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Original Research Paper

# Production of lipopeptide iturin a using novel strain *Bacillus* iso 1 in a packed bed bioreactor

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ABSTRACT

In the present study, the influence of volumetric airflow rate and rice husk as a bulking agent were evaluated in packed bed bioreactors to the production of lipopeptide iturin A. The iturin A is a powerful antifungal composed by seven α-amino acid rings linked to a β-amino fatty acid chain with an alkyl chain that can be linear or branched. The solid state fermentations (SSF) were carried out using a new strain *Bacillus* iso 1 as ferment and wheat bran and soybean meal as substrates at 30 °C for 96 h. It was found that the concentration of rice husk and volumetric airflow rate in the range of 20–40% (w/w) and 0.4–0.8 L/min are important operational parameters for the production of iturin A in packed bed bioreactors. The highest iturin A production (6.88 g/kg of dry substrate) was achieved when SSF was carried out with a 22.9% (w/w) of rice husk and a volumetric airflow rate of 0.46 L/min. These promising results show the potential of the new strain *Bacillus* iso 1 to produce high concentrations of lipopeptide iturin A using cheap agro-industrial substrates in packed bed bioreactors with forced aeration, in order to develop an industrial production processes.



## 1. Introduction

The iturin A belongs to a group of cyclic lipopeptides produced by *Bacillus subtilis* strains (Raaijmakers et al., 2010). The chemical structure is almost always heptapeptidic with seven α-aminoacids linked to a β-amino fatty acid with an alkyl chain that can be linear or branched (Maget-Dana and Peypoux, 1994; Falardeau et al., 2013) (Fig. 1). Moreover, *B. subtilis* produces other compounds from the same category: mycosubtilin, other iturins (A–C) and bacillomycins (D, F, L). These molecules have a common structure, but they differ in their aminoacid composition, aminoacid position, as well as in the length of the fatty acid chain (Bonmatin et al., 2003; Ongena and Jacques, 2008). Iturin A is a powerful antifungal agent than inhibits pathogenic fungal species as: *Rhizoctonia solani*, *Glomerella cingulata*, *Sclerotium rolfsii*, *Botrytis cinerea*, *Aspergillus* spp, *Fusarium oxysporum*, *Fusarium graminearum*, *Phomopsis persea*, *Fusicoccum aromaticum* and *Colletotrichum gloeosporioides* (Arrebola et al., 2010; Hsieh et al., 2008; Velho et al., 2011). This lipopeptide is regularly produced as a mixture of eight isomers ($A_1$–$A_8$), due to the variation in both the length of β-hydroxy fatty acid chains, which varies from $C_{13}$ to $C_{18}$, and the *n*- or iso-positioning (Bland, 1996; Iwase et al., 2009).

Commercially, these lipopeptide surfactins, including iturin A, are not sold as bulk chemicals (Slivinski et al., 2012) but in quantities as low as 1–5 mg at high market prices. For example, the cost of 1 mg of iturin A (95% purity) available from SIGMA-ALDRICH (St, Louis, MO, USA) is approximately 123.50 dollars. The high cost and small production quantities of this lipopeptide hamper research on production and purification.

Most of secondary metabolites (SM) such as iturin A are produced in submerged fermentation processes (SmF) (Robinson et al., 2001). However, solid state fermentation (SSF) may be an alternative to produce these molecules due to the high similarity in fermentation conditions and microorganism environments (Barrios-González et al., 2003; Ali and Zulkali, 2011). The main advantages of SSF over SmF are: reduced cost of fermentation equipment, use of low cost raw materials, low energy consumption, small volume of fermentation, low technology, no foam generation and production of higher yields of secondary metabolites and enzymes (Ali and Zulkali, 2011; Barrios-González, 2012). Even so, the majority of studies on iturin A production are carried out in submerged fermentation (Iwase et al., 2009; Khan et al., 2009; Rahman et al., 2006; Akpa et al., 2001). There are studies about iturin A production on solid state fermentation using chambers (Yao et al., 2012), glass flasks (Mizumoto et al., 2006), polypropilene bottle (Shih et al., 2008), glass petri dishes (Ohno et al., 1993) or an agitated reactor (Ano et al., 2009) to contain the fermentation medium. However, there are not works on iturin A production in an aerated packed bed column bioreactor. A aerated packed bed bioreactor design is a good alternative for production of

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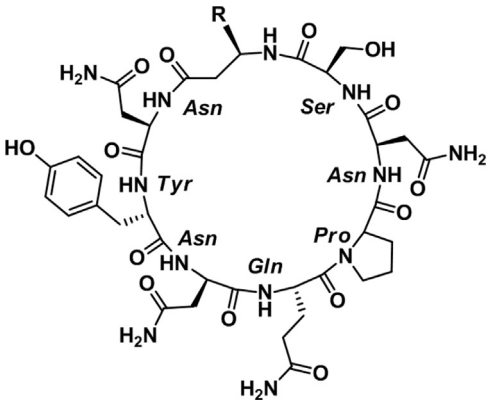


**Fig. 1.** Chemical structure of Iturin A.

iturin A at lower costs because it allows the control of the air rate that influences oxygen supply, temperature and moisture content (Lu et al., 1998).

The utilization of agro-industrial residues as substrates for production of iturin A is a good alternative, because these low-cost substrates allow for increased economic competitiveness while offering environmental benefits compared to conventional substrates. Traditionally, use of agro-industrial residues by solid state fermentation of iturin A has been limited to okara, wheat bran, rapeseed meal/wheat bran and rice bran/gluten flour (Ohno and Ano, 1992; Shih et al., 2008; Ano et al., 2009; Yao et al., 2012). However, the use of a mixture of soybean meal/wheat and bran/rice husk for production of iturin A with solid state fermentation has not yet been reported. In our present work, the technical feasibility of using this substrate alternative is explored which can lead to additional reduction of cost price and increased environmental benefits. Design of experiments (DOE) is a statistic tool that allows researchers to obtain reliable information about the process, minimizing empiric efforts as trial-and-error techniques and reducing time and costs (Rodrigues and Iemma, 2009; Aguiar-Oliveira et al., 2012; Forte et al., 2012; Hirata et al., 2013; Zemolin et al., 2013).

In this current study, the aim of this work was to investigate the effects of air flow rate and proportion of rice husk as a bulking agent on antifungal lipopeptide iturin A production in a aerated packed bed reactor by a new, strain *Bacillus* iso 1.

## 2. Material and methods

### 2.1. Microorganism and culture conditions

The iturin producing strain *Bacillus* iso 1 used in this study was isolated from soy roots collected from soybeans cultivated in Campinas-Brazil. Soy roots (2 g) were resuspended in 30 mL of a 1% NaCl solution and agited for 20 min. Subsequently, the resultant supernatant was diluted and one milliliter of each dilution was spread on defatted soybean meal and wheat meal agar. The soybean/wheat meal agar contained (w/v) 4% defatted soybean meal, 2% wheat meal and 1% agar–agar. The plates were incubated at 37 °C for 48 h until biofilm growth. The biofilm was then transferred to brain heart infusion agar (BHIA) and maintained at 5 °C. The bacterial inoculum was prepared using 30 g/L defatted soybean meal infusion and 1% agar in a 250 mL Erlenmeyer slant flask at 37 °C for 48 h, i.e. until the formation of a biofilm.

### 2.2. Solid state fermentation in packed bed bioreactor

The bacterial biofilm was then suspended with 50 mL of sterile distilled water. For inoculation, 10% (v/w) of formed suspension was used to inoculate a mixture of substrates: defatted soybean meal, wheat meal and rice husk (88 g in dry basis) in a polypropylene bag that was then packed into a jacketed column bioreactor (50 mm internal diameter and 300 mm high). The average diameter of particles was $0.55 \pm 0.05$ mm. Before autoclaving at 120 °C for 20 min, the moisture was adjusted to 70% dry basis (w/w) with distilled water, the composition of nutrient solution was (g/L): $KH_2PO_4$ 2.0; $MgSO_4.7H_2O$ 0.6; $(NH_4)_2SO_4$; and a modified trace elements solution described by Akpa et al. (2001) that contained (g/L): $CuSO_4$ 0.001; $FeCl_3$ 0.005; $NaMnO_4$ 0.004, KI 0.002; $ZnSO_4$ 0.014; $H_3BO_3$ 0.01; $MnSO_4$ 0.0036.The pressure drop in the medium was measured through a U-tube manometer with a water level resolution of 2 mm of water. The fermentation was carried out for 96 h at 30 °C with continuous injection of filtrated and humidified air, that was conditioned to a relative humidity and temperature of 95–100% and 30 °C, respectively (Fig. 2).

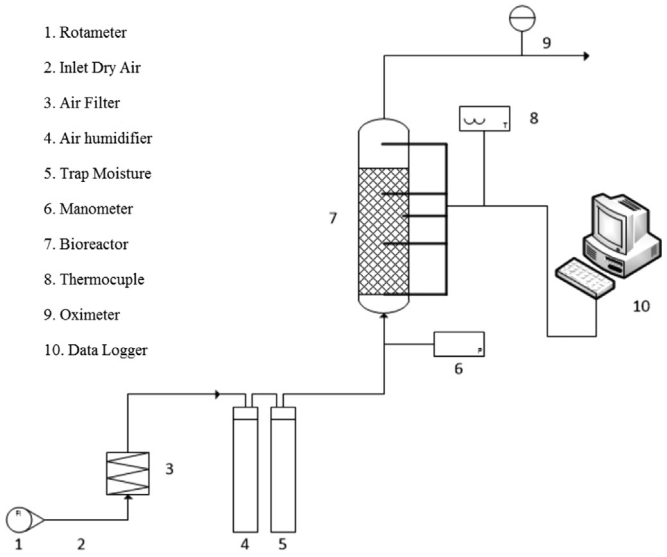


**Fig. 2.** Schematic of fermentation in packed bed bioreactors.

On the basis of a preliminary investigation two factors were employed in a central composite rotatable design (CCRD) to determine synergy of variables in iturin A production, totaling 11 experimental runs, defining air flow rate and rice husk (bulking agent) as independent variables ($X_1$ and $X_2$, respectively). The results were analyzed using Aexd.net, Alleviating Science (Zautsen, 2013).

### 2.3. Extraction and purification of iturin A

Twenty grams of the fermented material were added to 200 mL of ethanol and the mixture was stirred in a rotary shaker (Marconi, Mod. MA 830, Piracicaba, Brazil) at 30 °C, 150 rpm for 1 h. The extract was centrifuged and filtered through a paper filter and dried in a rotary evaporator at low pressure. The solid residue was dissolved in 20 mL of methanol (methanolic extract) and purified using the next methodology. Briefly, 500 μL was passed through the glass chromatography column (300 × 10 mm) packed with silica gel 60. The iturin A was purified by solvent system by 20 mL of a chloroform–methanol–water mixture (fraction $P_1$) with volume ratios 65:25:4, followed by 20 mL of chloroform–methanol–water (fraction $P_2$) with volume ratios 30:50:10, and finally 10 mL of chloroform–methanol–water (fraction $P_3$) with volume ratios 20:60:15. After addition of each solvent mixture, a volume of 20 mL was collected, followed by evaporating the solvent mixture under nitrogen flow and dissolving the obtained residue in 1 mL of methanol before analysis by HPLC-DAD. The chromatographic analysis was carried out using a ODS Hypersil® THERMO RP-18 column (150 × 4.6 mm, 3 μm, Thermo Scientific, USA) and