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Original Article

STUDIES ON β-CYCLODEXTRIN PRODUCTION FROM CGTase PRODUCING BACTERIA AND ITS EFFECT ON DRUG SOLUBILITY

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ABSTRACT

Objective: To produce cyclodextrin (CD) from Cyclodextrin glycosyltransferase (CGTase) producing bacteria isolated from various samples and to study its effect on drug solubility.

Methods: CGTase producing bacteria were isolated from various samples *viz*. Soil, rotten potatoes, and stale corn flour dough using Horikoshi - phenolphthalein agar. All the isolates were screened for CGTase activity. Isolate showing highest CGTase activity was characterized morphologically and biochemically. CD was produced using CGTase which was further used for drug solubility studies.

Results: A total of fifty samples were studied of which 20 bacterial isolates showed the presence of halo around them. Upon further screening, the culture supernatant of 12 isolates showed cyclization activity, of which CD 18 strain produced highest amount of CGTase. Morphological and biochemical characterization revealed that the isolate belonged to *Bacillus* sp. The isolate showed maximum growth, reducing sugars 0.724 mg/ml and maximum enzymatic activity for dextrinisation as well as cyclisation *viz*. 9.45 U/ml and 7.41 U/ml respectively after 48 hrs of incubation. Thus, *Bacillus* sp. CD 18 was used to produce CGTase, which was further used for the production of CD. CD increased the solubility of acclofenac and paracetamol.

Conclusion: Bacteria are regarded as important sources of CGTases. New strains might suit better for industrial production of CGTase after optimizing the various cultures and process parameters.

Keywords: CGTase, Cyclodextrins, *Bacillus* sp.

INTRODUCTION

Cyclodextrin glycosyltransferase (CGTase), EC 2.4.1.19 is an important enzyme that can convert starch to cyclodextrins (CDs) as product [1]. CDs are oligosaccharides consisting of 6 to 12 glucose units, the three most common forms of CDs are α-CD, β-CD and γ-CD consisting of six, seven and eight glucose molecules respectively joined by α- 1, 4 glucoside linkages. They are also known as cycloamyloses, cyclomaltoses and Schardinger dextrins respectively having molecular mass of 70 to 75 kDa and shaped into a conical doughnut shape [2]. Of the three kinds of CDs, β-CD is of the most practical use because its inclusion complexes are easily prepared and stable, Moreover β-CD is easily separated from reaction mixtures because of its low solubility in water.

CDs produced by the action of CGTase on starch and related compounds are cyclic, non hygroscopic and crystalline substances. They are conical doughnut shaped molecules with their OH groups facing outside making them water soluble while the interior cavity is relatively apolar and hydrophobic so they can easily form an inclusion complex with many organic hydrophobic substances [3]. Therefore, CDs are becoming increasingly popular in various industries like food industry, cosmetic industry, pharmaceutical field as stabilizers for flavoring agents, to reduce unpleasant odor, taste as stabilizers of chemically labile compounds, to obtain prolonged action, to decrease local irritation and to reduce unpleasant odors [4, 5].

In pharmaceuticals CDs have mainly been used as complexing agents to increase the aqueous solubility of poorly water-soluble drugs and to increase their bioavailability and stability. In addition, CDs have been used to reduce or prevent gastrointestinal or ocular irritation, reduce or eliminate unpleasant smells or tastes, prevent drug-drug or drug-additive interactions, or even to convert oils and liquid drugs into microcrystalline or amorphous powders [6].

Due to the above mentioned novel properties the industrial demand for β-CD has increased many folds within a few past years. The isolation and screening of microorganisms from natural sources have always been the most powerful means for obtaining useful and genetically stable strain for industrially important products. In view of the above lacunae, the present study was planned to produce β-CD from CGTase producing bacteria isolated from various samples and to study its effect on drug solubility.

MATERIALS AND METHODS

Isolation of CGTase producing microorganisms

Fifty samples from different sources such as soil, rotten potatoes and stale corn flour dough were used for the isolation of CGTase producing bacteria. The samples were collected in sterile bags and immediately transferred to biotechnology laboratory of Shaheed Udham Singh College of Research and Technology, Tangori, Mohali (Punjab) to isolate CGTase producing microorganisms for further processing. Each sample was suspended in normal saline, serially diluted and inoculated on Horikoshi - phenolphthalein agar containing (w/v) 1.0% soluble starch, 0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, 0.02% $MgSO_4 \times 7H_2O$, 0.02% phenolphthalein, 1.0% Na_2CO_3 and 1.5% agar [7]. The plates were incubated at 37°C and were observed daily for 1 week. Bacterial colonies with yellowish clearance zone around them were selected as β-CGTase producing isolate and were sub-cultured on Horikoshi - phenolphthalein agar plates for purification. The presence of halos as a result of bacterial growth on the plates containing phenolphthalein suggests the presence of β-CGTase producing bacteria [8]. The purified isolates were stored on nutrient agar slants at 4° C and were screened for CGTase activity.

Screening of isolates for CGTase activity

Flasks containing 50 ml of sterile Horikoshi liquid production media containing 1.5% soluble starch, 0.4% yeast extract, 0.15g of KH_2PO_4, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, 0.12g of 20% KCl, and 0.9% NaCl [7] were inoculated with each isolate. Each flask was then incubated at 37°C in a rotatory shaker at 120 rpm. Samples from each flask were withdrawn at specific intervals of 24 hrs, 48 hrs and 72 hrs to analyze bacterial growth, amount of reducing sugars produced, dextrinising activity and cyclisation activity.

The bacterial growth was determined by recording the absorbance for samples at 600 nm at intervals of 24, 48 and 72 hrs. Thereafter the samples were centrifuged at 5000 rpm for 30 mins and the supernatant was used for determining amount of reducing sugars, dextrinising activity and cyclisation activity in order to isolate the best growing CGTase producing microorganisms. Concentration of reducing sugars in the supernatant was determined using the dinitrosalicylic acid method.

Dextrinising activity

Dextrinisation was assayed using soluble potato starch as substrate and by measurement of the decrease in iodine-staining power [9]. Briefly, 0.1 ml of supernatant was mixed with 0.5 ml of 1% starch solution and 0.4 ml of 0.1 M citrate buffer (pH 6.0). The tubes were incubated in a water-bath at 50°C for 10 min. The reaction was terminated with 0.5 ml of 1M HCl. Then 0.1 ml of 4 mM iodine in 30 mM potassium iodide was added and then diluted to 10 ml with water. Besides the samples, a negative control was also maintained which did not contain the enzyme solution. The starch-iodine complex absorption was read at 620 nm using UV-VIS spectrophotometer. Dextrinising activity was defined as the number of dextrin units produced by the breakdown of starch by the enzyme per unit time.

Cyclisation activity

Cyclisation activity was measured in each sample using phenolphthalein [10]. Briefly 5 ml of the supernatant and 5 ml of 1% starch solution were mixed in a test tube and kept in a water bath at 50°C for 30 mins. Samples were taken periodically from the test tubes and inactivated in water bath at 100°C for 5 mins. The concentration of cyclodextrin was measured by the addition of 2.5 ml of 3 mM phenolphthalein solution to 0.5 ml of inactivated samples. The absorbance of the final solution was analyzed in spectrophotometer at 550 nm. The amount of β-CD produced was estimated from the standard graph prepared by varying concentration of β-CD. A unit of enzymatic activity was defined as the quantity of enzyme that produces one µmol of cyclodextrin per minute under standard conditions. Maximum CGTase producing microorganism was identified and characterized on the basis of both morphological as well as biochemical tests.

Characterization of isolates

The bacterial isolates were examined for colony morphology, cell shape as per standard procedures [11]. A series of biochemical tests were performed for characterization of bacterial strain [12].

β-Cyclodextrin Production

Cyclodextrin production using CGTase was carried out using soluble potato starch [13]. Briefly soluble potato starch was dissolved in phosphate buffer (pH 7) to which culture supernatant containing CGTase was added. The reaction mixture was incubated at 45°C for 24 h in shaking conditions. After 24 h the enzyme reaction was stopped by boiling the mixture for 10 mins in a water bath. To eliminate oligosaccharides the mixture was filtered through a 0.45 µm membrane filter.

The filtered sample was analyzed by HPLC system using Aminex-HPX-42-A column. The elute containing β-CD was crystallized using toluene [14]. The precipitants obtained were stored at 4°C and were used for studying the effect of β-CD on the solubility of two drugs i. e. aceclofenac and paracetamol.

Estimation of drug solubility

Effect of CD on drug solubility was studied by phase solubility method [15]. 5 mg of each drug was added to 10 ml of distilled water containing variable amount of CD (0-10 mg). The suspensions were then shaken in the rotary shaker for 72 h. After shaking, the solutions were filtered through 0.45 µm membrane filter and the concentration of the drug was determined spectrophotometrically at 248 nm. The amount of drug was calculated from standard curve prepared with the increasing concentration of drug dissolved in distilled water.

Preparation of inclusion complexes and estimation of drug content

Inclusion complexes of drug and cyclodexrin were prepared by kneading method. Initially cyclodextrin was added to the mortar and then the small quantity of 50% ethanol was added while triturating to get slurry like consistency. Then drugs were incorporated into the slurry and tirturation was further continued for one hour. Slurry was then air dried at 25°C for 24 h. The drug content in drug-cyclodextrin complex was analysed spectrophotometrically at 248 nm.

RESULTS AND DISCUSSION

Cyclodextrin glycosyltransferase (CGTase) are extracellular bacterial enzymes that are closely related with the rest of amylolytic enzymes which cleave α-1, 4 bonds in starch and concomitantly linking the reducing and non-reducing ends to produce cyclic molecules known as CDs [16]. CGTase involve intermolecular transglycosylation that consists of coupling and disproportionation reactions, as well as the hydrolytic action on starch and cyclisation reactions. CDs can encapsulate other hydrophobic organic substances, adding solubilisation in water. The present study was planned to isolate a CGTase producing bacteria from different sources with a high yield followed by its characterization. After production of CGTase, β-CD was prepared, which was further used to study its effect on drug solubility. A total of 50 samples were processed for isolation of CGTase producing bacteria of which 20 (40%) isolates showed halos around them. These 20 strains were further screened for CGTase production. The culture supernatant of 12 isolates did not show much hydrolytic activity, however showed cyclization activity. Of these strains CD 18 strain produced the highest amount of CGTase. Thus, CD 18 was selected as CGTase producer for further studies.

As CD 18 was found to show maximum CGTase activity, the time course optimum for the enzyme production was analyzed. It was found that CD 18 showed maximum growth of 2.273, producing maximum amount of reducing sugars 0.724 mg/ml and possess maximum enzymatic activity for dextrinisation as well as cyclisation *viz.* 9.45 U/ml and 7.41 U/ml respectively after 48 hrs of incubation (Fig. 1). After 24 hrs cyclization activity was however not observed. The enzymatic activity of the isolates increased as the incubation time increased to 48 hrs and thereafter it again decreased.

Table 1: Morphological and Biochemical characters shown by CD 18

Biochemical test	Reaction
Colony colour	Yellow
Colony elevation	Slighty raised
Colony margin	Regular
Gram reaction	+ve
Cell shape	Rod
Spore staining	+ve
Motiliy test	+ve
Catalase test	+ve
Methyl red test	+ve
Voges-proskeaur test	-ve
Indole test	-ve
Nitrate reduction test	+ve
Citrate utilization test	+ve
Gelatin liquefication test	+ve

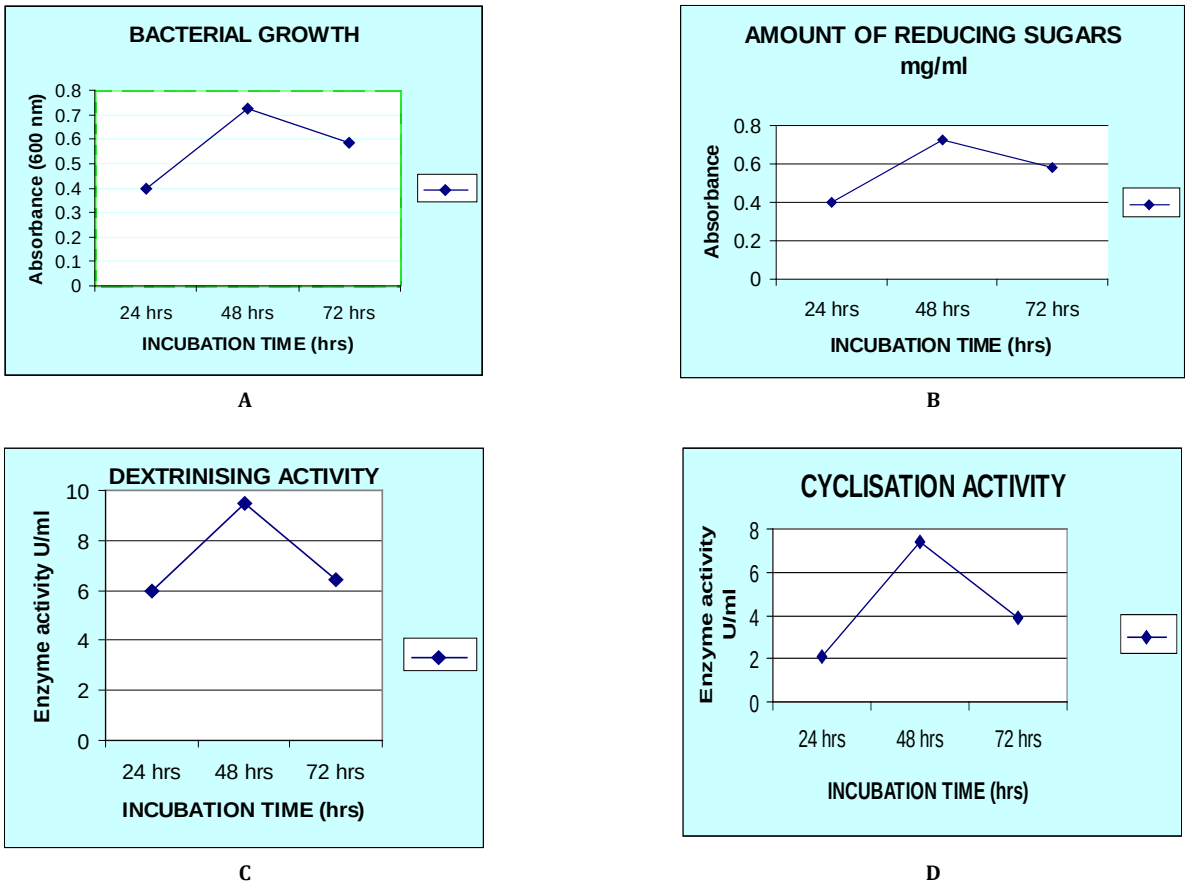

Fig. 1: Bacterial growth (A), amount of reducing sugars (B), dextrinising activity (C) and cyclization activity (D) shown by CD 18

The morphological and biochemical characters shown by CD 18 are shown in Table 1. The result indicated that the strain was Gram positive. Rod shaped and produced endospore. The isolate was motile, was positive for catalse, methyl red, nitrate reduction and citrate utilization but was negative for indole test and voges-proskeur test. The isolate hydrolysed gelatin and starch. The isolate was found to belong to *Bacilus* sp.

CGTase producing micro organisms have also been isolated by various other researchers from different sources like rotten potatoes [17], alkaline soil [8], Soda Lake [18], maize and amaranth starch [19], roots of cassava, corn, potato, bean, sugar cane, soya and pumpkin [9]. The genus *Bacillus* sp. Has remained the main source of bacterial CGTase that can be isolated from various sources.

The results contained in literature follow a different pattern where the enzyme synthesis began from the early exponential phase and maximum CGTase production was observed at 22-36 hr of incubation [20]. CGTase production by *B. cereus* was at its peak during the 16-20 hr of incubation period [21]. A similar results for cell growth and CGTase activity of *B. circulans* was reported by [17]. While *B. circulans* exhibited maximum cyclisation activity after 40 hr of incubation of growth with a long lag period [10] which was found to be in alignment with our results. A study by [22] suggested 36 hr of incubation to be the optimum time period.

CGTase enzyme was produced from *Bacillus* sp. CD 18 and was used for β-CD production. β-CD was successfully produced after 24 h of incubation by using soluble potato starch as substrate. The cyclodextrin produced was purified using HPLC system (Aminex-HPX-42-A column). Elute was precipitated by using toluene for the production of CD. Cyclodextrin has been produced in many studies by researchers using starch as substrate. CDs were produced after 24 hrs of incubation by using soluble starch as substrate [23]. In an another study by [18] produced β-CD using *Amphibacillus* sp. NPST-10. Cyclodextrin thus obtained was used to study its effect on drug solubility as CDs have unique property to form inclusion complexes due to their cyclic structure.

The phase solubility diagrams of Aceclofenac and paracetamol versus varying concentrations of CD were prepared. As shown in Fig. 2, it was found that solubility of Aceclofenac increased with increasing concentration of β-CD. Phase solubility diagram was studied by [24] and have shown that the aqueous solubility of nimodipine was increased linearly with the increased concentration of CD. The increase in solubility of paracetamol was also observed with the increasing concentration of CD as shown in Fig. 3.

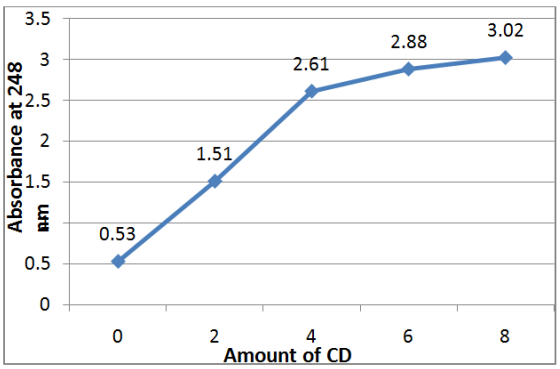

Fig. 2: Phase solubility diagram for Aceclofenac

The drug content of the sample was calculated. The drug content of aceclofenac was found to be 2.6 µg/ml (Fig. 4). The drug content of

paracetamol was found to be 2.9 µg/ml as shown in Fig. 5. Cyclodextrins are able to form inclusion complexes with poorly water soluble drugs and have been shown to improve pharmaceutical properties like solubility, dissolution rate, bioavailability, stability and even palatability without affecting their intrinsic lipophilicity or pharmacological properties [25].

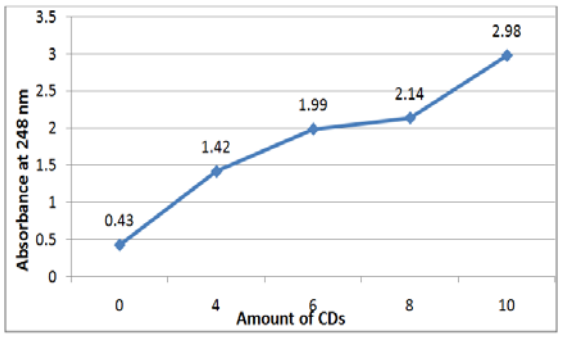

Fig. 3: Phase solubility diagram for Paracetamol

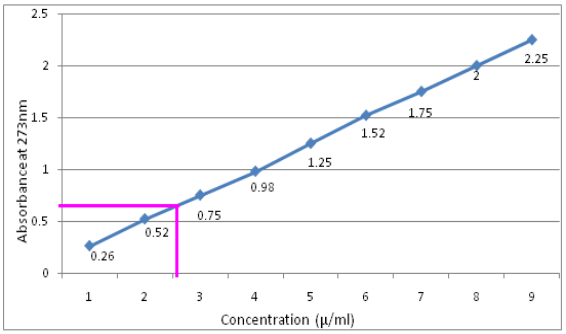

Fig. 4: Drug content estimation of Aceclofenac

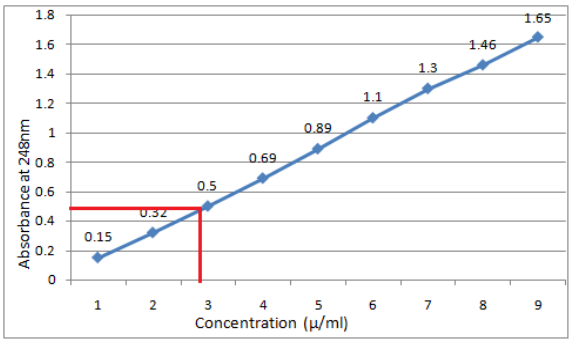

Fig. 5: Drug content estimation of Paracetamol

Several methods have been reported in the literature to enhance the aqueous solubilities of poorly water-soluble drugs. A number of poorly water-soluble drugs have been solubilized by use of various hydrotropic solutions and organic solvents. But the organic solvents suffer from drawbacks such as higher cost, toxicity and sources of pollution. The two salts aceclofenac and paracetamol are poorly soluble in water [26]. Aceclofenac has been shown to have potent analgesic and anti-inflammatory activities and due to its preferential cox-2 blockade it has better safety than conventional NSAIDs with respect to adverse effects on gastrointestinal and cardiovascular system. Paracetamol is a widely used over-the-counter analgesic (pain reliever) and antipyretic (fever reducer). It is commonly used for the relief of headaches, other minor aches and pains. pH dependent aqueous solubility and dissolution of nevirapine via complexation with β-CD was reported. It has been reported by [28] that CD enhanced solubilization of poorly soluble drugs sulfamethoxazole and trimethoprim by CD. The aqueous solubility of cyclosporine A was increased by 10 and 80 fold in the presence of α-CD and HP β-CD, respectively [29].

CONCLUSIONS

In the present study, 20 isolates showing halo zone in Hrikoshi-phenolphthalein media were screened for CGTase production. CD 18 showed highest CGTase production as shown by bacterial growth, hydrolytic activity, dextrinising activity and cyclization activity and was found to belong to *Bacillus* sp. CD was prepared on lab scale and was precipitated. The CD thus obtained was found to increase solubility of aceclofenac and paracetamol.

CONFLICT OF INTEREST

None

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