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Full Length Research Paper

Isolation and characterization of β-glucosidase producing bacteria from different sources

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β-Glucosidase producing microorganisms are potential sources that can be employed for bioconversion of cellulose. In the present study, nine morphologically different bacterial isolates were isolated from dairy effluent and seven were isolated from fermented barley. Four of the bacteria from the dairy effluent and five from barley source were found to possess β-glucosidase activity. This activity was tested by growth in medium supplemented with esculin and ferric ammonium citrate. The esculin positive strains from both the sources were characterized biochemically and checked for their ability to transform ginsenoside Rb1. The growth medium and the pH for maximum growth were optimized.

Key words: β-glucosidase, dairy effluent, barley, esculin.

INTRODUCTION

β-Glucosidases have been the focus of much research recently because of their important roles in a variety of fundamental biological and biotechnological processes (Czjzek et al., 2000). β-Glucosidase [β-glucoside glucohydrolase, EC 3.2.1.21] is one of the members of cellulase enzyme system, along with endoglucanase and cellobiohydrolase. β-glucosidase catalyzes the hydrolysis of the β-glucosidic linkages of aryl and alkyl β-glucosides, β-linked oligosaccharides and several other oligosaccharides with the release of glucose. It is involved in the last step of cellulose saccharification cleaving cellobiose to glucose (Yun et al., 2001).

β-Glucosidases are known to be widely distributed among animals, plants, fungi, yeasts and bacteria. β-glucosidase activity has been observed in various plant species such as maize and sorghum (Verdoucq et al., 2003) and microbes like roots of *Panax ginseng* plant (Zhang et al., 2001), *Penicillium purpurogenum* (Dhake and Patil, 2005), *Ceriporiopsis subvermispora* (Magalhaes et al., 2006), *Flavobacterium johnsonae* (Okamota et al., 2000), *Trichoderma harzianum* type C-4 (Yun et al., 2001), *Lactobacillus plantarum* (Spano et al., 2005) and *Dyella koreensis* spp. (An et al., 2005). β-Glucosidases find application in ethanol production from agricultural biomass substrates, for synthesis of useful glucosides, in flavor industry for the release of aromatic compounds from fruits and in deinking of printing ink from waste paper (Dhake and Patil, 2005).

β-Glucosidase is useful in increasing isoflavone aglycones during fermentation of soymilk (Otieno and Shah, 2007) and in the bioconversion of phenolic anti-oxidants from defatted soybean powder (McCue and Shetty, 2003). Micro-organisms producing this enzyme have been employed directly for the bioconversion of major ginsenoside Rb1 from *Panax ginseng* to minor ginsenosides of more therapeutic interest. These have been isolated from soil in a ginseng field (Kim et al., 2005) or performed by employing food grade micro organisms (Chi and Ji, 2003). β-Glucosidases of intestinal microflora in lower bowel can hydrolyze the glucoside isoflavones to aglycones and promote their absorption (Hendrich, 2002). Therefore, bacteria with β-glucosidase activity are potentially important in the production of compounds with higher estrogenecity and better absorption, facilitating the bioavailability of isoflavones (Hu et al., 2007).

Chromogenic substrates for the detection of β-glucosidase have been used in bacteriological culture media for almost a century (Harrison and van der Leck, 1909). The most commonly exploited substrate for β-glucosidase assay has been the naturally occurring glucoside esculin. James and Yeoman (1987) evaluated 8-hydroxy-

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quinoline-β-D-glucoside and found it to be equi-valent to esculin for the detection of β -glucosidase in gram-negative bacteria. Perry et al. (2006) have analyzed 3,4-dihydroxyflavone-β-D-glucoside, alizarin-β-D-gluco-side, 3',4'-dihydroxyflavone-β-D-glucoside to assay β-glucosi-dase activity. β-Glucosidase can also be assayed using ginsenosides as they are capable of converting major ginsenosides to minor ginsenosides (Zhang et al., 2001).

MATERIALS AND METHODS

Selection of source for isolation of micro organisms

The dairy effluent sample (source A) was collected from Aavin Dairy industry, Coimbatore. The sample was collected in a clean sterile plastic container and stored at 4°C until the analysis was carried out. The barley grains (source B) were purchased from local market.

Isolation of micro organisms from selected sources

1.0 g of soil sample from sample A was weighed and the microorganisms were isolated by employing standard serial dilution plating technique (Jensen, 1968). 0.1 ml of aliquot from each dilution was plated on R2A agar medium for the isolation of bacterial colonies and the plates were incubated at 37°C for 2-3 days.

Barley sample (10 g) was soaked in water (100 ml) and allowed for fermentation for a period of 4 days. For the isolation of micro organisms from this source, varying volumes (10, 100, 200 and 1000 µl) of the fermented supernatant was plated on R2A agar. The plates were incubated at 37°C for a period of 7 days. Morpho-logically different colonies were identified from each source.

Identification of bacteria with β-glucosidase activity

The morphologically different bacterial colonies obtained from source B were spotted on MRS agar and from source A on R2A agar, both supplemented with esculin (3 g/l) and ferric ammonium citrate (0.2 g/l). The plates were incubated at 37°C for about 48 h and colonies producing browning or blackening of the medium were noted as esculin hydrolyzing bacteria. The esculin positive bacterial colonies were identified and single colonies were obtained on MRS agar and their morphological features noted.

Biochemical characterization of the isolated bacterial cultures

The four esculin positive cultures from source A (2W, 3X, 4W, U5Z) and five bacterial colonies with β-glucosidase activity from source B (Bf1, Bf2, Bg, Bdt and Bdw). The cultures were streaked on R2A agar and MRS agar respectively to obtain single colonies. The cultures were maintained at 4°C. The isolated single colonies were studied for their morphological, physiological and biochemical characteristics by performing various biochemical tests.

Selection of nutrient solution for growth

The bacterial strains with β-glucosidase activity were inoculated in four media broth namely LB, NB, MRS and R2A. The cultures were incubated overnight at 37°C with 160-170 rpm. The optical density for each strain was measured at 600 nm against respective media blank.

Optimization of pH for growth

The bacterial strains with β-glucosidase activity were inoculated in LB broth with pH ranging from 4.0 to 10.0. The tubes were maintained in shaking at 160 - 170 rpm at 37°C and left for over-night growth. The optical density for each strain was measured at 600 nm against respective media blank.

RESULTS

Isolation of micro organisms from selected sources

Nine morphologically different bacteria were identified from source A as a result of serial dilution. They were identified on the basis of shape of the colony, colour and the appearance on their growth medium.

Barley grains were allowed to ferment and screened for microorganisms. The Barley grains were soaked in water for a period of 3 days and when the supernatant was used for isolation, morphologically different bacteria were identified on R2A agar. Neither the paste of soaked barley nor raw powdered barley when plated resulted in any colony growth suggesting the absence of any endophytic bacteria in barley. After identification of mor-phologically different colonies from sample A (barley water) each colony was spotted to verify its growth on MRS (de Mann Rogosa Sharpe) agar. Previously, food grade bacterial cultures have been maintained on this medium and used for bioconversion experiments. MRS agar is specific for the growth of *Lactobacillus* and related species which are predominant in fermented food (Chi and Ji, 2005). For further studies of source B, MRS medium was used.

Identification of bacteria with β-glucosidase activity

Morphologically different bacteria from source A were screened for the production of β-glucosidase enzyme by using esculin R2A agar. Four reddish brown colonies on the esculin R2A agar indicated that of the nine isolated bacteria four of them possess β-glucosidase activity. In order to check for the β-glucosidase activity of the isolated bacteria from source B, each individual bacterial colony was spotted on MRS agar supplemented with esculin and ferric ammonium citrate. Five bacteria from barley source were found to be esculin positive cultures as they resulted in the blackening of the medium around the colony. Figures 1 and 2 shows esculin positive cul-tures isolated from source A and B. The principle of esculin in MRS medium is represented in Figure 3.

Biochemical characterization of the isolated bacterial cultures

The cultures isolated from both the sources were rod shaped and negative for urea hydrolysis. Strains 3X and 4W were gram positive whereas the other two were gram negative. Stain 2X showed positive results. For strain

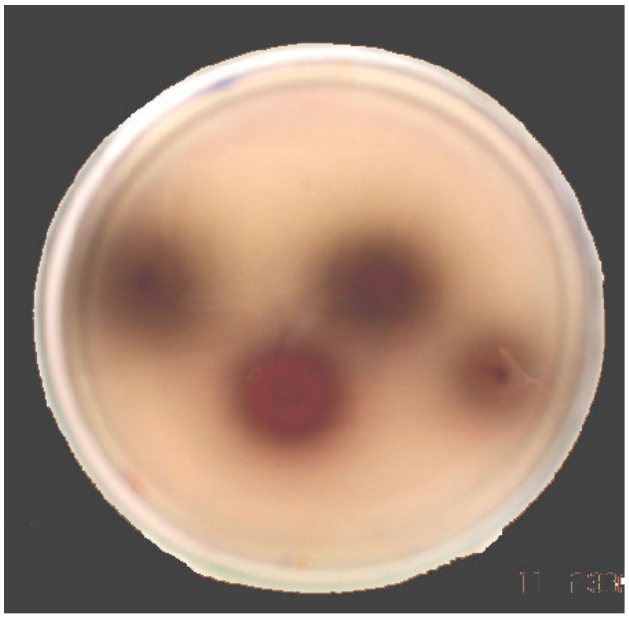

Figure 1. Esculin positive cultures on R2A agar isolated from dairy effluent source (Source A).

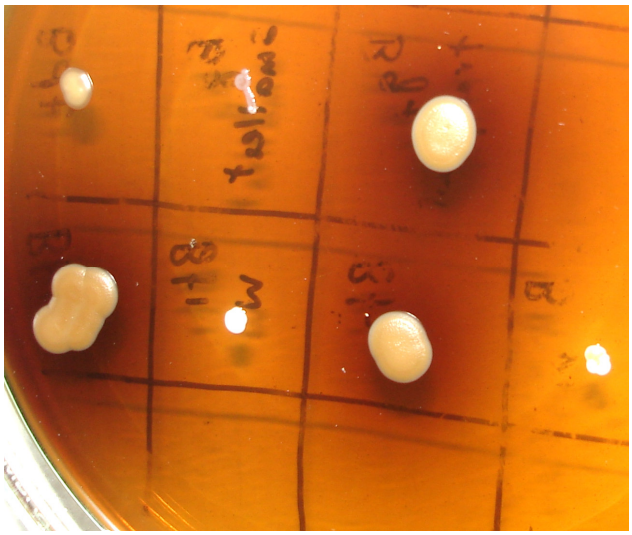

Figure 2. Esculin positive cultures on MRS agar isolated from fermented barley source (source B).

U5Z voges proskauer, citrate utilization, nitrate reduction, carbohydrate (glucose) fermentation and triple sugar ion tests. Strain 3X was found to be positive for indole production, methyl red, citrate utilization, catalase production, nitrate reduction and starch hydrolysis. In case of Strain 4W indole production, methyl red, citrate utilization, catalase, and starch hydrolysis tests showed positive results. For U5Z voges proskauer, citrate utilization, nitrate reduction, carbohydrate (glucose) fermentation and Triple sugar ion tests were found to be positive.

The five bacterial cultures from source B were citrate, starch, indole and negative. Therefore, the isolated cultures do not utilize citrate as the sole carbon source; do not contain α-amylases and cannot oxidize tryptophan. The bacteria were catalase positive and produced acids when allowed for fermentation with glucose as the carbon source. Except for the culture Bf2, the other four cultures were gram positive.

From Methyl red and triple sugar iron test, it is noted that cultures Bf2, Bg and Bdw produce acids like acetic, lactic and succinic acids, thereby reducing the pH of the medium. The cultures Bf1 and Bdt produce neutral and alkaline end products.

All the isolated bacterial strains have similarities and differences among them. Therefore, the strains may belong to the same or related genus. From the morphological and biochemical analysis, it is obvious that the strains are distinct from each other. The biochemical characteristics of all the bacteria are tabulated (Table 1).

Selection of nutrient solution for growth

All the esculin positive cultures were employed for bioconversion. All the barley cultures showed positive results for bioconversion. The strains 3X and 4W were also able to convert major ginsenoside Rb1 (result not shown). Only the cultures that can be employed for bioconversion were optimized for growth medium and pH.

The cultures Bdt and Bdw showed a slow growth compared to others. The cultures Bf1, Bf2 and Bg showed maximum growth in MRS medium followed by R2A, NB and LB. Bdt showed a similar growth on R2A and LB media whereas Bdw had maximum growth on LB. MRS medium was chosen for the optimization of pH as the bioconversion analysis will be carried out only in MRS broth as used by Chi and Ji (2005) for food grade microorganisms (Figure 4).

In R2A broth Strain 3X and 4W showed moderate growth. But in MRS there is no growths of Strain 3X and very small amount of growth of Strain 4W was observed. There are large amount of nutrients and solutes in R2A and MRS media compared to LB and NB. These results clearly reveal that the bacterial growth and enzyme production is more economical in LB and NB broth since they contain minimal number of constituents (Figure 5).

Optimization of pH for growth

Cultures Bf1, Bf2 and Bg showed a better growth profile in all pH range compared to Bdt and Bdw. A pH of 6.5 was found to be optimal for the culture Bdt and Bdw showed the best growth at the pH of 7.5. The bacteria Bdt and Bdw did not show any significant growth in the other pH ranges. In case of strains 3X and 4W maximum growth was observed at pH 7 - 8. This was measured by determining the optical density at 660 nm.

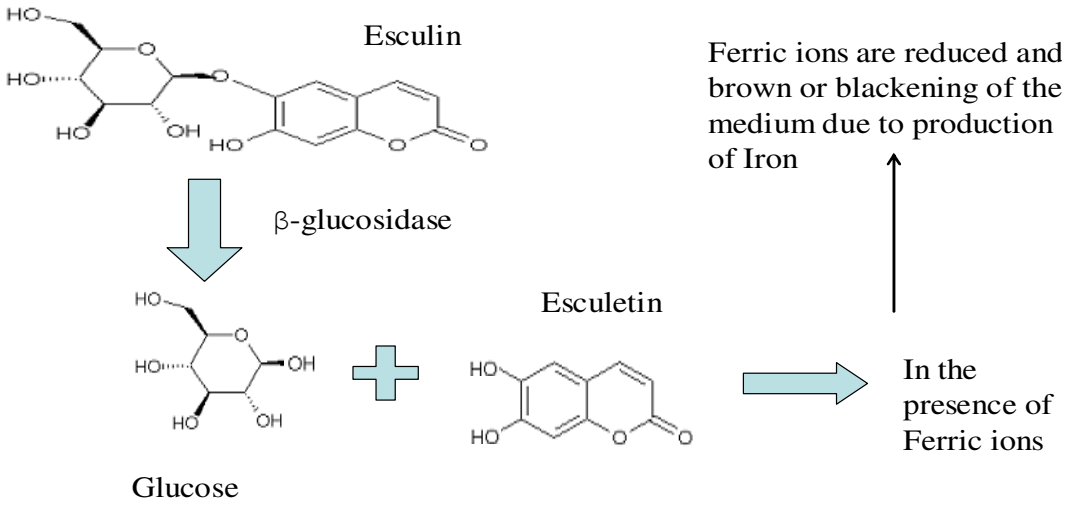

Figure 3. Principle of esculin for identification of β-glucosidase activity.

Table 1. Biochemical characteristics of the isolated bacteria from dairy effluent and fermented barley.

S/N	Biochemical test	Bf1	Bf2	Bg	Bdt	Bdw	2X	3X	4W	U5Z
1.	Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
2.	Grams staining	+	-	+	+	+	-	+	+	-
3.	Indole Production	-	-	-	-	-	-	+	+	-
4.	Methyl Red	-	+	+	+	-	-	+	+	-
5.	Voges-Prousker	+	-	-	-	+	+	-	-	+
6.	Citrate Utilization	-	-	-	-	-	+	+	+	+
7.	Starch hydrolysis	-	-	-	-	-	-	+	+	-
8.	Urea hydrolysis	-	-	-	-	-	-	-	-	-
9.	Nitrate reduction	+	+	+	-	+	+	+	-	+
10.	Catalase	+	+	+	+	+	-	+	+	-
11.	Carbohydrate fermentation	Acid	Acid	Acid	Acid	Acid	Acid	-	-	Acid
12.	Triple sugar Iron	F-	G+	G+	G+	G+	G+	F-	F-	G+

+: Positive; F: fermentation of carbohydrates; - : negative; G, fermentation of glucose alone.

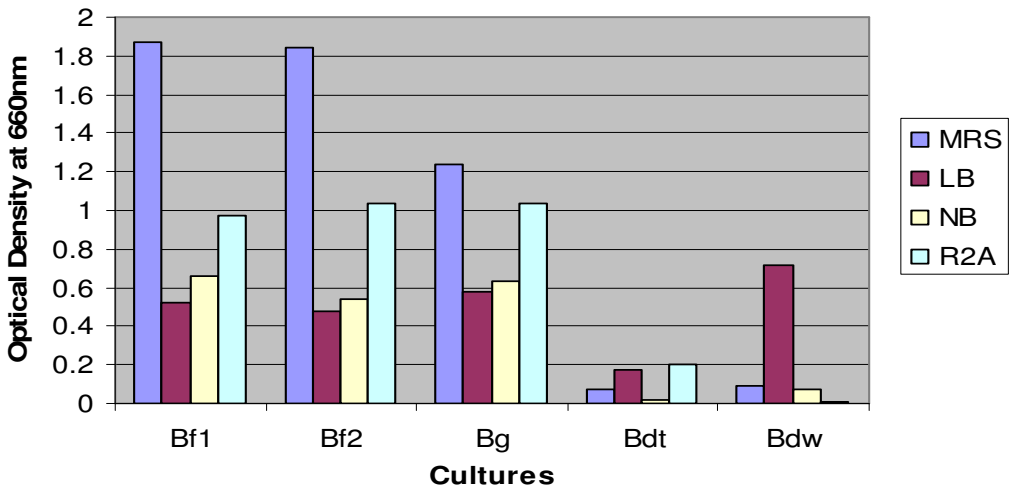

Figure 4. Optimization of growth media for cultures from source B.

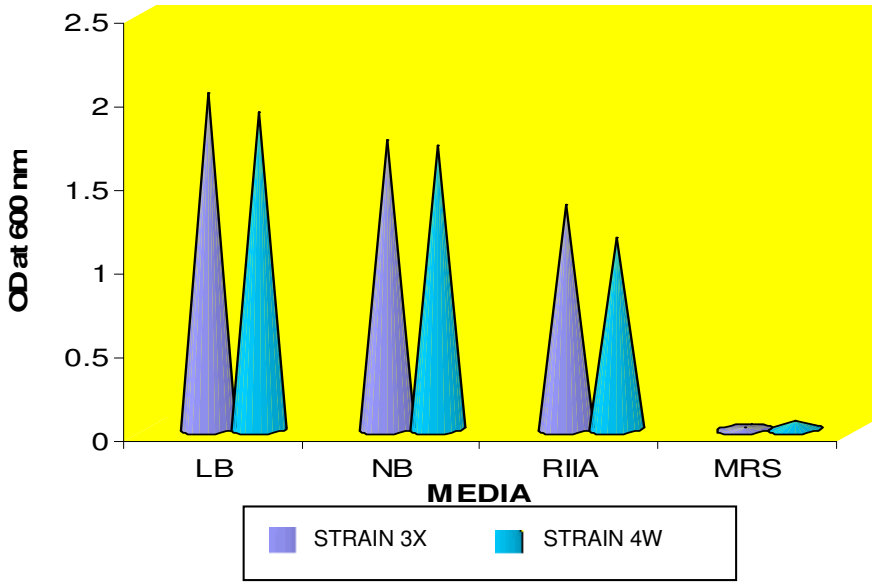

Figure 5. Optimization of growth media for cultures from source A.

DISCUSSION

Cheng et al. (2006) have isolated 77 types of β-glucosidase producing microorganisms on esculin R2A agar plates from soil around the ginseng roots and 20 of the isolates had ginsenoside conversion activity. Kim et al. (2005) used R2A agar for isolation of bacteria from soil samples as these strains will require only less amount of nutrients for their growth. In this study, R2A agar was used for isolation of bacteria from food sources as these bacteria will require minimum nutrients for their growth.

The source of the enzyme β-glucosidase will be the bacterial cultures isolated from barley since; the sugars present in barley are in the β-glucan form (Robertson et al., 1996). When barley grains are allowed to ferment, the bacteria that grow on it during fermentation process can produce the enzyme β-glucosidase to utilize constituents of barley for their survival.

Supplementing the growth media with esculin can identify the presence of the enzyme β-glucosidase. Esculin is cleaved by β-glucosidase to yield esculetin and glucose molecule. The hydrolytic product esculetin reduces the ferric ions (provided by ferric ammonium citrate in the medium) and produces iron, which results in the browning of the medium. Therefore, the colonies producing browning of the medium are esculin positive and possess β-glucosidase activity.

Among the various chromogenic substances available http://dx.doi.org/10.1111/j.1745-4514.2003.tb00597.xlike arbutin, alizarin, esculin etc., available to detect β-glucosidase, esculin was used in the present study.

Though indoxylic substrates are highly effective, they are relatively difficult to prepare and have other limitations such as their requirement for oxygen in order for the colored complex to develop (Perry et al., 2006).

The bacteria isolated from various sources can be employed for bioconversion of ginsenosides and may find other suitable applications. The phylogenetic position of the bacteria, the characterization of the enzyme β-glucosidase from them may lead to the improvisation in the enzyme production by the bacterial cultures. If the application of the enzyme is successful in one or more fields, then the gene responsible may be isolated, cloned and used for the large scale production of the enzyme.

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