http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 13 (23): 1151-1155, 2010
ISSN 1028-8880
© 2010 Asian Network for Scientific Information

Production of Desferrioxamine B (Desferal) using Corn Steep Liquor in *Streptomyces pilosus*

M. Chiani, A. Akbarzadeh, A. Farhangi and M.R. Mehrabi
Department of Pilot Biotechnology, Pasteur Institute of Iran, Tehran, Iran

Abstract: The aim of this study was evaluation of corn steep liquor as an alternative or substitution medium for production of desferrioxamine B in *streptomyces pilosus*. Desferrioxamine B is the major siderophore of *Streptomyces pilosus*. The genus Streptomyces are Gram positive and GC (Guamine/Cytosine) rich bacteria that are important for production of many antibiotics and secondary metabolites. These metabolites get more attention in industrial and medical fields. Desferrioxamine B is used clinically to treat disorders related to iron overload and pathological iron deposition in human. Corn Steep Liquor (CSL) is a by-product of corn wet-milling. It is an excellent source of organic nitrogen and important constituent of some culture media. Nowadays CSL was mainly used for feeding of livestock. In this study we substitute the conventional media with CSL and surveyed its effect on production of desferrioxamine B. The CSL is cheaper than other media and its availability is so easy. In this research, for the first time we used a cheap material for production of a valuable product. Our results showed that the use of CSL solely, increased the production of Desferal more than three times in comparison with conventional media such as soybean.

Key words: *Streptomyces pilosus*, desferrioxamine, desferal, corn steep liquor, siderophore

INTRODUCTION

Actinomycetes are an important group of microorganisms, not only as degraders of organic materials in the natural environment, but also as producers of many useful compounds of commercial interest (Saugar *et al*., 2002; Bentley *et al*., 2002; Basilio *et al*., 2003). 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 Mycobacterim have received an increasing amount of attention, particularly in the industrial fields. They exhibit potential advantages in the synthesis of secondary metabolites of industrial and medical importance (Tokiwa and Buenaventurada, 2004) and are active in the production of amino acids by fermentation and in bioconversion processes. Actinomycetes are important for the synthesis of enzymes, such as peptidases, chitinase, amylases, cellulases, proteases, xylanases, pectinase, ligninases, sugar isomerases, hemicellulase and keratinase (Solans and Vobis, 2003). They are also produced various types of antibiotics (Weber *et al*., 2003).

Siderophores are avid iron chelating agents synthesized by numerous microorganisms to sequester iron in environments where it is typically available in an insoluble form (Guerinot, 1994). Considerable attention has been devoted to the nutritional importance, genetic regulation, virulence factor and uptake function of these molecules (Guerinot, 1994).

Among the great number of desferrioxamines known from the literature desferrioxamine B is the most valuable one and is marketed as the mesylate salt (Fig. 1a, b) under the trade name Desferal. Desferal is a selective iron chelating agent and can be used in the treatment of different anemia and iron intoxications. For the industrial production of desferrioxamine B the *Streptomyces pilosus* strain is used, which produced mostly desferrioxamine B in a culture medium poor in iron (Bergeron and Brittenham, 1994).

CSL is a viscous liquid mixture consisting entirely of the water soluble components of corn steeped in water,

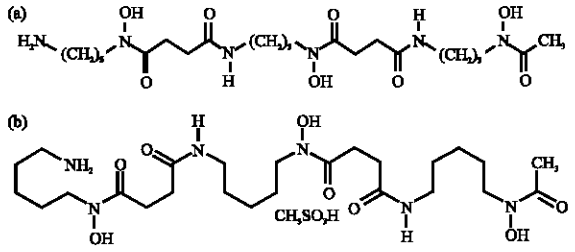

Fig. 1: Structure of (a) desferrioxamine B and (b) its mesylated salt

Corresponding Author: Mohammad Reza Mehrabi, Department of Pilot Biotechnology, Pasteur Institute of Iran, No. 358, 12 Farvardin Street, Jomhoori Avenue, Tehran, 13169-43551, Iran
Tel: +98 21 6696 8856 Fax: +98 21 6646 5132

Table 1: Corn steep liquor composition

General properties	Values	General	Values
Protein	20.5%	Humidity	47.5%
Carbohydrate	13-22%	Fiber	1%
Lipid	1%	Ash	8.8%
Dry mass	52.5%	pH	4-5
Brix	54.5		
Amino acids	**Values**	**Amino acids**	**Values**
Arginine	3.3%	Glycine	5.1%
Isoleucine	3.6%	Methionine	1.9%
Threonine	4%	Leucine	11.3%
Tryptophan	0.2%	Lysine	2.5%
Tyrosine	5.8%	Histidine	2.8%
Cysteine	1.9%	Valine	3.4%
Phenylalanine	4.4%		
Minerals	**Values**	**Amino acid**	**Values**
(Ca) Calcium	1%	(K)Potassium	4.5%
(Mg) Magnesium	1.5%	Sodium (Na)	0.2%
(P) Phosphor	3.3%	Chlorine (Cl)	0.18%
Sulfur (S)	0.58%	Iron (Fe)	0.10%
Vitamins	**Values**	**Vitamins**	**Values**
(B1) Thiamine	1 mg kg^{-1}	(B5)Pantothenic Acid	8 mg kg^{-1}
(B2) Riboflavine	5 mg kg^{-1}	(B6)Pyridoxine	2 mg kg^{-1}
(B3) Niacine	30 mg kg^{-1}		

along with a small amount of sulfurous acid (<0.01%). All constituents are naturally occurring nutritive materials such as crude proteins, amino acids, vitamins, reducing sugars (e.g., dextrose), organic acids (e.g., lactic acid), minerals and other elemental nutrients. In addition to being rich in nutrients, it is an economical fermentation nutrient source (Parekh *et al*., 1998). CSL composition has been indicated in Table 1. Hence, in our belief CSL could be a good option because it is a rich source of nutrients and minerals.

So far, no studies have been performed about the using of alternative media for the production of siderophores in these microorganisms. It is logical that finding a new culture medium that was cost effective and more efficient on the growth of the bacteria was very important.

MATERIALS AND METHODS

Streptomyces pilosus ATCC 19797 was purchased from microbial collection of U.S.A. All reagent-grade chemicals were provided from Merck comany. Desferrioxamine B was obtained as the mesylate salt (Desferal; Ciba-Geigy, Inc., Basel, Switzerland). CSL was kindly prepared from Glucozan Inc., Qazvin Province, Iran. This research project was conducted from February 2010 to September 2010.

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).

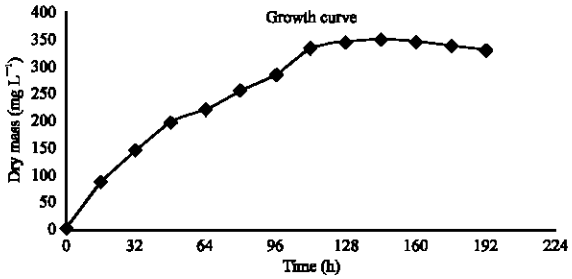

Fig. 2: Growth curve of *Streptomyces pilosus* (ATCC 19797) during a period of 192 h. Cultures were performed in soybean medium at 29°C and 150 rpm. Sampling was done every 8 h and filtered bacterial masses were weighed after drying in a 60°C oven for 3 days

Verify, assay and extraction of Desferal production: To confirm the production of desferrioxamine B, a single colony of the bacteria was cultured on nutrient agar (NB plus 2% agar). After 4 days, a piece of Whatmann 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 desferrioxamine B (Schupp *et al*., 1988).

The amount of Desferal in culture was measured spectrophotometerically. In this regard, the bacterium was cultured in soybean broth medium for 8 day 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

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

comparing the ODs with the standard curve prepared with different concentrations of Desferal.

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 (Gaeumann and Prelog, 1964) 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, 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 ad water and acetone (Zoltan *et al*., 1994).

Desferrioxamine B is generally used in the form of the methanesulfonate salt (mesylated form) which is readily soluble in water. The methanesulfonate is prepared from desferrioxamine B 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 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 pharmacopeia 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 sulfate solution was added and vortexed. The dark yellow color indicated the presence of Desferal in our product.

We inspected the effectiveness of CSL as a new culture medium and compared it with one of the best standard culture media (soybean) in growth of *S. pilosus* and Desferal production thereof. For this purpose in each onset, different dilutions of CSL were applied and the best concentration was obtained.

For using the CSL as a culture medium, we diluted it one to fifth with distilled water and centrifuged for 15 min at 3500 rpm. After centrifugation the supernatant was transmitted to new flask and the debris was discarded. Due to acidic components the supernatant pH was 4.5 to 5. Different proportions of CSL and soybean were prepared, the pH were adjusted to 7.8 with KOH and then were autoclaved for 15 min at 121°C. After that, each medium was added with same amount of refreshed spores of *S. pilosus* and placed in shaker incubator for 6 days (150 rpm, 29°C). All conditions such as temperature, shaking rate, amount of bacteria in each flask and incubation time throughout the experiment was constant. After this period, the content of each flask was centrifuged, the supernatant was transmitted to new flask and the pH was measured and its content of Desferal was measured by spectrophotometer.

RESULTS AND DISCUSSION

Several standard media were experimented and concluded that among them soybean is the best medium and has the highest productivity. Different dilutions of

Table 2: Different combinations of soybean with CSL and their effects on Desferal production

Medium No.	Soybean medium	20% CSL	Desferal (g L^{-1})
1	100%	0%	0.63
2	80%	20%	0.82
3	60%	40%	1.03
4	40%	60%	1.22
5	20%	80%	1.55
6	0%	100%	1.82

CSL in distilled water were prepared and their effects on production of Desferal were examined. It's cleared that the best dilution of CSL was 20%. In subsequent steps we used this medium (20% CSL) in combination with different concentration of soybean and compared their effectiveness on increasing the yield of Desferal production in *S. pilosus* (Table 2). We found that whatever the concentration of CSL in medium is increased, the yield of Desferal is enhanced. The most production of Desferal was obtained with 20% dilution of CSL alone. The results showed that 20% dilution of CSL without any additive or modification can be a good medium for growth of the bacterium. This medium increased the production of Desferal more than three times compare to soybean. We repeated the experiment several times and got the same results.

CSL is a rich source of nutrients (Formanek, 1998) and has been successfully used for other fermentations including ABE (Parekh *et al*., 1999) and CMA (Witjitra *et al*., 1996). In the present study, the possibility of substituting soybean with CSL (by-product of cornstarch extraction process) as a source of proteins, vitamins and minerals was investigated. CSL was selected mainly because it's cheaper than standard media (e.g., soybean) and can be easily available with standard and constant characteristics. *S. pilosus* is selective bacterium for industrial production of Desferal. As mentioned earlier its production on the best standard medium was 0.63 g L^{-1} (Table 2) but using 20% CSL its efficiency enhanced more than 3 times (1.82 g L^{-1}). In a study conducted by Kona *et al.* (2001) the production of glucose oxidase using Aspergillus niger and CSL was inspected. Their results also showed that using CSL solely can increase the production of glucose oxidase about 30% (Kona *et al*., 2001). In another study, Parekh and colleagues applied glucose/corn steep water (CSW) medium for production of acetone, buthanol and ethanol by *C. beijerinckii* NCIMB 8052 and BA101 in 200-1. Their findings represent a 40% increase in buthanol production by the *C. be-ijerinckii* BA101 mutant strain when compared to the 8052 parent strain (Parekh *et al*., 1999). In addition, Pyung Cheon Lee *et al* evaluated the production of Succinic Acid using Glucose and CSL by *Anaerobiospirillum succiniciproducens*. Their results suggesting that succinic acid can be produced more economically using glucose and CSL (Lee *et al*., 2000).

These finding propose that CSL can be used as a perfect medium for production of valuable products in microorganisms. Undoubtedly, using fermentation system in large scale that enable more control on growth conditions will further increase the desired product(s).

ACKNOWLEDGMENTS

The authors are thankful to the manager of Glucozan Corporation for providing CSL for the study. The authors are also grateful to all colleagues in Pilot Biotechnology Department especially Mahboubeh Mazinani for their sincere cooperation and their assistance in this project.

REFERENCES

Basilio, A., I. Gonzalez, M.F. Vicente, J. Gorrochategui, A. Cabello, A. Gonzalez and O. Genilloud, 2003. Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. J. Appl. Microbiol., 95: 814-823.

Bentley, S.D., K.F. Chater, A.M. CerdeÃ±o-T rraga, G.L. Challis and N.R. Thomson *et al*., 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature, 417: 141-147.

Bergeron, R.J. and G.M. Brittenhanı, 1994. The Development of Iron Chelators for Clinical Use. CRC Press, Boca Raton, FL., ISBN 10: 0849386799.

Formanek, J.A., 1998. Genetic manipulation and characterization of solvent-producing clostridia. Ph.D. Thesis, University of Illinois, IL, USA.

Gaeumann, E. and V. Prelog, 1964. Methods for Producing Ferrioxamine. United States Patent Office, USA.

Guerinot, M.L., 1994. Microbial iron transport. Annu. Rev. Microbiol., 48: 743-772.

Kona, P.R., N. Qureshi and J.S. Pai, 2001. Production of glucose oxidase Using *Aspergillus niger* and corn steep liquor. Bioresour. Technol., 78: 123-126.

Lee, P.C., W.G. Lee, S.Y. Lee, H.N. Chang and Y.K. Chang, 2000. Fermentative production of succinic acid from glucose and corn steep liquor by *Anaerobiospirillum succiniciproducens*. Biotechnol. Bioprocess Eng., 5: 379-381.

Parekh, M., J. Formanek and H.P. Blaschek, 1998. Development of a cost-effective glucose-corn steep medium for production of butanol by *Clostridium beijerinckii*. J. Ind. Microbiol. Biotechnol. 21: 187-191.

Parekh, M., J. Formanek and H.P. Blaschek, 1999. Pilot-scale production of butanol by *Clostridium beijerinckii* BA101 using a low-cost corn steep water based fermentation medium. Applied Microbiol. Biotechnol., 51: 152-157.

Saugar, I., E. Sanz, M.A. Rubio, J.C. Espinosa and A. Jimenez, 2002. Identification of a set of genes involved in the biosynthesis of the aminonucleoside moiety of antibiotic A201A from *Streptomyces capreolus*. Eur. J. Biochem., 269: 5527-5535.

Schupp, C., C. Toupet and M. Divers, 1988. Coloning and expression of two genes of *Streptomyces pilosus* involved in the biosynthesis of the siderophore desferrioxamine B. Gene, 64: 179-188.

Solans, M. and G. Vobis, 2003. Saprophytic actinomycetes associated to the rhizosphere and rhizoplane of Discaria trinervis. Ecologia Austr., 13: 97-107.

Tokiwa, Y. and P.C. Buenaventurada, 2004. Degradation of microbial polyesters. Biotechnol. Lett., 26: 1181-1189.

Weber, T., K. Welzel, S. Pelzer, A. Vente and W. Wohlleben, 2003. Exploiting the genetic potential of polyketide producing streptomycetes. J. Biotechnol., 106: 221-232.

Witjitra, K., M.M. Shah and M. Cheryan, 1996. Effect of nutrient sources on growth and acetate production by Clostridium thermo-aceticum. Enz. Microbial. Technol., 19: 322-327.

Zoltan, K., K. Vilmos and K. Antal, 1994. Process for the preparation of high-purity deferoxamine salts. United State Patent, No. 5,374,771.