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Optimization of Culture Medium to Increase the Production of Desferrioxamine B (Desferal) in *Streptomyces pilosus*

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Abstract: The aim of this study was optimization of culture medium in direction of increasing the production rate of desferrioxamine B. Streptomycetes are the most widely studied and well known genus of the actinomycete family. Streptomycetes usually inhabit soil and are important decomposers. The genus *Streptomyces* are Gram-positive and GC rich bacteria that are important for production of many antibiotics and secondary metabolites. These metabolites are important in industrial and medical fields. Deferoxamines (also known as desferrioxamine B, desferoxamine B, DFO-B, DFOA, DFB or desferal) are low-molecular-weight, iron-chelating compounds (siderophores) produced and secreted by many actinomycetes, including species of *Streptomyces*, *Nocardia* and *Micromonospora*. *Streptomyces pilosus* synthesizes the siderofore desferrioxamine B. Desferrioxamine B is used clinically to treat disorders related to iron overload and pathological iron deposition in human. Our results revealed that the use of soybean as a base medium plus additives such as $Na_2HPO_4.12H_2O$, NaH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, $CaCl_2.2H_2O$, NaCl, $MnSO_4$, NH_4Cl, KH_2PO_4, K_2HPO_4, some of the amino acids and vitamins increased the production of desferrioxamine B about 8 times in comparison with the control.

Key words: *Streptomyces pilosus*, desferrioxamin B, soybean medium, optimization

INTRODUCTION

At present, desferrioxamine B is produced by fermentation of *S. pilosus*. Desferrioxamine B as a siderophore is synthesized in a series of steps from L-lysine amino acid. Early steps of desferrioxamine B biosynthesis in *S. pilosus* involve decarboxylation of L-lysine and hydroxylation of the resulting cadaverine to give N-hydroxycadaverine (Schupp *et al*., 1987, 1988).

Streptomyces is the largest genus of Actinobacteria (Actinomycetes) and the type genus of the family Streptomycetaceae (Kampfer, 2006). Over 500 species of *Streptomyces* bacteria have been described (Euzeby, 2008). As with the other Actinobacteria, streptomycetes are Gram-positive and have genomes with high GC-content. Found predominantly in soil and decaying vegetation, most streptomycetes produce spores and are noted for their distinct earthy odor which results from production of a volatile metabolite, geosmin (Madigan and Martinko, 2005). Actinomycetes are highly attractive as cell factories or bioreactors for applications in industrial, agricultural, environmental and pharmaceutical fields. In actinomycetes, the genera *Streptomyces*, *Rhodococcus*, corynebacterium and mycobacterium have received an increasing amount of attention, particularly in industrial fields (Demain and Fang, 2000). They exhibit potential advantages in the synthesis of secondary metabolites of industrial and medical importance, in the production of amino acids by fermentation and in bioconversion processes. The compounds they produce as secondary metabolites are valuable for industrial and pharmaceutical purposes (Tokiwa and Buenaventurada, 2004) and the enzymes themselves are also valuable. For example, *Streptomyces* sp. produces various types of antibiotics (Weber *et al*., 2003) and some *Rhodococcus* sp. are being used for the industrial production of acrylamide (Komeda *et al*., 1996).

The ferrioxamines are an important class of iron transport agents that are produced by several species of *Nocardia*, *Streptomynces* (Yang and Leong, 1982), *Micrornonospora* (Emery and Hoffer, 1980), *Arthrobacter, Chromobaciterillm* and *Pselidomonas*.

Although the ferrioxamines are an important and well-known class of siderophores produced by several species of microorganisms that mentioned before, no studies have

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been performed about the improvement of media for increasing the production of siderophores (e.g., desferrioxamines) in these microorganisms.

Hundreds of structurally distinct siderophores are known, typically with ligating catecholate, carboxylate, α-hydroxycarboxylate, or hydroxamate functional groups (Boukhalfa and Crumbliss, 2002; Albrecht-Gary and Crumbliss, 1998). Most siderophores are hexadentate and form 1:1 Fe (III)-complexes (Martell *et al*., 2001; Murakami *et al*., 1989; Kalinowski *et al*., 2000). Desferrioxamine-B is an example of a cationic (pH<8) trihydroxamate siderophore found in both terrestrial and marine systems (Borgias *et al*., 1989). Desferrioxamine B is the main siderophore of *Streptomyces pilosus* (Schupp *et al*., 1988). Its production is induced in response to iron limitation. Siderophores as iron chelators have been investigated as potential therapeutic agents for diseases of iron overload such as thalassemia. Desferrioxamine (DFO) is the only chelator in regular clinical use for the treatment of iron overload.

MATERIALS AND METHODS

Reagent-grade chemicals were used in all experiments (all of them were provided from Merck Corporation). Desferrioxamine B (Desferal or DFO) was obtained as the mesylate salt (Desferal; Ciba-Geigy, Inc., Basel, Switzerland). FeC13 was purchased from New England Nuclear Corp., 1998. *Streptomyces pilosus* ATCC 19797 was used in this study which was done in 2009.

A characteristic structural feature of the ferrioxamines (Fig. 1a-c) is repeating units of α-amino-ω-hydroxyaminoalkane and succinic or acetic acid, so that a thermodynamically stable octahedral ferric complex may be formed with three hydroxamate groups.

Organism and growth condition: Lyophilized *S. pilosus* ATCC 19797 was cultured in several media such as malt-yeast extract broth (MYB) containing 1% malt extract, 0.4% yeast extract and 0.4% glucose; Nutrient Broth (NB) containing 0.5% peptone and 0.3% meat extract; Luria Bertani (LB) containing 1% Bacto-tryptone, 0.5% yeast extract and 1% NaCl and soybean containing 2% soybean flour and 2% mannitol. After 48 h incubation in shaker incubator at 29°C and shaking at 150 rpm, the refreshed bacteria were stocked in 10 mL vials with 15% glycerol and kept on 4°C for 24 h and then were transferred to -20°C. The best growth was observed in the soybean medium. Therefore this medium was selected as a base medium for subsequent experiments. The bacterium was cultured in 24 similar flasks containing 25 mL soybean medium under the above-mentioned conditions. Samples were drawn every 8 h for 8 days. Contents of the flasks were filtered and dried in an oven at 60°C for 3 days. Dry mass of bacteria was weighted and the growth curve was drawn (Fig. 2).

To confirm the production of desferrioxamine B (Desferal), a single colony of the bacteria was cultured on nutrient agar (NB plus 2% agar). After 4 days, a piece of Whatman No. 1 filter paper, soaked in 1% ammonium ferric sulfate ($FeNH_4(SO_4)_2.12H_2O$) in 1% sulfuric acid, was placed on the single colonies. The appearance of a brown or reddish brown halo around the colony in agar after 15 min indicated the presence of Desferal (Schupp *et al*., 1988).

The amount of Desferal in culture was measured spectrophotometerically.

In this regard, the bacterium was cultured in soybean broth medium (2% soybean flour and 2% mannitol) for 8 days at 29°C under shaking (150 rpm). Every 8 h, 1 mL sample was withdrawn and centrifuged at 4000 rpm (4°C). The supernatant was diluted ten times with distilled water and 5 mg mL^{-1} of ammonium ferric sulfate in 1% sulfuric acid was added to a final concentration of 20%. The absorbance of Desferal was read at 430 nm and its concentration was found by comparing the ODs with the standard curve prepared with different concentrations of Desferal.

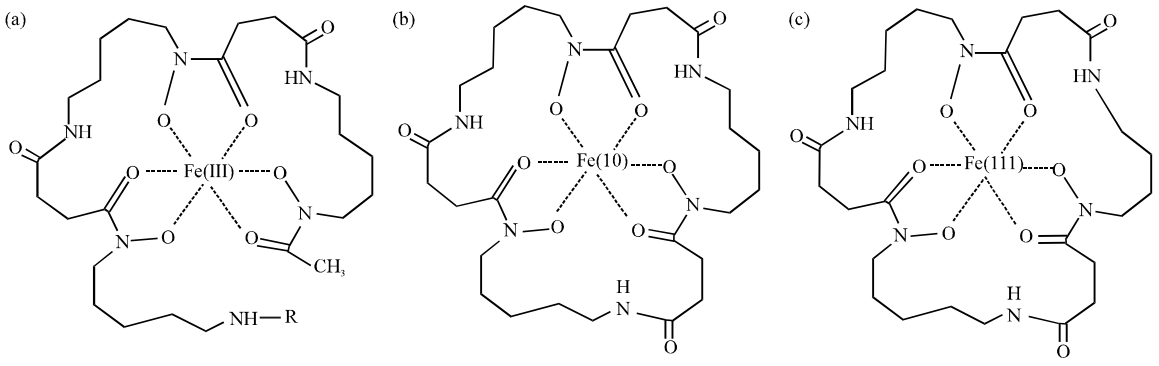

Fig. 1: Structure of ferrioxamines (a) B and D1(for B, R = H; for D1, R = $COCH_3$), (b) E and (c) D2

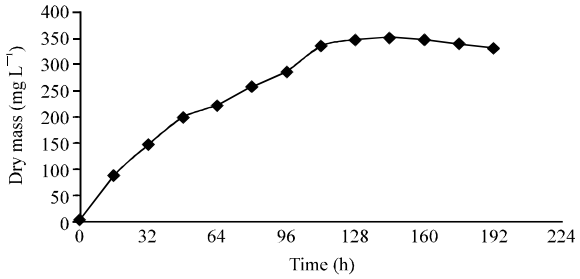

Fig. 2: Growth curve of *Streptomyces pilosus* (ATCC 19797) during a period of 192 h. Cultures were performed in soybean medium at 29°C and 150 rpm. Sampling was done every 8 h and filtered bacterial masses were weighed after drying in a 60°C oven for 3 days

To verify that the synthesized Desferrioxamine (Des) was B type, Des secreted into a six-day culture in soybean broth was extracted using Gaeumann's method and run on a chromatography paper along with standard Desferal. Chromatography solvent contained 40% (v/v) n-butanol and 10% (v/v) glacial acetic acid in water (Gaeumann and Prelog, 1964). For developing the spots, the paper was dried in an oven at 70°C and then soaked into 0.2% ninhydrin in acetone and baked at 105°C for 4 min. Purple spots confirmed the presence of Desferal (Fig. 3).

For extraction and purification of desferrioxamine B, a solution of 8-hydroxychinoline in methanol is added to the liquid medium in order to decompose the ferrioxamine complex formed by the iron-(III) ions of the culture medium with desferrioxamine. The liquid medium is then filtered and the excess of 8-hydroxychinoline is removed from the filtered liquor with the aid of AMBERLITE IR-45 ion exchange resin. The active ingredient is adsorbed by AMBERLITE IRC-50 ion exchange resin and eluted with 0.2 M hydrochloric acid. Thus a great volume of diluted eluate is obtained. In order to concentrate and purify the active ingredient in the eluate the solution is extracted at pH 5 with benzyl alcohol or with a 1:1 mixture of chloroform and phenol. The extract is treated again with 8-hydroxychinoline and methyl isobutyl ketone is added to it and the mixture is re-extracted with water. The excess of 8-hydroxychinoline is removed by extraction with chloroform. In order to isolate desferrioxamine B hydrochloride the aqueous solution is concentrated in vacuum and the separated crystals are recrystallized from the mixture of water and methanol and water and acetone (Zoltan *et al*., 1994).

Desferrioxamine B is generally used in the form of the methanesulfonate salt which is readily soluble in water. The methanesulfonate is prepared from desferrioxamine B

Fig. 3: Paper chromatography of extracted Des. from *S. pilosus* (ATCC 19797) (2) and standard Desferal (1). Rfs are 0.457 and 0.462, respectively. These results show that the produced Des in *S. pilosus* is B-type

hydrochloride in such a manner that an aqueous solution of the latter is passed through an anion exchange resin in the hydroxyl form to be converted first to base and then a solution containing an equivalent amount of methanesulfonic acid is added to the aqueous solution of the desferrioxamine B base. The solution is evaporated and the thus-obtained desferrioxamine B methanesulfonate is recrystallized from an aqueous alcohol or from an aqueous mixture of methanol and acetone (Zoltan *et al*., 1994).

Produced Desferal also confirmed with pharmacopoeia USP 2008 guidelines. For this purpose, initially 5 mg of Desferal was dissolved in water and then 2 mL tri-sodium phosphate dodecahydrate (5 g L^{-1}) and 0.5 mL sodium naphtoquinon sulphate solution was added and vortexed. The dark yellow color indicated the presence of Desferal in our product.

In this study, we surveyed the effects of additives (such as biotin, thiamin, arginine, methionine, lysine, glycine, serine, tyrosine, alanine, threonine, $Na_2HPO_4.12H_2O$, NaH_2PO_4, $MgSO_4.7H_2O$, $ZnSO_4.7H_2O$, $FeSO_4.7H_2O$, $CaCl_2.2H_2O$, NaCl, $MnSO_4$, NH_4Cl, KH_2PO_4, K_2HPO_4, Tris-base and 2,2'-dipyridyle, EDTA and 8-hydroxyquinoline) on increasing the yield of Desferal in culture media. For this purpose in each onset, different concentrations of an additive were applied and the results were compared with the control (containing water in the same amount of additive). All conditions such as

temperature, shaking rate, amount of bacteria in each flask and incubation time was the same.

RESULTS

In this study we focused on the media ingredients and tried to modify its composition in direction with improvement for increasing the yield of Desferal production. In this regard, we experimented several media and concluded that the soybean is the best medium and has the highest productivity. This medium has considered as base medium and the additives that mentioned above with distinct quantities were added to the medium.

Present results showed that some additives with distinct concentration have the most significantly effects on Desferal synthesis. Compare to control, KH_2PO_4 (32 g L^{-1}), $Na_2HPO_4.12H_2O$ (4 g L^{-1}), $MgSO_4.7H_2O$ (0.6 g L^{-1}), $CaCl_2.2H_2O$ (2 g L^{-1}), $ZnSO_4.7H_2O$ (0.004 g L^{-1}), Tris-base (100 mM) and threonine (0.1 g L^{-1}) increased the desferal production about 7.6, 5, 5, 4, 3, 2 and 2 times respectively (Fig. 4). Arginine (0.1 g L^{-1}) and glycine (0.1 g L^{-1}) also to some extent had a positive effect on Desferal production. Combination of positive- effect additives also increased the Desferal synthesis (about 7.8 times in compare to control).

Other studies have shown that the removal of excess iron from the culture medium has an increasing effect on Desferal production. For this reason we decided to apply 2, 2'-dipyridyle, EDTA and 8-hydroxyquinoline (as iron chelators, in different quantities and in several experiments) into the media. Our results indicated that 2,2'-dipyridyle (50-250 μM), EDTA (5 mM) and 8-hydroxyquinoline (5 mM) have a negative effect on Desferal production.

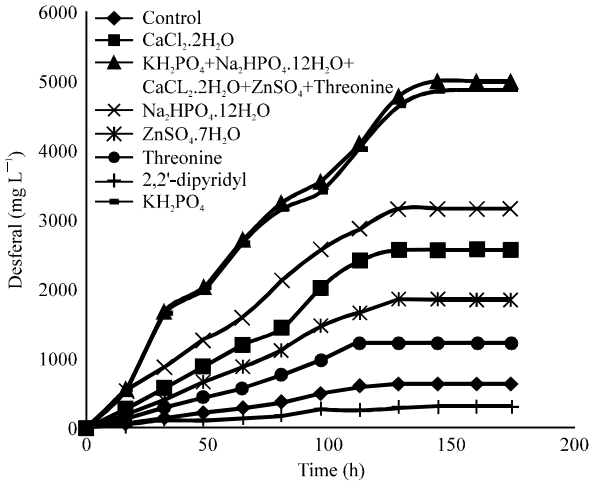

Fig. 4: Desferal production curve of *S. pilosus* (ATCC 19797) with different additives

As indicated in the results section, some additives with distinct concentration have the most increasing effect on Desferal synthesis in the culture medium during a period of 192 h. Culturing was performed in soybean medium. Sampling was done every 8 h and samples were centrifuged to remove the mycelia and the supernatant was diluted 10 folds with distilled water, then 5 mg mL^{-1} ammonium ferric sulphate in 1% sulphuric acid was added to the solution to a final concentration of 20%. Optical density of Desferal was read in 430 nm and concentration was calculated by comparing the ODs with that of the standard Desferal and its related concentration.

DISCUSSION

It is well known that Desferal is the most important drug which is being used for treatment of acute iron poisoning and iron-overload anemia, such as thalassaemia major, as well as aluminum poisoning associated with chronic renal dialysis. This drug is exclusively produced by Novartis Company. The aim of this investigation was to improve the culture medium for increasing the yield of Desferal production in *Streptomyces pilosus* (ATCC 19797). In this regard we examined the effect of some minerals and vitamins on the growth and the amount of Desferal production in *S. pilosus*. As mentioned earlier, KH_2PO_4, $NaH_2PO_4.12H_2O$ and $MgSO_4.H_2O$ have the most positive effect on increasing secretion of Desferal in the media. The addition of these materials resulted in the increasing of pH and it seems that elevated pH accompanied by element composition of these materials play an important role in this regard. EDTA and 8-hydroxyquinoline are iron chelating agents, but they can chelate other cations such as Al^{3+} and Cu^{2+}. These chelating agents diminished the production of Desferal in the medium. Although in a study it has been reported that concentrations equal to or greater than 10 μM of $FeCl_3$ in the liquid medium inhibit the synthesis of desferrioxamine B nearly completely (Schupp *et al*., 1987), but we observed that chelating the Fe and to some extent other elements such as Cu and Al also decreased the Desferal production. It seems that the minimum concentration of Fe and other elements are necessary for the bacteria and complete removal of the elements from the culture medium decreased the growth of bacteria and thereof Desferal synthesis. Although the combination of positive- effect additives increased the Desferal synthesis, but this increase was not satisfactory. This might be due to the interaction between the constitutive components of the medium.

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