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ISOLATION FROM SOIL OF BACTERIA PRODUCING EXTRACELLULAR ALPHA GALACTOSIDASE

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Abstract : Extracellular alpha galactosidase (alpha galactosidase galactohydrolase EC 3.2.1.22) producing bacteria were isolated from soil by the enrichment culture technique using raffinose as the inducer. Six bacterial species were isolated by this method based on their morphological characteristics. Their raffinose utilization rate varied from 11 mg/h to 27 mg/h. Enzyme acivity present in the supernatant varied from 2-11 mU/ml. Two of the isolated species did not show any alpha galactosidase activity. Three bacterial species having high alpha galactosidase activity were identified as *Klebsiella pneumoniae*, *Citrobacter freundii* and *Escherichia coli* by their colony morphology and biochemical tests. They were cultivated at different pH values with peptone and ammonium sulphate as the nitrogen source. From those studies, it was shown that the highest extracellular alpha galactosidase activity of 14.7 mU/ml could be obtained from *Citrobacter freundii* after 18h of cultivation in a culture medium with an initial pH of 8 containing peptone as the nitrogen source. Extracellular enzyme production was increased up to 19mU/ml by the cultivation of *Citrobacter freundii* in pH8 phosphate buffer for 36h with peptone as the nitrogen source.

Key Words: Alpha galactosidase, *Citrobacter freundii*, extracellular, raffinose hydrolysis

INTRODUCTION

Alpha galactosidase (alpha-D-galactoside galactohydrolase EC 3.2.1.22) is an exocarbohydrase. It hydrolyses the terminal alpha 1-4 galactosidic linkages of galactose containing oligosaccharides and polysaccharides.

Alpha galactosidase has been reported to occur widely in microorganisms, plants and in animals.[1] This enzyme is required for the degradation of galactomannans and the reserve oligosaccharides during seed germination.[2]

It has been isolated from several bacterial species e.g. *Bacillus subtilis*,[3] *Bacillus stearothermophilus*,[4] *Klebsiella* sp. No. PG-2[5] and also from many species of fungi e.g. *Mortierella vinacea*,[6] *Monascus pilosus*,[7] *Trichoderma reesei* Rut C-30.[8]

Industrial applications of alpha galactosidase are in beet sugar industry, soy milk processing, pulp and paper industry and manufacture of gelling agents. Attempts have been made in medicine to use alpha galactosidase in the treatment

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of Fabry's disease.[9] In industrial applications, fungi and bacteria are used as the enzyme source as they can be easily grown and it is usually not difficult to scale up the production process. There is no seasonal variation in the enzyme production with microorganisms. The majority of enzymes that have so far been used in industry are extracellular enzymes produced by microorganisms. With microbial enzymes, it is often possible to increase the yield by changes in the growth conditions, addition of inducers, or strain selection including increasing the number of gene copies by genetic engineering.

In this paper we report the isolation of extracellular alpha galactosidase producing bacteria from soil by using raffinose as the inducer and the optimization of alpha galactosidase production.

METHODS AND MATERIALS

Materials: Analytical grade Serva Fein Biochemicals and Sigma Chemicals were used. Absorbance measurements were carried out using a Shimadzu UV 120-02 spectrophotometer. A bench centrifuge (MSE) and a high speed (Beckman model J2-21) centrifuge were used for centrifugation. Fisherbrand Hydrus 300 pH meter was used for pH measurements. Sterilization of culture media was done by autoclaving them at 121^0C for 15 minutes.

Enzyme Assay: To 1ml of culture supernatant, 1ml of 0.15M McIlvaine buffer (pH 5.0) was added and mixed well. 1ml of this mixture was incubated with 0.5ml 1mM p-nitrophenyl alpha-D-galactophyranoside solution for 30 minutes at 29^0C. The reaction was terminated by the addition of 5ml of 1.0N Na_2CO_3 solution. Absorbance was measured at 405 nm. In enzyme assays at pH 6.0 & 7.0, McIlvaine buffer was used.

A unit of enzyme activity is defined as the amount of enzyme that hydrolyses one micromole of substrate per minute under the conditions specified above.

Raffinose Estimation: Raffinose concentration was determined by the method described by Tanaka et al.[10]

Isolation of bacteria producing extracellular alpha galactosidase: Soil samples were collected from topsoil from humus rich places. Soil samples were pooled and approximately 2ml of soil sample was incubated in a culture medium containing 1% raffinose, 3% peptone, 0.05% yeast extract and 10% (v/v) salt solution (1% K_2HPO_4, 0.9% $MgCl_2.6H_2O$, 0.1% $CaCl_2$, 1% NaCl). pH of the culture medium was adjusted to 7.0 with 0.1% NaOH. Culture medium (25ml) in 100ml conical flasks were incubated at room temperature in an orbital shaker at 115 r.p.m. for four days. After four days, 1ml of each culture was transferred to a fresh culture medium of the same composition and incubated for two days under the same conditions.

The process was repeated twice. Afterwards a loopful of culture was transferred on to an agar plate containing the same culture medium solidified with 3% agar and streak plate isolation procedure was performed. Six bacterial species were isolated and inoculated into fresh culture media separately. After 24hrs cultivation the following parameters were tested with each culture.

(i) Optical Density at 510nm (ii) Wet weight (iii) Raffinose Utilization Rate (iv) Enzyme Activity of the supernatant at different pH values.

Determination of wet weight: The weight of pellets obtained by centrifugation of 10ml of each bacterial culture at speed 5 for 15 minutes using a MSE bench centrifuge was determined.

Determination of Raffinose Utilization Rate: Before inoculating sterilized enrichment culture medium in different flasks with six bacterial species, 2ml of culture medium from each flask was taken out for raffinose estimation. After 24hrs of incubation 2ml of the supernatant obtained after centrifugation of 10ml of each culture broth was also subjected to raffinose estimation.

1ml of the culture medium without raffinose was used to prepare the blank solution. 1ml of the culture supernatant to be tested for raffinose estimation was mixed with 1ml of 0.02 M thio-barbituric acid. 1ml of concentrated HCI was added to each mixture and kept in a boiling waterbath for 6 minutes. After cooling, the absorbance was measured at 432.5nm.

Raffinose utilization rate mg/h for each bacterial culture was calculated using the difference obtained for the raffinose contents of each culture medium.

Three selected bacterial species were cultured at 30°C, 45°C and 60°C and the raffinose utilization rates were measured with each culture.

Identification of the isolated bacterial cultures: Three bacterial species which showed high extracellular alpha galactosidase production were identified by their morphological and biochemical characteristics as described by Barrow and Felthan[11] and Lennette[12] as *Escherichia coli, Klebsiella pneumoniae* and *Citrobacter freundii.* No tests were done to determine whether the bacteria were pathogenic, as they were isolated from soil.

Optimization of enzyme production by Bacteria: The three bacterial species which were identified as *Escherichia coli, Klebsiella pneumoniae* and *Citrobacter freundii* were cultivated in a medium with different nitrogen sources (ammonium sulphate and peptone) and with different initial pH values 5, 7, 8. A small decrease in initial pH value was observed after autoclaving.

Table 1 : Cell growth, Raffinose utilization and α- Galactosidase production by six different bacterial species

Bacterial Culture (24 hrs. old)	Growth			O.D. at 510nm	Wet wt.	R.U.R. (mg/h)		E.A.S. (mU/ml)		
	30C⁰	45C⁰	60C⁰		(mg/10ml)	30C⁰	45C⁰	5	6	7
S	+	+	-	0.776	12.784	22.20	1.8	3.1	9.5	11.1
M	+	+	-	1.675	53.696	27.15	6.375	1.0	1.5	1.8
H	+	-	-	1.233	27.441	0	0	0	0	0
J	+	-	-	1.407	22.207	0	0	0	0	0
F	+	-	-	1.589	28.018	11.12	0	2.1	5.1	7.2
W	+	+	-	1.258	40.757	28.12	3.13	2.0	3.6	9.5

Abbreviations:

O.D - Optical Density
R.U.R - Raffinose Utilization Rate
E.A.S. - Enzyme Activity of the Supernatant
F - *Escherichia coli*
W - *Klebsiella pneumoniae*
S - *Citrobacter freundii*
+ - Growth
- - No growth
M - Unidentified Bacteria
H - Unidentified Bacteria
J - Unidentified Bacteria

Table 2: Growth Characteristics and Biochemical Test Results for three bacterial species

Character/Biochemical Test	Bacterial Species F	W	S
Colony Morphology On Nutrient agar	Well grown Colourless Smooth colonies	Large Extremely mucoid colonies	Smooth convex mucoid colonies
On MacConkey agar	Large,circular,smooth pink colonies	Small pink colonies	Light yellow colonies
Motility	Motile	Non-motile	Motile
Gram Stain	Gram negative rods	Gram negative bacilli	Gram negative bacilli
Biochemical Tests			
Carbohydrate Fermentation	Acid, Gas	Acid,Gas	Acid,Gas
Glucose			
Sucrose	+ve	+ve	+ve
Maltose	+ve	+ve	-ve
Lactose	+ve	+ve	-ve
Mannitol	+ve	+ve	-ve
Eesculin Hydrolysis	-ve	ND	ND
Oxidation Fermentation			
Glucose	Fermentation	Fermentation	Fermentation
Utilization of Amino Acids			
Decarboxylase			
Lysine	+ve	+ve	-ve
Arginine	+ve	+ve	+ve(48hrs)
Ornithine	+ve	+ve(48hrs)	+ve
Deaminase			
Phenylalanine	-ve	ND	ND
Indole	+ve	-ve	+ve
Methyl Red	+ve	-ve	+ve
Voges-Proskauer	-ve	+ve	-ve
Simmon's citrate	-ve	+ve	+ve
Hydrogen Sulphide (TSI)	-ve	-ve	+ve
Urea	-ve	+ve	+ve
Lead Acetate	-ve	-ve	+ve
Catalase	+ve	+ve	+ve
Oxidase	-ve	-ve	-ve

Symbols : (+) - Positive within 24-48hrs.
(-) - 10% or less positive within 24-48
ND - Not Determined
F - *Escherichia coli*
W - *Klebsiella pneumoniae*
S - *Citrobacter freundii*

Table 3 : Optimum Enzyme Activity of Bacterial Cultures under different culture conditions

Species	Initial pH	Nitrogen source	Culture Time required for maximum E.A.S. (h)	Wet weight g/10ml	Enzyme activity cells mU/G super	Enzyme activity supernatant mU/ml
F	5.0	Peptone	6	55	2.3	6.6
F	7.0	Peptone	6	46	2.3	10.2
F	8.0	Peptone	6	67	0.9	12.3
W	5.0	Peptone	28	127	0.2	6.8
W	7.0	Peptone	12	48	0.5	3.1
W	8.0	Peptone	120	315	0.3	5.8
S	5.0	Peptone	18	189	0.6	1.0
S	7.0	Peptone	12	161	0.7	13.2
S	8.0	Peptone	18	200	1.6	14.7
F	7.0	Ammonium Sulphate	6	64	1.4	1.2
W	7.0	Ammonium Sulphate	12	34	0.4	1.2
S	7.0	Ammonium Sulphate	12	116	1.5	2.5

Abbreviations: E.A.S. - Enzyme Activity of the Supernatant
F - *Escherichia coli*
W - *Klebsiella pneumoniae*
S - *Citrobacter freundii*

For each bacterial species under each culture condition the following parameters were estimated at 6h intervals.

(i) Optical density at 510nm (ii) Wet weight
(iii) pH of the supernatant (iv) Enzyme activity of the supernatant at pH 7.
(v) Enzyme activity of the cells at pH 7.

Culture medium was the same as used in the isolation of bacteria. Culture volume was 500ml (culture flasks 2L) and cultures were inoculated with 50ml of bacteria culture medium cultivated for 24 h. Cultures were incubated in an orbital shaker at a speed of 115 rpm at room temperature. 20ml samples were removed at 6 h intervals aseptically using a laminar flow. 3%ammonium sulphate was used instead of 3% peptone when ammonium sulphate was used as the nitrogen source.

Enzyme activity of the cells: Enzyme activity of the cells was determined by washing the pellet obtained from wet weight determination in 0.15M McIlvaine buffer (pH7). The pellet was suspended in final volume of 2ml using 0.15M McIlvaine buffer (pH7) and 0.1ml if this suspension was incubated with 0.4ml of 0.15M McIlvaine buffer (pH7) and 0.5ml 10 mM p-nitrphenyl-alpha-D-galactpyranoside solution for 30 minutes at 29°C. The reaction was terminated by adding 5ml of 0.1N Na_2CO_3 solution. Absorbance was measured at 405nm, using a Shimadzu UV 120-02 spectrophotometer.

C. freundii was also grown on a culture medium containing pH 8 phosphate buffer and the above parameters were estimated at 6h intervals.

RESULTS

Six bacterial species were isolated from soil samples after cultivation for an initial period of four days and cultivation in fresh culture medium for two days three times. The bacterial species were labelled as S,M,H,G,F and W. Detailed studies indicated that supernatant of species H and J have no enzyme activity (Table 1). They also do not utilize raffinose. Other four bacterial species have extracellular enzyme activity. Their raffinose utilization rate varied from 11-28mg/h (Table 1). In all cases, the enzymes showed high activity at neutral pH. The lowest wet weight and highest activity of 11mU/ml were observed in *Citrobacter freundii*.

The three species which showed high production of extracellular enzyme were identified as *E. coli, Citrobacter freundii and Klebsiella pneumoniae* (Table 2).

According to the results obtained from three bacterial species grown in the culture medium containing ammonium sulphate, *Citrobacter freundii* gave the highest enzyme production of 2.5 mU/ml after 12h of cultivation when compared

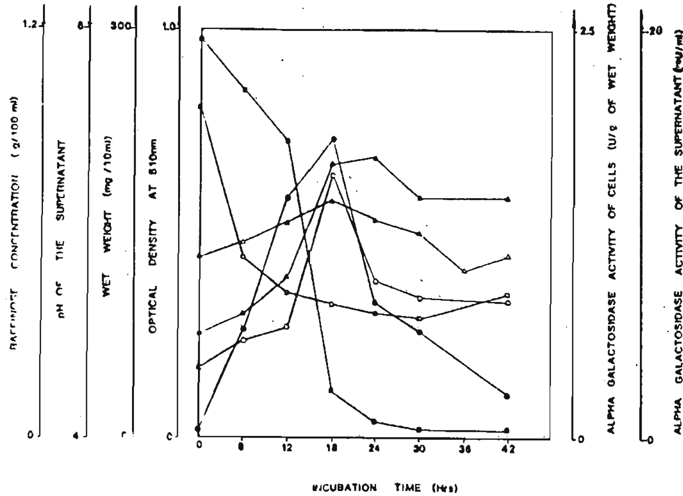

Figure 1: Alpha galactosidase production by *Citrobacter freundii* grown in peptone culture medium with an initial pH of 8.

Symbols : (▲) Wet weight (●) Optical density at 510 nm
(△) Raffinose concentration (○) pH of the supernatant
(■) Alpha galactosidase activity of the supernatant at pH 7
(□) Alpha galactosidase activity of the cells at pH 7

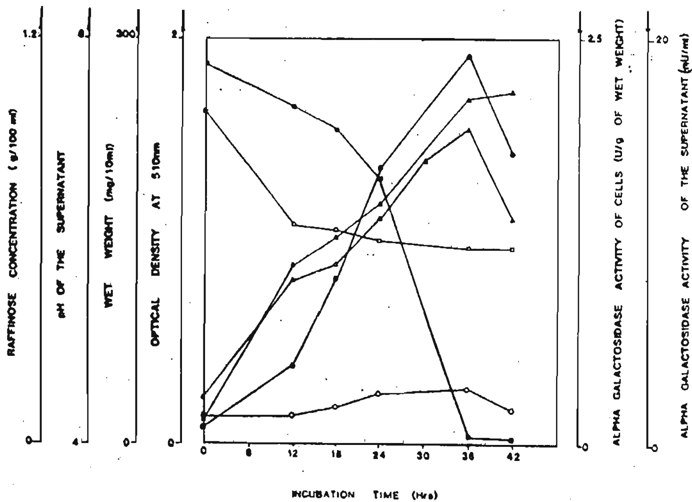

Figure 2: Alpha galactosidase production by *Citrobacter freundii* grown in pH 8 phosphate buffer culture medium containing peptone.

Symbols : (▲) Wet weight (●) Optical density at 510nm
(△) Raffinose concentration (○) pH of the supernatant
(■) Alphagalactosidase activity of the supernatant at pH 7
(□) Alpha galactosidase activity of the cells at pH 7

with highest activity of 1.2 mU/ml for *E.coli* and *Klebsiella pneumoniae* at 6h and 12h of cultivation respectively (Table 3). Inorganic nitrogen has led to poor cell growth and low alpha galactosidase production when grown in culture medium with peptone as the nitrogen source. With different initial pH, again higher enzyme production of 14.7 mU/ml was observed with *C. freundii* after 18h of cultivation at pH 8.0 (Figure 1). The enzyme production could be further increased up to 19mU/ml after 36h when grown in pH 8 phosphate buffer (Figure 2). As shown in the graph, the pH of the medium does not change significantly with time as observed under other culture conditions.

DISCUSSION

In this study, initial pH of the medium was adjusted to 7 because most bacteria grow well at neutral pH values and also earlier reports showed that bacterial alpha galactosidases are more stable at neutral pH values.[13] Raffinose was used as the inducing sugar in the medium. The results show that *C. freundii* grown in pH 8 phosphate buffer culture medium containing peptone as the nitrogen source is suitable for the production of extracellular alpha galactosidase. In future studies the alpha galactosidase from *C. freundii* will be purified and characterized to determine its suitability as an industrial enzyme for removal of raffinose sugars from food products.

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