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Original Research Article

ONE-FACTOR-AT-A-TIME (OFAT) OPTIMIZATION OF B-GALACTOSIDASE PRODUCTION FROM *ASPERGILLUS FLAVUS* GR. NFCCI-2728 IN SUBMERGED FERMENTATION.

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Abstract: Production of β-galactosidase enzyme under submerged fermentation was carried out using *Aspergillus flavus* gr. NFCCI-2728. Optimization of different cultural conditions viz., incubation time, nitrogen source and its concentration, carbon source and its concentration, pH and inoculum size were carried out using one factor at time approach (OFAT) in Czapek's-Lactose (CL) Medium for enhanced production of β-galactosidase enzyme. The highest yield of β-galactosidase was achieved 843.75 Umg^{-1} which was 4.75 times higher than unoptimized medium.

Keywords: *Aspergillus flavus*, OFAT, Submerged fermentation, Czapek's-Lactose broth.

Introduction: Submerged fermentations (SmF) for enzymes production are typically applied with a substrate, that is either dissolved or remains suspended in an aqueous medium. For majority of the massive scale enzyme production SmF technology is most popular because of higher observance and simple handling (Pandey *et al*., 2010). Optimization of fermentation conditions, significantly physical and chemical parameters, is vital within the development of fermentation processes because of their impact on the economy and usefulness of the method. The growth and enzyme production of the organism are powerfully influenced by medium composition therefore improvement of media elements and cultural parameters is that the primary task during a process (Pandey *et al*., 2003). To satisfy the growing demands within the industry it's necessary to enhance the performance of the system and therefore increase the yield while not increasing the value of production. Enzyme prices are often reduced by finding optimum conditions for their production (Ponce-Noyola and de la Tom, 2001). β-galactosidase belongs to the cluster of saccharides converting enzymes within the family of hydrolases. It's unremarkably referred to as lactase. They're widespread distributed in various biological systems, e.g. microorganisms, plants and animal tissues. Compared to animal and plant sources

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of catalyst, microorganisms turn out catalyst at higher yields, resulted in decrease value of β-galactosidase (Nagy *et al*., 2001; Kang *et al*., 2005; Lu *et al*., 2007; Raol *et al*., 2010; Raol *et al*., 2014). For the event of economical medium to extend microbial metabolites yields, needs choice of carbon, nitrogen, phosphorous, potassium, and chemical element sources. Such nutritional demands are often manipulated by the traditional or statistical strategies. Standard technique involves ever-changing one experimental variable at a time whereas keeping the others at fixed level (OFAT). This approach offers a plus point regarding identification of great method parameters on enzyme production (Prajapati *et al*., 2013).

Present work is targeted on production of β-galactosidase enzyme victimisation Czapek's-Lactose broth beneath submerged fermentation exploitation fungus isolate *Aspergillus flavus* and improvement of method parameters exploitation one factor-at-a-time approach. The aim of improvement is to work out appropriate conditions for the economically and/or technologically necessary method variables like product concentration, yield, property and raw material price for increased enzyme production.

Materials and Method:

Chemicals and Media:- The substrate o-nitrophenyl-β-D-galactopyrranoside (ONPG) and all other chemicals were of analytical grade and procured from HiMedia and SRL, Mumbai. Wheat Bran was obtained from Lisa Roller Mill Pvt. Ltd., Gandhinagar and CSL from Maize Products, Ahmedabad.

Isolation, Identification and Screening of Microbial strain:- Isolate fungus strain *A. flavus* gr. NFCCI-2728 was kindly procured from Department of Microbiology, Shri A. N. Patel P. G. Institute of Science & Research, Anand and was identified and deposited to National Fungal Culture Collection of India, NFCCI, Pune. Isolate was screened for β-galactosidase enzyme using following screening medium.

1) 0.004% 5-bromo -4- chloro-3-indolyl β-galactopyranoside (X-Gal) with 2% lactose

Composition of Screening Medium (g/L):-

Urea	: 1.4
$CaCl_2$	: 0.3
$MgSO_4$	: 0.3
Protease Peptone	: 1.0
Lactose	: 20
$FeSO_4.7H_2O$	: 0.005
$MnSO_4.7H_2O$	: 0.0016
$ZnSO_4.7H_2O$	: 0.0014
$CoCl_2$	: 0.002
Agar	: 30
pH	: 5.0

Above medium was sterilized at 15 psi for 15 min. After sterilization 0.004 % of X-gal was added in to medium and plates were prepared by pouring method.

2) PNPG medium:-

Composition of media:-

PNPG	: 5mM
Aspartate	: 0.015 %
Bile Salt	: 0.45 %
Agar	: 3 %

Above medium sterilized at 15 psi for 15 min. and plates were prepared by pouring method.

Maintenance of the screened isolate:- *A. flavus* gr. NFCCI-2728 was maintained on potato dextrose agar slants and plates and was stored at 4^oC in a refrigerator. After every month culture was transferred on new slants. Before preparing the inoculum the purity of culture was checked under microscope.

Production of β-galactosidase enzyme:-

***Inoculum preparation*:-** For inoculum preparation PDA medium was used. From slant pure culture of *A. flavus* gr. NFCCI-2728 transferred on to sterile PDA medium and incubated at 28^oC for several days to obtain enough spores. After incubation spores were harvested by adding sterile distilled water containing 0.01% triton-X-100 and counted in to Neubauer's chamber.

Calculation of spore counting:-

1 square dimension of Neubauer's is:-

- Length – 0.05 mm
- Width – 0.05 mm
- Depth – 0.1 mm
- Volume of center square = 0.1 cu mm

So, Volume acquired by 5 cubes = 0.1 x 5 /25
0.2 cu mm volume = X (No. of spores)
100 cu mm volumes = 1000X/0.02
=X/ 20 x 10 spores / mL

***Culture media and condition*:-** Two liquid culture media were evaluated for production of β-Galactosidase activity.

1) Yeast Lactose (YL) Medium (g/L)

Lactose	: 20
Yeast Extract	: 10
pH	: 5

2) Czapek's -Lactose (CL) Medium (g/L)

Lactose	: 20
$NaNO_3$	: 3
K_2HPO_4	: 1
NaCl	: 0.5
$MgSO_4.7H_2O$	: 0.5
$FeSO_4.5H_2O$	: 0.01
pH	: 5

Above medium sterilized at 15 psi for 15 min

***Inoculation and Incubation of sets*:-**

Submerged fermentation:- In order to achieve the best β-galactosidase production,100mL of each production medium in 250mL Erlenmeyer flasks were inoculated with $1x10^5$ spores per mL. The operational conditions were 28°C and under shaking condition at 150 rpm on orbital shaker. After 24, 48, 72, 96, 120, 144 and 168 hour of time interval appropriate sample was withdrawn, centrifuged at 8000 rpm for 10 min under cooling condition to remove biomass and supernant was taken to analyze enzyme assay and protein content.

β-Galactosidase assay:- The β-galactosidase activity was measured spectrophotometrically by using o-nitrophenyl-β-D-galactopyranoside (ONPG) (Miller, 1972). A 20µL of supernant after centrifugation was taken and final volume was make up to 1 mL with 75 mM Citrate-Phosphate buffer (pH-5.0) and mixed with 1 mL of 2 mM ONPG prepared in Citrate-Phosphate buffer (14.41 g/L of citric acid, 13.35 g/L Na_2HPO_4, pH-5.0), and incubated for 5 min at 50°C. The reaction was stopped by adding 3 mL of 0.1 N NaOH. Liberated o-nitro phenol (ONP) was measured spectrophotometrically at 420 nm using UV-Visible SL164 ELICO, India spectrophotometer. One unit of enzyme activity (IU) was defined as the quantity of enzyme necessary to liberate 1 µM of o-nitro phenol per min per mg of protein under stated conditions.

Protein Content:- The concentration of protein was measured by the method of Lowry using BSA (bovine serum albumin) as a standard (Lowry *et al*., 1951).

Optimization of cultural condition:- Optimization of various parameters and development of media are the most important criteria for the overproduction of the enzyme. Several parameters were studied for optimization of β-galactosidase production medium by using CL medium as follows.

***Effect of incubation time on β-galactosidase production*:-** 1 mL of $1x10^5$ spores/mL of inoculum was inoculated in to 100mL Sterile CL medium in 250mL conical flask and was incubated at 28°C under shaking condition at 150 rpm. After each 24 h, sample was withdrawn up to 168 h and maximum β-Galactosidase activity was observed as mentioned above.

***Effect of Nitrogen source on β-galactosidase production*:-** For determination of the best nitrogen source, sodium nitrate from CL medium was replaced with Urea, Corn Steep Liquor (CSL), Soya bean flour and $(NH_4)_2HPO_4$, respectively. After each 24h of incubation sample was withdrawn to check enzyme activity and protein content as per the standard methods.

***Effect of Carbon source on β-galactosidase Production*:-** To determine the best carbon source, Lactose from CL medium was replaced with 1% of Galactose, Sucrose, Maltose, Cellobiose and Wheat Bran (WB), respectively in to Erlenmeyer flasks. Samples were withdrawn at time interval of 24h and β-galactosidase enzyme production and total protein concentration were determined.

***Effect of pH on production of β-galactosidase*:-** To determine optimum pH for β-galactosidase production by *A. flavus* gr. NFCCI-2728 Culture was inoculated in 100 mL Sterile CL medium at different pH 4.0, 4.5, 5.0,

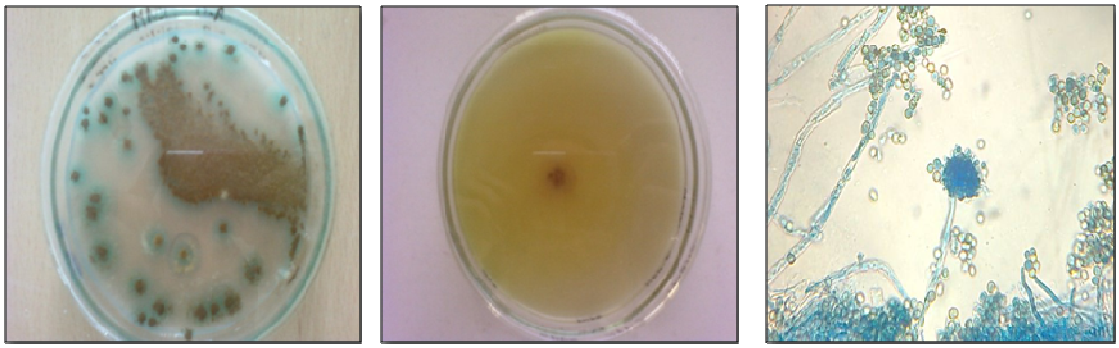
5.5 & 6.0. pH of medium was adjusted with 1N HCl and 1N NaOH. β-galactosidase production and total protein content were determined at time interval of 24h by standard methods, respectively.

***Effect of inoculum size on production of β-galactosidase*:-** Effect of inoculum size on β-galactosidase production was determined by inoculating spore suspension of $1x10^4$, $1x10^5$, $1x10^6$, $1x10^7$ spores per mL in to 100mL sterile optimized media. β-galactosidase production and total protein content were determined at time interval of 24h by standard methods, respectively.

Statistical analysis: All experiments were performed in triplicate and the results reported are the mean of the three values in which 2-6% variability was observed.

Results and Discussion

Screening and identification of β-galactosidase producing Fungi:- Isolation of fungus was carried out on Sabraud's Dextrose Agar medium. Based on the colonial and microscopic morphological characteristics isolate was identified. Identified isolate was deposited to NFCCI and named as *A. flavus* gr. NFCCI-2728, which was further screened for β-galactosidase enzyme production. Various authors reported the screening methods for β-galactosidase production (Khan *et al*., 2008 and Gheytanchi *et al*., 2010). Here, we selected a synthetic substrate *viz.* PNPG and X-Gal to screen β-galactosidase producing fungus. As shown in Figure 1 (A), fungus grown on X-Gal plate secrete extra cellular β-galactosidase enzyme that utilize X-Gal and converts it in to 5-Bromo-4-chloroindigo, which is a deep blue colored compound that appears surrounding the colony. Similarly, another synthetic substrate PNPG was also metabolized by isolate. β-galactosidase act on β-galactopyrranoside of PNPG and released þ-nitrophenol, which appears yellow in color [Figure 1(B)]. Both screening methods are easy to perform and give reproducible results to screen β-galactosidase producing microorganisms. Figure 1 (C) shows microscopic view of screened β-galactosidase producing fungus *A. flavus* gr. NFCCI-2728. The screened colonies were used for further work.



(A) **(B)** **(C)**

Figure 1 Screening of *A. flavus* gr. NFCCI-2728 for β-galactosidase production on (A) X-Gal medium and (B) PNPG medium. (C) Microscopic examination of screened fungus using lactophenol blue mounting method.

Production of β-galactosidase:- β-galactosidase was produced from various agro industrial and dairy wastes. Many researchers reported different agro wastes e. g. wheat bran, rice straw, soya bean meal, corn steep liquor, wheat steep liquor for production of β-galactosidase (Joker *et al*., 2008; Miramadi *et al*., 1997; Jadwiga and Wtodzimierz, 2003; Raol *et al*., 2010; Raol *et al*., 2014). Production of β-galactosidase on to undefined YL medium and defined CL medium was performed and results are presented in Figure 2. Maximum β-galactosidase activity of 177.78 Umg^{-1} was obtained in CL medium at 72h of incubation, whereas under the same condition and time interval YL medium shows only 67.65 Umg^{-1} β-

galactosidase activity using *A. flavus* gr. NFCCI-2728. Our result proves that the chemically defined trace element in the medium favors the β-galactosidase production. Similar results were also reported by Gindy *et al*., (2009) using *Chaetomium thermophile* and *Thermomyces lanuginosus.* Therefore, CL medium was selected for further optimization.

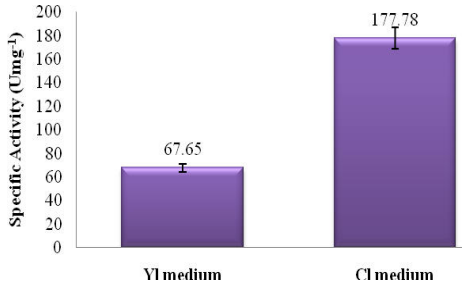


Figure 2 Effect of YL and CL medium on β-galactosidase production.

Effect of Incubation period on β-galactosidase production:- The optimum time interval for β-galactosidase production was determined under submerged fermentation condition. Accordingly, samples were withdrawn at different time intervals, it was determined that the optimum incubation time for maximum β-galactosidase production was 177.78 Umg^{-1} at 72 h (Figure 3). As incubation time increases more than 72h to 120h enzyme activity decreases and beyond this period, the enzyme yield was not increased. This may be because culture was reached to its stationary phase as a consequence of metabolism. Keeping above results in view, the incubation time of 72 h was considered for maximum production of β-galactosidase.

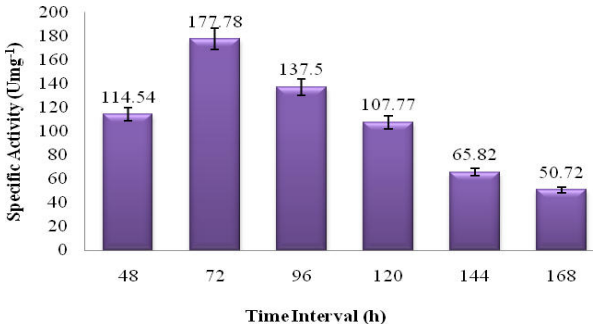


Figure 3 Effect of incubation Time (h) on β-Galactosidase production.

Effect of Nitrogen source on β-galactosidase production:- Nitrogen sources play an important role in the production enzyme. Inorganic nitrogen sources can be quickly used, while organic nitrogen sources can supply many cell growth factors and amino acids, which are needed for cell growth, metabolism and enzyme synthesis. In present experiment we compared the effect of organic and inorganic nitrogen sources on production of β-galactosidase enzyme. The influence of various nitrogen sources including $NaNO_3$, corn steep liquor (CSL), di-ammonium hydrogen phosphate, soya bean flour and urea on production of β-galactosidase by *A. flavus* gr. NFCCI-2728 results are shown in Figure 4. Corn steep liquor, one of the by-products of starch industry and least expensive nitrogen source shows highest enzyme activity of 335.59 Umg^{-1}, followed by soya bean flour, $NaNO_3$ and urea. Enzyme activity reduced in medium containing di-ammonium hydrogen phosphate and urea. Awan *et al*., (2010) reported highest enzyme activity of 168 IU/l/h with CSL with solid state fermentation and Miramadi *et al*., (1997) reported CSL as good source of β-galactosidase with enzyme activity of 1.54 IU/h/l.

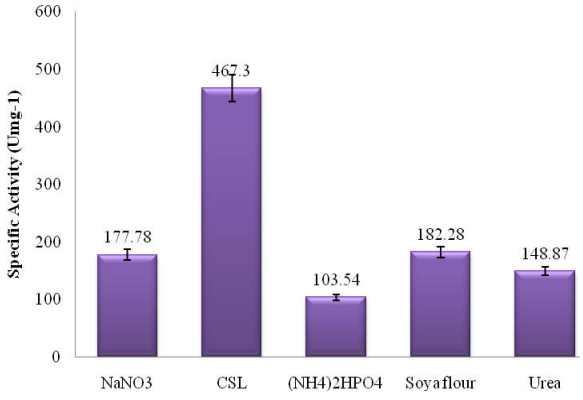


Figure 4 Effect of different nitrogen sources on β-galactosidase production.

Optimization of CSL concentration for maximum β-galactosidase production:- After optimization of nitrogen source, which was CSL, effect of different concentration of CSL on β-galactosidase production was studied. It was found that different concentration of CSL

also affects enzyme activity. As shown in figure 5, as CSL concentration increases up to 0.3% β-galactosidase activity was increased up to 335.39 Umg^{-1} but by further increasing CSL concentration enzyme activity was gradually decreased. Corn seep liquor contains large amounts of readily fermentable carbohydrates such as glucose which may decrease enzyme production by catabolic repression (Miramadi *et al*., 1997). Hence, 0.3% CSL was used for further experiments as a nitrogen source. Supplementation of CSL in production medium increase β-galactosidase production was also reported by Miramadi *et al*., 1997; Nizamuddin *et al*., 2008 and Awan *et al*., 2010.

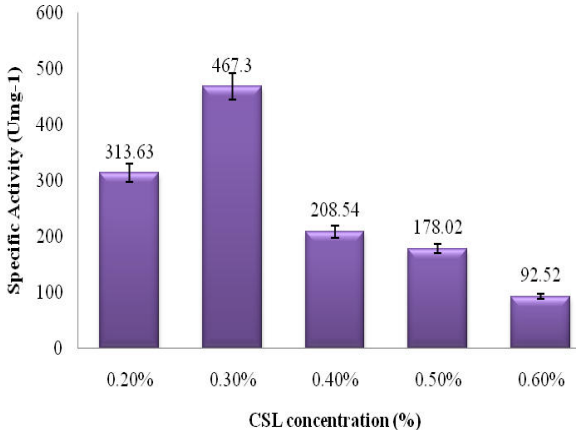


Figure 5 Effect of different CSL concentration on β-Galactosidase production.

***Effect of carbon source on β-galactosidase production*:-** The cost of substrate has a significant role in the economics of enzyme production. The utilization of agro industrial wastes as potential substrates for the production of different enzymes has attracted much attention because it reduces the cost of enzyme production and also reduces environmental pollution. Nature and amount of carbon source in culture medium is important for growth and production of extra cellular β-galactosidase in fungi. Carbon source regulates biosynthesis of β-galactosidase in various microorganisms (Nagy *et al*., 2001; Akolkar *et al*., 2005; Hue *et al*., 2005; Konsoula and Liakopoulou-Kyriakides, 2007). All indicated that the role of carbon source in the biosynthesis of β-galactosidase may vary and depend on the microorganisms tested. Concentration of carbon source in the medium may affect the expression of β-galactosidase by microorganisms. In this work, effect of different carbon sources *viz.* cellobiose, galactose, maltose, sucrose, lactose and wheat bran on β-galactosidase production was studied with optimized nitrogen source, CSL.

Maximum yield was detected in case of wheat bran followed by lactose, galactose and maltose (Figure 6). Miramadi *et al*., (1997) reported maximum enzyme activity of 1.54 IU/h/l in medium containing WB & CSL by *Aspergillus oryzae*. In this work, maximum activity was reported of 578.57 Umg^{-1} in medium containing WB & CSL while Nurullah, (2011) reported maximum enzyme activity of 1713 Umg^{-1} using wheat bran from *Bacillus licheniformis* ATCC 12759. The presence of oligosaccharides from wheat bran in the culture medium seems to have more influence on the production of enzyme (Raol *et al*., 2014).

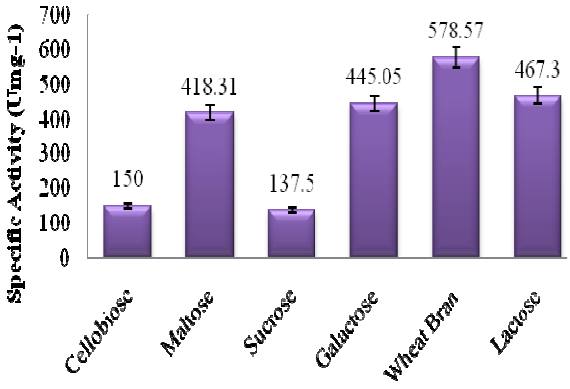


Figure 6 Effect of different carbon sources on β-Galactosidase production.

***Optimization of wheat bran concentration on β-galactosidase production*:-** Wheat bran was found good source of β-galactosidase production when supplemented with corn steep liquor. It contains large amounts of hemicelluloses, which influence production of enzyme β-galactosidase. To determine optimum concentration of wheat bran for β-galactosidase yield, different concentration of WB *viz*. 1%,

2%, 3%, 4% and 5% was added in to production medium. At 2% concentration maximum β-galactosidase production (720 Umg^{-1}) was obtained but as concentration increases production of β-galactosidase was decreases (Figure 7). WB contains galactan based hemicelluloses (30%) which is susceptible to hydrolysis by β-galactosidase and releases various fermentable sugars (Miramadi *et al*., 1997). So, as concentration increases up to 2% production was higher but at higher WB concentration released products may serves as repressor for activity of β-galactosidase enzyme. Our results are also in tune with Nizamuddin *et al*., (2008), Nurullah *et al*., (2011) and Raol *et al*., (2014).

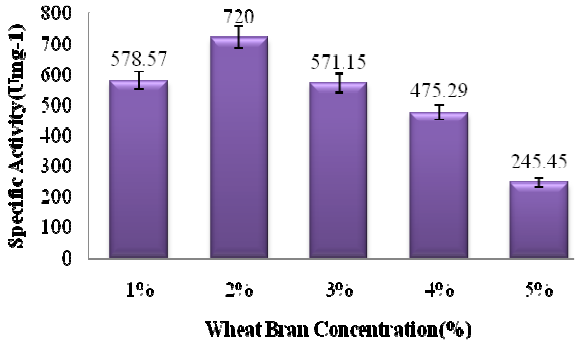


Figure 7 Effect of different WB concentration on β-galactosidase production.

***Effect of pH on β-galactosidase production*:-** The proton concentration of surroundings has the utmost influence on the microbial growth and catalyst production. The foremost favorable hydrogen ion concentration value is that the point wherever the enzyme production is highest. Because the pH will increase or decreases from that time, the production of the enzyme decreases. Till the hydrogen ion concentration gets therefore extreme that the enzyme begins to degrade and loses all function. Totally different pH (pH-4.0, 4.5, 5.0, 5.5 and 6.0) values of the medium were adjusted and were accustomed study the impact of it on enzyme production. Enzyme activity was higher at acidic pH and reduced below the alkaline condition. Maximum enzyme activity was found at pH-4.0 (746.81 Umg^{-1}) (Figure 8). numerous authors (Nizamuddin *et al*., 2008; Panesar, 2008; Pavani *et al*., 2011) reported pH 5.0 as optimum pH for production of β-galactosidase however our results with pH 4.0 indicates that this isolate is appropriate to provide enzyme underneath acidic condition.

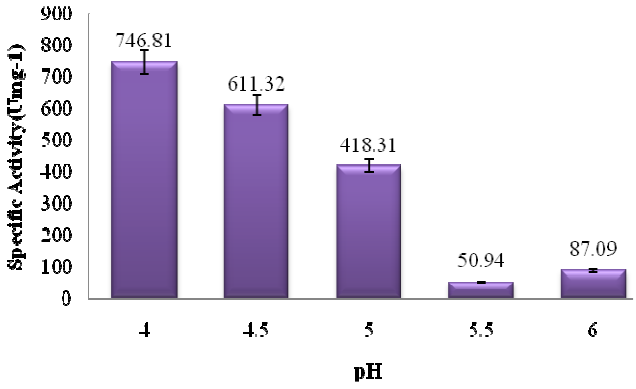


Figure 8 Effect of different pH on β-galactosidase production.

***Effect of inoculum size on β-galactosidase production*:-** Different spore inoculum concentrations were added into the medium, to study the influence of inoculum size on the β-galactosidase activity. The enzyme activity increased with the increase in inoculum size up to $1x10^6$ spores/mL thereafter, no improvement in enzyme activity was observed (Figure 9). The maximum enzyme activity of 843.75Umg^{-1} was observed with $1x10^6$ spores/mL inoculum. The low enzyme activity at $1x10^4$ spores/mL inoculum level may be attributed to the low density of starter culture. At $1x10^7$ spores/mL enzyme production was decreased may be due to higher biomass concentration compared to low nutrients available. Therefore, an inoculum of $1x10^6$ spores/mL can be considered optimal for achieving maximum enzyme activity.

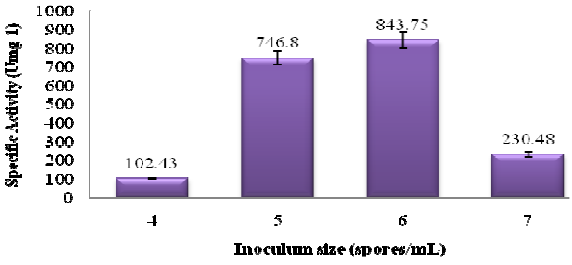


Figure 9 Effect of different inoculum size on β-Galactosidase production.

Comparison between optimized and unoptimized medium:-

After optimizing various process parameters for β-galactosidase production it was found that enzyme activity was about 4.7 times raised than the enzyme activity of unoptimized CL medium (Figure 10).

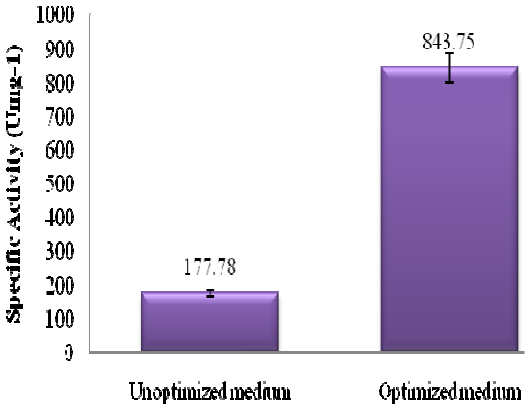


Figure 10 Comparison between optimized and unoptimized medium.

Conclusion: Present study shows improvement of β-galactosidase production using *A. flavus* gr. NFCCI-2728. Enzyme yield and production was found to be influenced by crude carbon and nitrogen source like wheat bran, corn steep liquor and not by pure form of carbohydrate and other organic and inorganic nitrogen source. Along with that acidic condition and proper inoculum size favors β-galactosidase production under submerged fermentation using *Aspergillus* sp. The data obtained after optimization has resulted in 843.75 U/mg compared to unoptimized medium (177.78 U/m) for enzyme production.

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