ON, Tokyo, Japan) for sampling. For the CFU count, collected culture samples were serially diluted with 0.85% NaCl and spread on L-agar plate. The plates were incubated at 37°C for 16 hr. To detect the spore numbers, samples from the culture were heated at 80°C for 10 min in water bath, serially diluted and spreaded on agar plate. Time course of pH were recorded at regular intervals.

1.4 Sample preparation and iturin A detection

For sample preparation, 100 μL submerged culture solution was transferred into 1.5 mL polypropylene tube and mixed with 900 μL of extraction buffer (acetonitrile:10 mmol/L ammonium acetate (35:65; *V/V*)). On the other hand, the entire biofilm produced during biofilm fermentation and medium in which the biofilm was produced were collected and homogenized together for sampling. For adequate dispersal of the cells the collected biofilm and medium was homogenized by using Physcotron homogenizer (NS-310E, NITI-ON, Tokyo, Japan) for 15 sec and this homogenized sample was used similarly as submerged sample for iturin A extraction. All the preparations were vortexed at room temperature for 20 min and then centrifuged at 15,000 $\times g$ for 10 min at

4°C. The supernatant was filtrated through 0.20 μmol/L polytetrafluoro ethylene (PTFE) membrane filter (Advantec, Tokyo, Japan). A 20-μL of the filtrate was injected into HPLC column by autosampler (AS-2055Plus, JASCO, Tokyo, Japan) for iturin A detection. Acetonitrile and 10 mM ammonium acetate (35:65, *V/V*) was used in mobile phase through ODS column (chromolith performance RP-18e 100-4.6mm, Merck KGaA, Darmstadt, Germany) at a flow rate of 2 mL/min and elution was monitored at 205 nm. HPLC Analysis and quantification was performed by using HSS-2000 and JASCO-Borwin software installed in a desktop computer.

2 Results

2.1 Effect of carbon source on the production of iturin A

In this investigation maltose was used as the carbon source on the basis of our previous observation where it offered the highest iturin A production over glucose, sucrose, fructose and mannitol (data not shown). When increased concentration of maltose (from 6% to 15%) was used in addition to fish protein, production of iturin A was improved. Interestingly, the amount of maltose uptake was not similar in submerged and biofilm fermentations. During submerged fermentation, iturin A production improvement was observed when maltose was supplemented up to 12% of its concentration (**Fig. 1**). Whereas, in biofilm fermentation, gradual progress of iturin A production was observed up to 15% maltose concentration (**Fig. 2**). However, if the maltose concentration was further enhanced, iturin A production was found hindered in both the fermentation conditions.

2.2 Effect of nitrogen source on the production of iturin A

Bacterial cells, according to their specificity, consume nitrogen from the ammonium, nitrate, organic compounds or from the peptones of the culture medium. Fish protein contains high amount of carbon and nitrogen sources (Zohora et al., 2011), which plays a great role in the composition of microbial cellular materials. In this study its concentration was investigated to optimize for better iturin A production in both submerged and biofilm fermentations. It was observed that at increased fish protein concentration from 3% to 5%, production of iturin A was enhanced as well in both the fermentation conditions. However, additional amount of fish protein (8%) showed adverse effect in both fermentation system (**Figs. 1** and **2**).

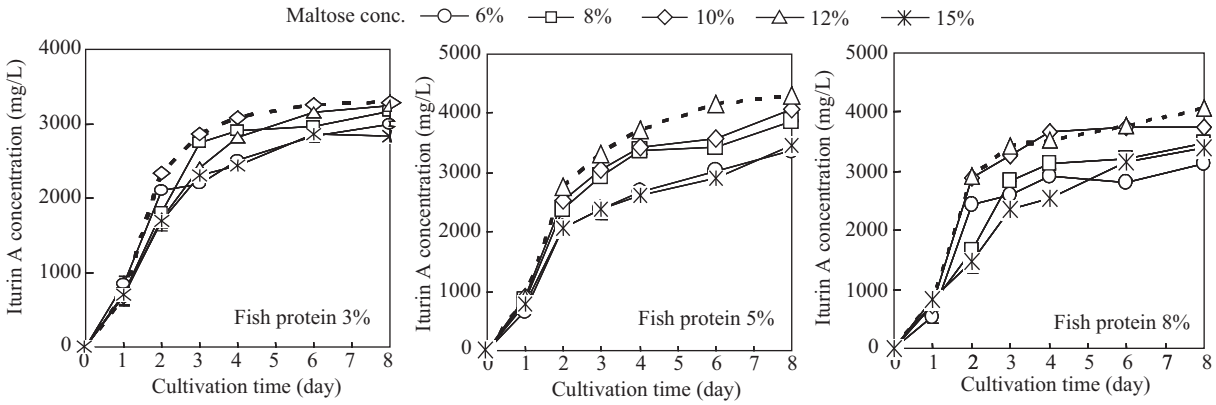

Fig. 1 Effect of nitrogen (fish protein) and carbon (maltose) on the production of iturin A in submerged fermentation.

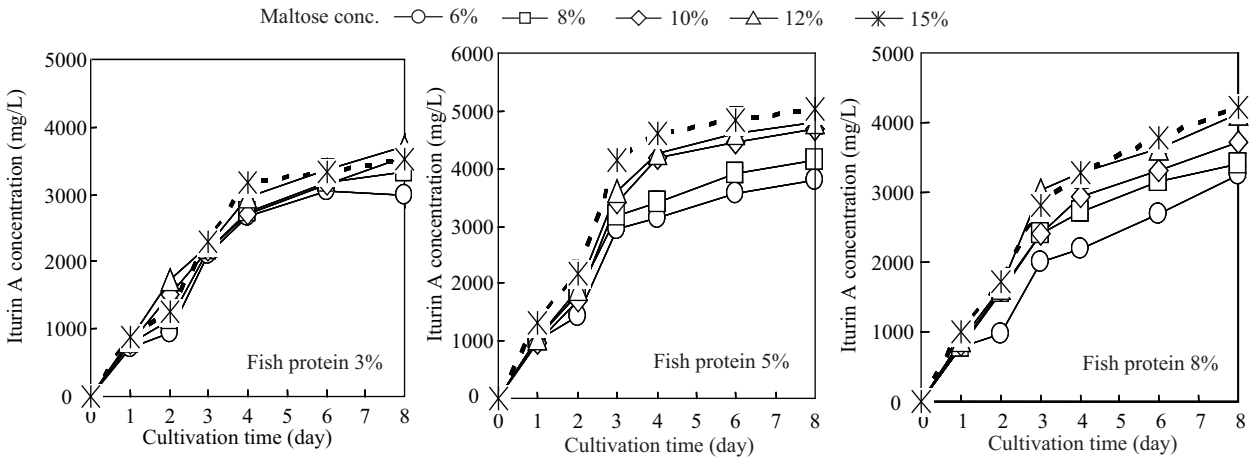

Fig. 2 Effect of nitrogen (fish protein) and carbon (maltose) on the production of iturin A in biofilm fermentation.

2.3 Comparative iturin A production

During the optimization of carbon (maltose) and nitrogen (fish protein) source, highest amount of iturin A production was observed with 5% fish protein in combination of 12% maltose in submerged fermentation. Further enhancement of maltose concentration (15%) resulted with reduced production. On the other hand, in biofilm fermentation significant amount of iturin A was produced with 5% fish protein and 15% maltose and it was 5050 mg/L. Interestingly, the production of iturin A at 5% fish protein with 12% maltose concentration in biofilm fermentation was found higher (4800 mg/L) compared to submerged fermentation (4450 mg/L) (**Fig. 3**).

3 Discussion

Carbon and nitrogen are the key elements of microbial cellular material. They play a great role in the constitution of most of the cellular component along with protein, DNA and RNA inside the microbial cells. In submerged fermentation, proper oxygen, heat and mass transfer induce rapid cellular growth and sporulation. On the other hand, sporulation was much delayed in the biofilm and the cells remained metabolically active for a long time which could utilize excess amount of maltose (15%) compared to submerged culture (12%).

B. subtilis produces the lipopeptide antibiotic iturin A by its exceptionally large non ribosomal peptide synthetase (NRPS synthetase) (Marahiel 1997, Tsuge et al., 2001) on the onset of sporulation. It is accepted that differential expression of the extra large NRPS synthetase requires higher oxygen transfer system for iturin A production. As a result production of iturin A starts earlier in submerged fermentation than that in biofilm fermentation. It has been reported that antibiotic production of *B. subtilis* takes place on the onset of sporulation. Therefore, delayed sporulation resulted with relatively higher iturin A production in biofilm fermentation. During the optimization of carbon and nitrogen, the higher amount of iturin A was obtained in 5% fish protein with 12% maltose in submerged fermentation (4450 mg/L). Whereas the highest amount of iturin A was produced in biofilm fermentation at 5% fish protein with 15% maltose concentration (5050 mg/L), which is notably higher, compared to any of the previous report of iturin A production (Jacques et al., 1999, Mizumoto et al., 2007). From this investigation it can be said that biofilm fermentation is efficient for the production of high amount of iturin A compared to submerged fermentation. Whereas the fermentation takes place in static condition it consumes little or no energy.

It was observed that 5% fish protein and 12% or 15% maltose addition improved the productions, for this reason cellular growth, pH in addition to iturin A profile were investigated. From the cfu numbers it shows that cell growth and sporulation was quicker in submerged fermentation compared to biofilm fermentation (**Fig. 4**). In biofilm, cellular growth continued for much longer period than submerged fermentation and sporulation was also found delayed. At the latter stage of cultivation all vegetative cells turned to spores in both fermentation conditions. As a result the number of vegetative cells and spores, 5×10^{10} found in biofilm was higher than the number, 2.3×10^{10}

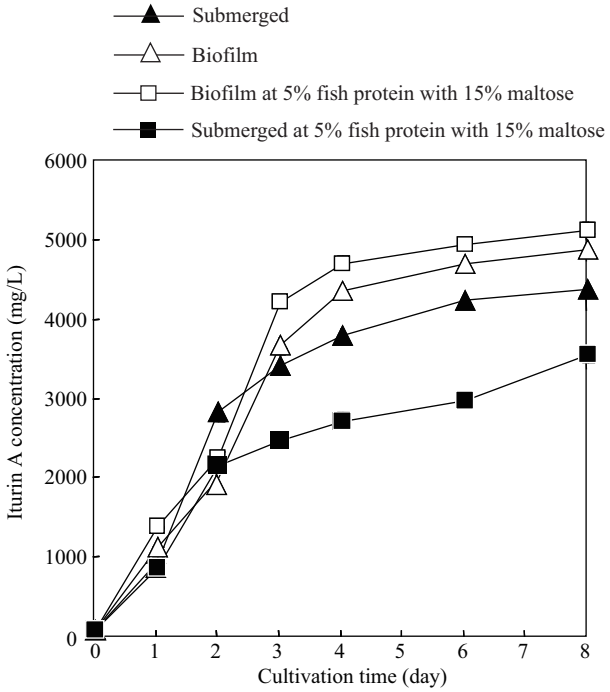

Fig. 3 Comparative iturin A production within submerged and biofilm fermentation. The maximum production in biofilm fermentation was observed at 5% fish protein with 15% maltose but in submerged fermentation showed significantly reduced production.

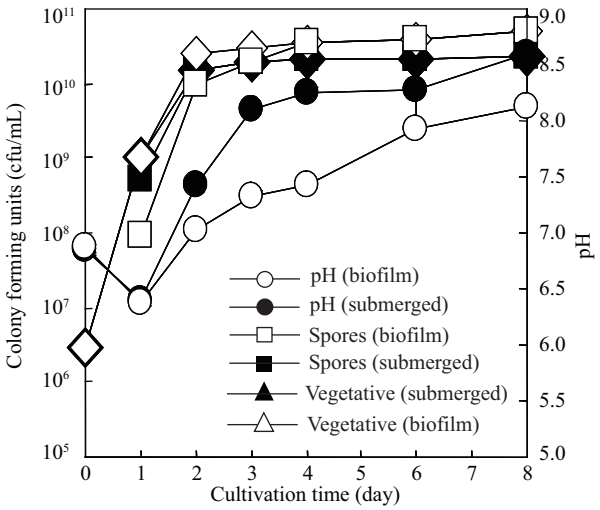

Fig. 4 Comparative cellular growth and pH profile within submerged and biofilm fermentation at 5% fish protein and 12% maltose concentration. The maximum cellular growth was observed in biofilm fermentation (vegetative cells and spores) compared to submerged fermentation (vegetative cells and spores).

found in submerged fermentation.

Although the initial pH of both submerged and biofilm cultures were similar, due to the rapid cellular growth and spore formation, the pH of the submerged culture became alkaline within 3 days. On the other hand, in biofilm fermentation culture medium possessed slightly over the neutral pH until the 3rd and 4th day of cultivation (**Fig. 4**)

During the growth of *B. subtilis* in submerged and biofilm fermentations were analyzed for their carbon sources utilization, evidently maltose, as a carbon source, was depleted comparatively earlier in submerged fermentations (**Fig. 5**). Due to the higher rate of oxygen transfer in the submerged fermentation resulted with quicker cellular growth and carbon source utilization. In comparison to submerged fermentation, the biofilm fermentation allows slower grouth which is due to the lower oxygen and mass (nutrient for instance) transfer, and thereby utilization of carbon source was slower.

It has been reported that sporulation of *B. subtilis* is comparatively delayed in biofilm than its submerged fermentation (Lindsay et al., 2005). Therefore, it can be said that the cells remain in the biofilm community in their metabolically active state for a longer period of time. Nevertheless, the initiation of iturin A production was started late when plotted against the sporulation (**Fig. 6**) that resembles our previous observation (Rahman et al., 2007). This supports our previous postulation that the cells remain in the metabolically active state for a longer period of time and maximum utilization of nutrient ultimately resulted with higher production of lipopeptide antibiotic iturin A in biofilm compared to the submerged fermentation.

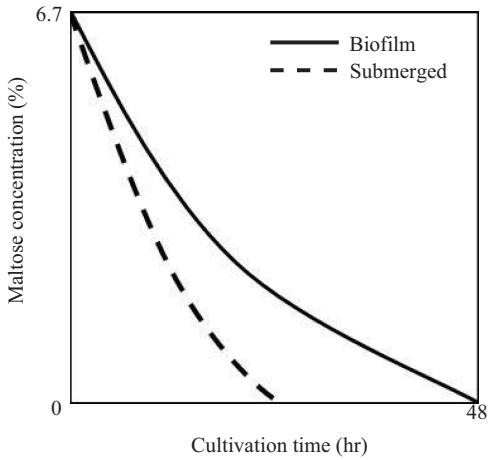

Fig. 5 Comparative utilization profile of maltose as a carbon source in submerged (- - -) and biofilm (—) fermentations.

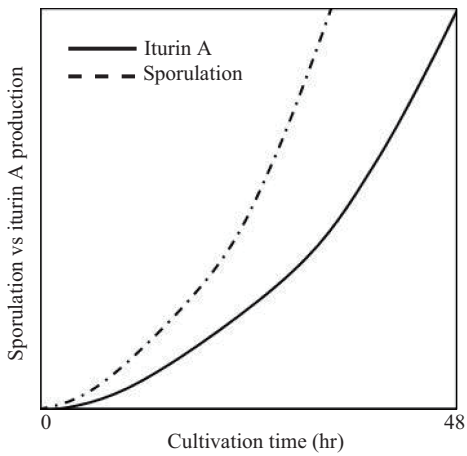

Fig. 6 Relative production of iturin A and sporulation of *B. subtilis* in biofilm fermentation.

4 Conclusions

During the investigation of the effect of carbon (maltose) and nitrogen (fish protein) source on the production of iturin A, it was observed that up to a certain level both maltose and fish protein addition enhanced the production. The optimum concentration of maltose was found different in respect of fermentation conditions while concentration of fish protein was remained similar. Fish protein contains higher amount of nitrogen than commercially available peptones like Polypepton S and Polypepton. This could be the reason to provide improved iturin A production with only 5% of fish protein in this investigation. The utilization of fish protein is beneficial due to its natural abundance and year round availability. At present only the powdered fish protein from cod fish was investigated but gradually other non edible fishes (trash fishes) can be used for this purpose which will not conflict with human food in future. In addition though it is difficult to obtain a firm idea of its likely commercial price; this will depend obviously on the cost of the raw material and the process used. Fish protein concentrate is for example about half the price of non-fat dried milk and about one-twentieth that of meat for a unit weight of protein. It has been estimated that the commercial price for the type of fish protein that is required for bacteriological use in industry would probably be close to just less than a US dollar per kilogram. These prices compare very favorably with those for proteins from other sources like milk or soybean.

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