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Isolation screening and identification of bile salt hydrolase producing bacteria from waste samples

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ABSTRACT

Recently the significance of bile salt hydrolase (BSH, EC 3.1.24) enzyme has been increased since it is highly effective in reducing serum cholesterol levels in humans and animals. High cholesterol level is found as an important reason of atherosclerosis which results in cardiovascular diseases (CVD's). In this study, the search for novel BSH producing bacteria leads to their finding in soil from fish waste- yards. The soil bacteria with potent bile salt hydrolase production and bile tolerant activity was isolated through sequential screening methods and identified based on its phenotypic and genotypic characteristics as *Staphylococcus saprophyiticus* ZABR2. The media and culture conditions of this selected isolate were optimized for the maximum production of the significant enzyme. The optimum production of the enzyme was observed in sodium glutamate medium, with glucose as carbon source, peptone as nitrogen source, medium pH 5 and when incubated at 45ºC, 160 rpm for 14 hours. A two fold increase in the production of enzyme was observed in the optimized media.

KEY WORDS: Bile salt hydrolase, Atherosclerosis, Cardiovascular diseases, Phenotypic identification, Genotypic identification.

1. INTRODUCTION

Bile salt hydrolase, a biologically significant enzyme involved in the enzymatic deconjugation of bile salts, has been associated with the cholesterol metabolism in mammals (Klaver & Van der Meer, 1993; Pereira, 2003). BSH belongs to the choloyglycine hydrolase enzyme family and is normally associated with the gastrointestinal bacteria of both humans and animals. This enzyme has been classified as an N- Terminal nucleophilic hydrolase which can recognize substrate at either the amino acid conjugate or steroid nucleus resulting in the release of amino acid residues and free bile acids (Patel, 2010). The free bile acids released are excreted via faeces there by enhancing the synthesis of bile salt from serum cholesterol which leads to the reduction of serum cholesterol levels or by lowering the solubility of cholesterol and thus reducing its uptake from the gut (Kumar, 2006).

Cholesterol is regarded as an important substance in the human body, but high serum cholesterol levels may lead to atherosclerosis and results in cardiovascular diseases (CVD's) (Tsai, 2014). According to the recent reports of World Health Organization (WHO), the CVD's are responsible for 30% of death globally and that by 2030, about 23.3 million people will be affected around the world. Most of the drugs in use, though may effectively reduce the cholesterol level, are expensive and known to have side effects (Sridevi & Prabhune, 2009). Hence the importance of using enzymatic deconjugation by BSH to lower serum cholesterol levels in hypercholesteremic patients and to prevent hypercholesteremia in normal people is increasing nowadays (De Smet, 1994). Microorganisms are found as important sources of this highly significant enzyme.

The BSH production by many gastrointestinal bacterial species has been reported earlier (De Smet,1998; Tanaka, 1999). In addition to this, deconjugation capacity was also reported from other bacterial species including an enteropathogenic strain of *Listeria monocytogenes* (Dussurget, 2002) and a bile adapted strain of *Xanthomonas maltophila* (Dean, 2002). Only a few attempts were made to isolate BSH producers from the environment source. So the present investigation was focused mainly to isolate the BSH producing bacteria from the environmental sources and to optimize the growth conditions for the maximum production of BSH.

2. MATERIALS AND METHODS

Sample collection: Soil samples were collected through sterile methods, from the soil of the waste-yards where mainly fish waste from market is dumped.

Isolation of microorganism: One gram of soil was suspended in sterile saline and 10 fold serial dilutions were prepared. 1 mL of aliquot from each dilution were spread on sodium glutamate agar plates containing (g/L) yeast extract – 5, peptone – 10, NaCl – 2, sodium glutamate- 5 and agar- 15. The plates were incubated for 24 hrs at 37°C. The colonies from the primary screening were further cultured on sodium glutamate agar plates containing 0.5 % sodium glycodeoxycholic acid. Colonies appeared on the agar plates after incubation were selected and further characterized (Sridevi & Prabhune, 2009).

Quantitative analysis of BSH producers: The modified method of Liong and Shah (2005) was used for the determination of the amino acids released from the deconjugation of bile salt by bile salt hydrolase enzyme. For this, the bacteria were grown in sodium glutamate broth for 20 h at 37ºC and 160 rpm and then centrifuged at 10000 rpm for 10 min. From this 100 µl of aliquot was added to 800 µl of reaction buffer (sodium phosphate buffer, pH 6)

containing 100 µl 6 mM sodium glycocolate or sodium taurocholate. In this study, sodium glycocolate and sodium taurocholate have been used in the medium to find out the best substrate for deconjugation. The reaction was carried out at 37ºC for 30 min. After 30 min the reaction was stopped by mixing with 1 mL of 15 % (wt/vol) trichloroacetic acid. Then the mixture was centrifuged at 10,000 rpm for 10 min and 0.5 mL of sample was taken. To this 1.5 mL of water and 1 mL of ninhydrin reagent (l.5 mL of 1% wt/vol ninhydrin in 0.5 M sodium citrate buffer, pH 5.5; 1.2 ml of 30 % glycerol, 0.2 ml of 0.5M) were added and boiled in a boiling water bath for 14 min and the absorbance was recorded at 570 nm. One unit of BSH activity is defined as the amount of enzyme that liberated 1 µ mol of amino acid from the substrate per minute. Protein concentrations were measured by the Lowry's method (Randall & Lewis, 1951), using bovine serum albumin as the standard.

Identification of the isolate:

Phenotypic characterization: The isolates were phenotypically characterized by morphological, physiological and also their carbohydrate utilization characteristics.

Genotypic characterization:

16S rRNA sequencing: The genomic DNA was isolate and the purity (Cunha, 2004) and quality of the DNA was analysed (Unal, 1992). Then the bacterial genome of the isolates was amplified using the universal primers 27 F (5'AGAGTTTGATCMTGGCTCAG3') and 1492 R (GGTTACCTTGTTACGACTT). The amplified product was then purified and analysed in 1.2 % agarose gel containing ethidium bromide. Sequencing reaction was done using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The obtained sequence was subjected to sequence alignment and required editing using Geneious Pro v5.6 (Kearse, 2012). The retrieved sequences (16S rDNA) were then used for identical searches using BLAST (Basic Local Alignment Search Tool) programme in the NCBI Gen Bank (www.ncbi.nlm.nih.gov) DNA database for identifying the bacterial strains. The sequence obtained was aligned by CLUSTAL W program (Thompson, 1994) and the phylogenetic tree was constructed by Neighbor Joining method (Saitou & Nei, 1987) using Kimura 2- parameter (Kimura, 1980) in Molecular Evolutionary Genetic Analysis (MEGA) version 7.0. Bootstrap analysis was calculated for 500 trees.

Optimization of culture conditions for maximum BSH production: The optimization of various physical and chemical parameters for BSH production was determined by one factor at a time method.

Effect of Carbon and Nitrogen sources: Different carbon source were supplemented to the sodium glutamate media and the effect on BSH production was estimated. Carbon compounds (0.5%) such as glucose, xylose, lactose, mannose, fructose, mannitol, galactose, glucose and sorbitol were the carbon sources used. The effect of nitrogen source on BSH yield was studied by using media with different nitrogen sources (0.6%) such as malt extract, yeast extract, peptone and beef extract. Media without carbon and nitrogen source served as control.

Effect of pH and Temperature: The effect of pH for BSH production was determined by varying the pH of sodium glutamate media ranging from 3 to 8 for 14 h at 37ºC and 160 rpm and for the influence of temperature the isolate was incubated at different temperatures ranging from 25ºC to 55ºC for 14 h at 160 rpm. The samples were then assayed for bile salt hydrolase activity.

Effect of incubation period: The isolate was grown in sodium glutamate media at 37ºC and 160 rpm. The samples were withdrawn after 12 hrs. With 1 hour interval up to 18 hrs. The samples thus collected were assayed for bile salt hydrolase activity.

Statistics: All assays were done in triplicate and the values are represented as mean with standard error. Microsoft excel (2010) was used for drawing graphs and the error bar indicates standard error of the mean.

3. RESULTS

Isolation of microorganism: Soil samples collected from the dumping site of fish wastes were screened for bile salt deconjugating bacteria. The isolates grown on sodium glutamate agar plates were selected and these 43 isolates were further screened for the bile tolerance on sodium glutamate agar containing 0.5 % sodium glycodeoxycholic acid. Of these, 35 isolates could grow on the media indicating bile tolerance.

Quantitative analysis of BSH producers: The bile tolerant isolates were quantitatively assayed by ninhydrin method with slight modification. All the isolates showed varying degrees of substrate specificity towards the bile salts such as sodium glycocholate and sodium taurocholate. Among them one of the isolate, named as ZABR2, showed highest deconjugation capacity by ninhydrin method and was selected for further study. The isolate ZABR2 showed maximum specificity towards the substrate sodium taurocholate with a total activity of 28 ± 0.9 U/mL. For sodium glycocholate it was 25.63 ± 0.48 U/mL.

Identification of the isolate:

Phenotypic characterization: The isolate ZABR2 was large pale orange pigmented colony with entire margin and convex elevation. It was gram positive cocci arranged in clusters. It showed positive test results to catalase, urease and was able to ferment sugars such as mannitol, glucose, maltose, fructose and sucrose. It showed a positive result

in mannitol salt agar media and negative for coagulase test. The obtained results were compared with Bergey's Manual of determinative Bacteriology (Bergey & Peter H Sneath, 1994) and the isolate ZABR2 showed similarity towards *Staphylococcus sp*.

Genotypic characterization: The 16S rDNA sequence of 1201 bp, obtained in the 16S rDNA sequencing, was used for identifying the bacterial strain using BLAST (Basic Local Alingnment Search Tool) programme in the NCBI Gen Bank (www.ncbi.nlm.nih.gov) DNA database. The sequence showed 99% similarity to *Staphylococcus saprophyticus strain* RCB560. The sequence was submitted to the database and accession number was assigned as KX068681.1. The new strain was then named as *Staphylococcus saprophyticus* ZABR2. The phologenetic tree of the isolate was constructed which showed the highest homology towards *Satphlococcus saprophyticus NTS-1*.

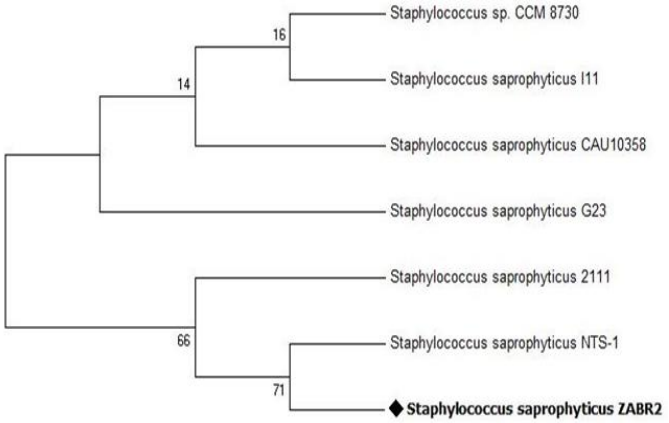

Figure.1. Phylogenetic tree of the isolate *Staphylococcus saprophyticus* ZABR2 based on 16S rDNA sequences with the closely related known bacterial strains

Optimization of culture conditions for maximum BSH production: The optimization of the culture conditions of *Staphylococcus saprophyticus* ZABR2 was carried out for getting the maximum production of the enzyme, BSH. Sodium glutamate medium was used for the optimisation study. When various carbon sources were used to find the best carbon source for the maximum production of BSH, glucose was found as the most suitable one followed by sorbitol and fructose (Fig.2). Among the various nitrogen sources, peptone was found to be the best source for the production of the enzyme (Fig.3). The influence pH on enzyme activity was studied on a wide range and pH 5 was observed as the optimum (Fig.4). Similarly the most suitable temperature detected for maximum BSH activity was 45 ºC and further increase or decrease in the temperature resulted in the reduction of the enzyme activity (Fig.5). The influence of incubation time was also studied here and a considerable increase in the activity was detected from the 12th hour onwards showing a maximum activity at 14th hour. Further increase in the incubation time showed a decrease in the activity (Fig.6).

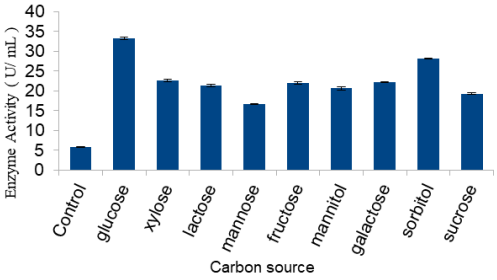

Figure.2. Effect of carbon source on BSH production

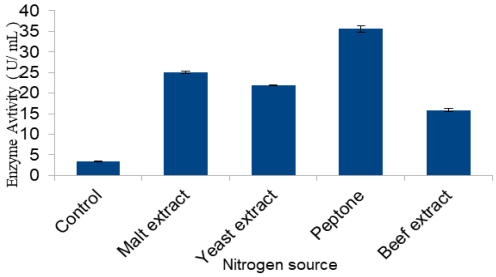

Figure.3. Effect of nitrogen source on BSH production

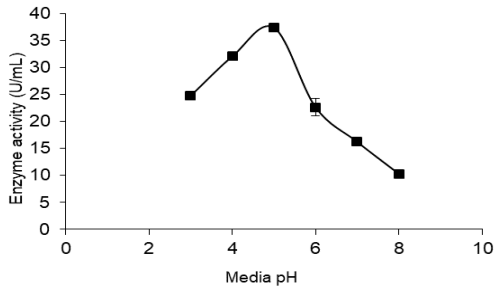

Figure.4. Effect of media pH on BSH production

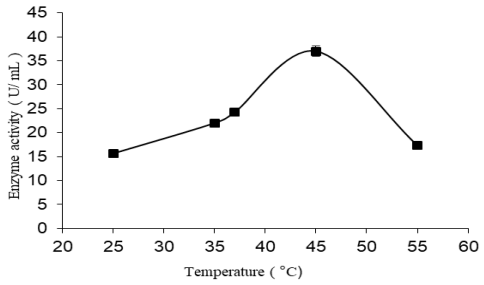

Figure.5. Effect of Temperature on BSH production

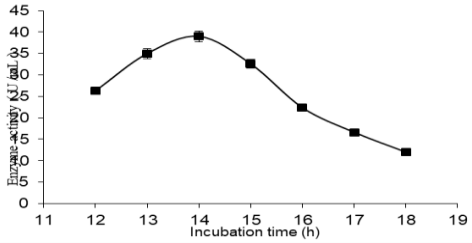

Figure.6. Effect of incubation time on BSH production

DISCUSSION

In the present study a sequential screening was used to isolate potent BSH producing bacteria from soil. A new strain ZABR2 with bile tolerant ability was isolated. Phenotypic characters were compared with Bergey's Manual which revealed that the isolate was similar to *Staphylococcus sp*. The isolate was subjected to genotypic characterization by 16S rRNA sequencing and the phylogenetic tree was constructed which showed that the isolate has the highest homology towards *Satphlococcus saprophyticus NTS-1*. The 16S rRNA sequencing is a rapid and accurate technique for the precise identification of unknown bacterial isolates and also it helps to study the diversity of the microbiological population (Drancourt, 2000; Greetham, 2002; Heilig, 2002). Since BSH is a pharmaceutically important enzyme, to explore the possibility of its large scale economically viable production the optimization of the media components and physical conditions are necessary. The optimum production of the enzyme was observed in sodium glutamate medium, with glucose as carbon source, peptone as nitrogen source, medium pH 5 and when incubated at 45ºC, 160 rpm for 14 hours. A two fold increase in the production of enzyme was observed in the optimized media.

4. CONCLUSION

The present study was intended to isolate potent bile salt hydrolase producing bacteria from environmental source, since the enzyme has a significant role in reducing cholesterol in human beings. A potent producer of BSH, *Staphylococcus saprophyticus* ZABR2, was isolated from soil and when the culture conditions were optimized for the maximum enzyme production, the isolate showed a two fold increase in the production. The findings of this study can be considered as novel since, to the best of our knowledge; there are no reports of bile salt hydrolase production by the organism, *Staphylococcus saprophyticus* ZABR2.

5. ACKNOWLEDGEMENT

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