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Original Research Paper

Improved Production of β-Galactosidase from the Mutated *Aspergillus* sp. on Deproteinized Cheese Whey

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ABSTRACT

Aspergillus sp. was isolated from the soil near the dairy region, Anand, Gujarat and screened for β-galactosidase production using minimal nutrient salt (MNS) medium with 0.004% 5-bromo-4-chloro-3-indolyl β-D-galactoside (x-Gal) with 2% lactose and deproteinized cheese whey agar. Using wild type strain, β-galactosidase production was carried out on deproteinized cheese whey medium with varying lactose concentrations. Maximum β-galactosidase was 49 specific activity (U mg^{-1} protein: 1 U is equivalent to 1nM o-nitrophenol produced min^{-1}) obtained after 72 hours of incubation at 1.5% lactose content using deproteinized cheese whey medium. To improve the production of β-galactosidase, two chemical mutagens *viz.,* ethyl methane sulfonate (EMS) and hypoxyl amine (HA) were used at different concentrations. The mutants were screened on the basis of development of blue colour on MNS agar containing x-Gal after 72 hrs incubation at 30°C whereas wild type strain showed the blue colour at 120 hrs incubation at 30°C. Both the mutants, *Aspergillus* sp. EMS and *Aspergillus* sp. HA were also grown on the deproteinized cheese whey medium with varying lactose concentrations for β-galactosidase production. *Aspergillus* sp. EMS and *Aspergillus* sp. HA showed 2 and 2.47 fold more β-galactosidase production compared to wild type strain after 72 hours of incubation at 1.5% lactose content, respectively.

INTRODUCTION

During the manufacturing of cheese and paneer a spent liquid waste is generated known as whey. Usually such whey is deproteinized to get casein protein or it is directly discharged into water bodies, which leads to increase in the BOD level and serves as major pollutant (Guimaraes et al. 1992). The carbohydrate reservoir of lactose in whey and presence of nutrients makes it a good natural medium for growth of microorganisms and potential substrate for β-D-galactosidase production. β-D-galactosidase E.C.3.2.1.23 (lactase, β-gal) catalyses the hydrolysis of lactose to glucose and galactose. It is useful for production of dairy products free of or with low lactose content suitable for use by lactose-intolerant people. Various populations suffer from β-gal deficiency (Bayless et al. 1971). Production of β-gal by GRAS (generally recognized as safe) microorganisms is very important. Among these *Bifidobacterium brevie, Bifidobacterium longum, Lactobacillus bulgaricus, Streptococcus thermophilus, Kluyveromyces marxianus, Kluyveromyces fragilis, Candida pseudotropicalis, Aspergillus niger, Penicillium chrysogenum* and *Alternaria alternata* are widely used for production of β-gal (Ibrahim & Sullivan 2000, Macris 1982, Bales & Castillo 1979, Saad 2004). Fungi excreting elevated quantities of extracellular β-gal so have been used for commercial production. This study has

been made to isolate a potent β-gal producing *Aspergillus* sp. and to evaluate the potential of exposure to two chemical mutagens for the isolation of β-gal over producing mutants of *Aspergillus* sp. on deproteinized cheese whey.

MATERIALS AND METHODS

Isolation of fungi: A fungus was isolated from soil and cheese whey effluent. Both the sources were procured from dairy region near Anand. Appropriate dilutions were prepared from both the sources and spread on to the Potato Dextrose Agar (PDA) medium. Pure cultures were maintained on PDA slants at 4°C for further use in screening.

Screening of β-D-galactosidase producing fungi: β-D-galactosidase producing fungi were screened by growing it onto following media:

- Minimal nutrient salt (MNS) with 0.004 % 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) and 2% lactose
- Deproteinized cheese whey agar.

Composition of Minimal Nutrient Salt (MNS):

Component	:	*g%*
1. $FeSO_4.7H_2O$	:	0.05
2. $MnSO_4.H_2O$	:	0.016
3. $ZnSO_4.H_2O$	:	0.014
4. $CoCl_2$	:	0.02
5. KH_2PO_4	:	20
6. $(NH4)_2SO_4$	:	14
7. Urea	:	3
8. $MgSO_4.7H_2O$	:	3

This 100X stock was diluted to 1 X and following ingredients were added in following 100 mL media:

1. Peptone	:	0.01 %
2. Lactose	:	2 %
3. Tween 80	:	0.01%
4. X Gal	:	0.004%

Identification of β-D-galactosidase producing fungi: Screened fungus was identified by mounting process using light microscopy. β-D-galactosidase producing fungi was identified from X-Gal plate. The deep blue-green colonies appeared due to release of dye, 5-bromo-4-chloroindigo and transparent zone surrounding the colonies on deproteinized cheese whey agar (Fig. 1).

Strain improvement using chemical mutagens: For improved production of β-gal, screened wild type *Aspergillus* sp. was mutated using two mutagens viz., ethyl methyl sulphonate (EMS) and hypoxylamine (HA).

Mutagenesis procedure: Mutagenesis using EMS: 1×10^5 spores per mL were suspended in sterile 0.05 M phosphate buffer pH 6 and 9 mL of spore suspension was distributed in seven different tubes. Now, in each tube, 1 mL of EMS was added to achieve final concentrations of 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mg per mL. All the tubes were incubated at 37°C for 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 240 minutes for mutagenesis. After each time interval, 1 mL sample was

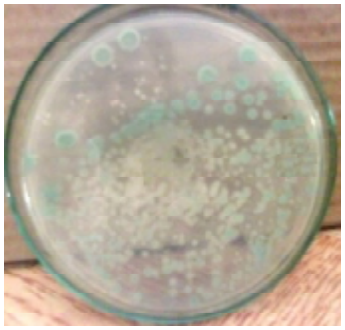

Fig. 1: *Aspergillus* sp. (wild type).

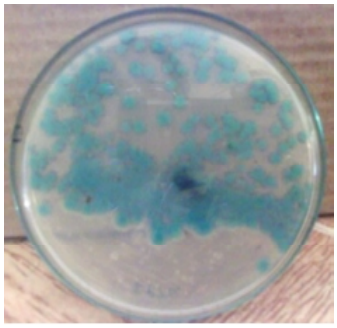

Fig. 2: *Aspergillus* sp. EMS.

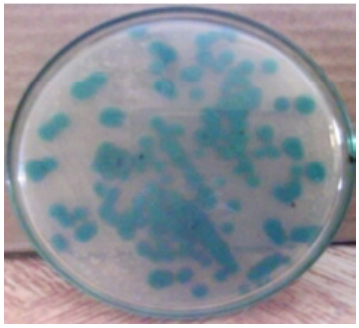

Fig. 3: *Aspergillus* sp. HA.

diluted to 100 mL with 0.05 M phosphate buffer pH 6 and from each tube 0.2 mL sample was plated on MNS plate with X-Gal. Untreated spores were also plated on the same media as a control. The plates were incubated for 120 hrs at 30°C.

Table 1: Chemical composition of cheese whey.

Component	Content
Water	95 %
Dry matter	5 %
Lactose	3.8 %
Lactic acid	Traces
Total proteins	1.0 %
Citric acid	0.1 %
Minerals	0.6 %
pH	5.5

Mutagenesis using HA: Same procedure as that used for EMS was followed but the concentration and time intervals were different i.e., 10, 20, 30, 40 mg per mL and 100 minutes, respectively.

Selection of mutants: Colonies that turned blue on X-gal agar were selected and stability of mutants was checked by repeated transfer up to several generations. Two stable mutants of *Aspergillus* sp. were selected and named as *Aspergillus* sp. EMS and *Aspergillus* sp. HA (Figs. 2 and 3).

Culture media and conditions: Deproteinized cheese whey was diluted to achieve lactose concentration @ 3%, 1%, 1.5%, 0.75% and each diluted 100 mL media (pH-5.5) were inoculated with 1 mL of 2×10^6 spores of each fungus. All the flasks were incubated at 30°C in an orbital shaker (150 rpm). After 24, 48 and 72 hours time interval, samples were withdrawn, centrifuged to remove biomass and supernatant were taken for different estimations.

Enzyme assays: The β-D-galactosidase activity was measured as per standard methods explained by Biswas (1985). One unit of enzyme activity was defined as nM of ONP (o-nitro phenol) liberated per mL of enzyme per minute.

Protein determination: Total soluble protein was estimated according to Lowery's method (Lowery et al. 1951) employing bovine serum albumin as a standard.

Reducing sugar estimation: Reducing sugar was estimated according to method of Cole (1949).

RESULTS AND DISCUSSION

β-D-galactosidase producing *Aspergillus* sp. were easily identified by observing blue colour surrounding the colonies (Fig. 1). This colour development was found due to breakdown of 5-bromo-4-chloro-3-indolyl β-D-galactoside into, 5-bromo-4-chloroindigo a blue colour complex. Table 1 shows the chemical constituent of the cheese whey. Usually cheese whey is acidic in nature and contains 3-5% of lactose content. Hence, it also serves as good inducers for the production of β-D-galactosidase enzyme. Earlier, it was reported that the fungal β-D-galactosidase from *Aspergillus* with acid pH optima (2.5-4.5) is especially suitable for hydrolysis of lactose in acid whey but it is more sensitive to product inhibition by galactose sugar (Budriene et al. 2005).

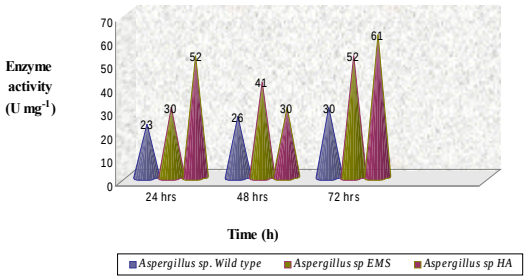

Fig. 1: Production of β-galactosidase on cheese whey at 3% lactose content.

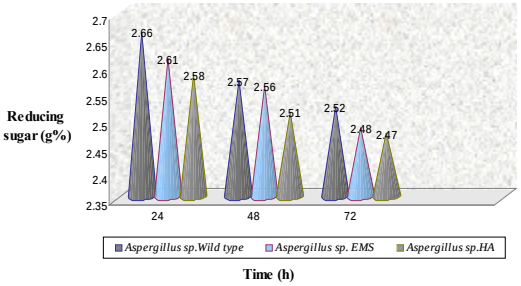

Fig. 2: Percentage decrease in reducing sugar @ 3% lactose content (g %).

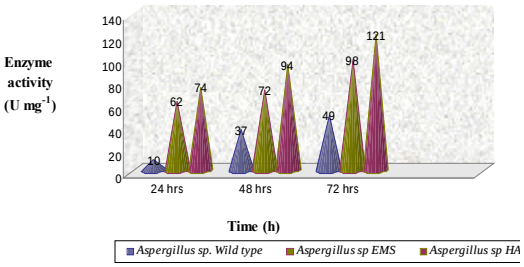

Fig. 3: Production of β-galactosidase on cheese whey at 1.5% lactose content.

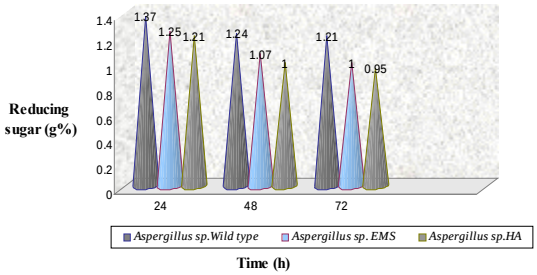

Fig. 4: Percentage decrease in reducing sugar @ 1.5% lactose content (g %).

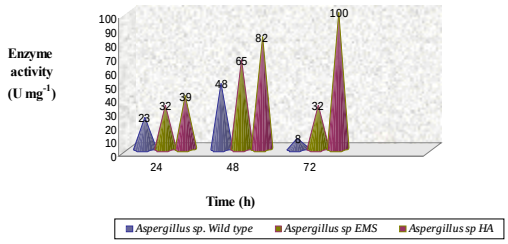

Fig. 5: Production of β-galactosidase on cheese whey at 1% lactose content.

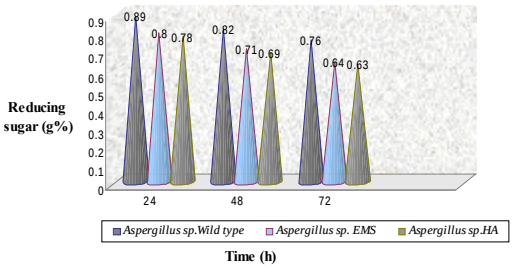

Fig. 6: Percentage decrease in reducing sugar @ 1% lactose content (g %).

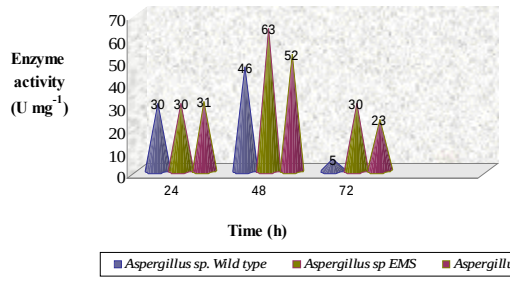

Fig. 7: Production of β-galactosidase on cheese whey at 0.75% lactose content.

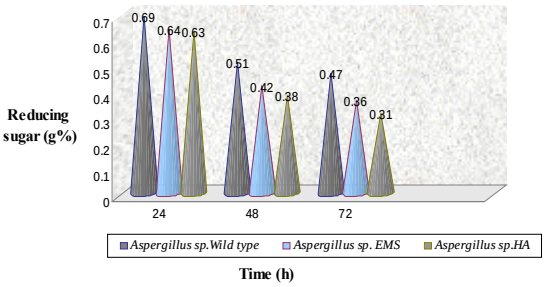

Fig. 8: Percentage decrease in reducing sugar @ 0.75% lactose content (g %).

Table 2: Production of β-galactosidase on cheese whey at 3% lactose content.

Time (h)	β-galactosidase Activity					
	Aspergillus sp.		*Aspergillus* sp. EMS		*Aspergillus* sp. HA	
	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}
24	30	23	50	30	91	52
48	12	26	20	41	27	30
72	9	30	19	52	22	61

Table 3: Percentage decrease in reducing sugar @ 3% lactose content (g %).

Time (h)	*Aspergillus* sp.	*Aspergillus* sp. EMS	*Aspergillus* sp. HA
24	2.66	2.61	2.58
48	2.57	2.56	2.51
72	2.52	2.48	2.47

Table 4: Production of β-galactosidase on cheese whey at 1.5% lactose content.

Time (h)	β- galactosidase Activity					
	Aspergillus sp.		*Aspergillus* sp. EMS		*Aspergillus* sp. HA	
	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}
24	24	10	30	62	35	74
48	30	37	39	72	46	94
72	19	49	20	98	31	121

Table 5: Percentage Decrease in reducing sugar @ 1.5% lactose content (g %).

Time (h)	*Aspergillus* sp.	*Aspergillus* sp. EMS	*Aspergillus* sp. HA
24	1.37	1.25	1.21
48	1.24	1.07	1.00
72	1.21	1.00	0.95

Table 6: Production of β- galactosidase on cheese whey at 1% lactose content.

Time (h)	β- galactosidase Activity					
	Aspergillus sp.		*Aspergillus* sp. EMS		*Aspergillus* sp. HA	
	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}
24	10	23	20	32	26	39
48	59	48	71	65	97	82
72	4	8	10	32	19	100

Table 7: Percentage decrease in reducing sugar @ 1% lactose content (g %).

Time (h)	*Aspergillus* sp.	*Aspergillus* sp. EMS	*Aspergillus* sp. HA
24	0.89	0.80	0. 78
48	0.82	0.71	0.69
72	0.76	0.64	0.63

Table 8: Production of β-galactosidase on cheese whey at 0.75% lactose content.

Time (h)	β- galactosidase Activity					
	Aspergillus sp.		*Aspergillus* sp. EMS		*Aspergillus* sp. HA	
	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}	U ml^{-1}	U mg^{-1}
24	9	30	18	30	23	31
48	39	46	51	63	71	52
72	2	5	9	30	7	23

Table 9: Percentage decrease in reducing sugar @ 0.75% lactose content (g %)

Time (h)	*Aspergillus* sp.	*Aspergillus* sp. EMS	*Aspergillus* sp. HA
24	0.69	0.64	0.63
48	0.51	0.42	0.38
72	0.47	0.36	0.31

In the present study an *Aspergillus* sp. that shows β-D-galactosidase enzyme production under acidic pH conditions was selected. Wild type fungus isolate produced very less amount of β-D-galactosidase enzyme production on whey media (Table 2 and Fig. 1). Mutation with chemical mutagens is the classical approach for the improvement of strains. By using two chemical mutagens viz., ethyl methyl sulphonate (EMS) and hypoxylamine (HA) isolated *Aspergillus* strain was subjected to mutation and best stable survivors and producers were screened, which showed higher β-galactosidase activity in less time compared to wild type strain (Fig. 2 and 3).

Lactose concentration higher or lower was also affecting the enzyme production. The similar finding was reported earlier (Macris 1982). Whey was diluted to achieve four different concentrations of lactose i.e., 3%, 1.5%, 1% and 0.75%. Using such a diluted whey, lactase production was checked at 24, 48 and 72 hours by two mutants and it was compared with lactase production by wild type strain (Tables 2, 4, 6, 8 and Figs. 4, 6, 8, 10). Data presented in Tables 3, 5, 7, 9 and Figs. 5, 7, 9, 11 reveal the remaining lactose content in production media.

At 0.75 % lactose containing whey media minimum enzyme activity i.e., 5 U per mg, 30 U per mg and 23 U per mg was observed by *Aspergillus* sp., *Aspergillus* sp. EMS and *Aspergillus* sp. HA at 72 h incubation, respectively. Maximum enzyme activity was found at 1.5% lactose containing whey media, i.e., 49 U per mg, 98 U per mg and 121 U per mg by *Aspergillus* sp., *Aspergillus* sp. EMS and *Aspergillus* sp. HA at 72 hrs incubation, respectively. Whereas, at higher concentration of lactose (3%) enzyme production was slightly low compared to 1.5% lactose containing media. Thus, 1.5% lactose concentration was served as good inducers for β-galactosidase production. These results are also in tune with Bales & Castillo (1979). Amongst these three species of fungi *Aspergillus* sp. HA was the superior producer and shows maximum production of 121 U per mg at 72 h incubation time.

CONCLUSION

Results of the experiment points towards following conclusions:

- Deproteinized cheese whey at 1.5% lactose content was best inducer for production of β-D-galactosidase.
- Wild type *Aspergillus* sp. was poor producers for β-D-galactosidase enzyme compared to mutants *Aspergillus* sp. EMS and *Aspergillus* sp. HA.

- *Aspergillus* sp. HA was the superior producer and showed maximum production of β-D-galactosidase enzyme, i. e., 121 U per mg at 72 h incubation time.
- Hypoxylamine serves as the best mutagen for the improvement of wild type *Aspergillus* sp. producing β-D-galactosidase.

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