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Media Optimization for the Production of B-Galactosidase by a Novel Actinomycetal Isolate Via OVAT Methodology

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Abstract

In this study β -Galactosidase producing Actinomycetes were isolated from dairy soil and screened by enzyme assay. The isolate with highest enzyme activity was selected for further growth optimization studies . Process variables namely incubation time, incubation temperature , pH of the medium, Carbon and Nitrogen sources, metal ions, age and volume of inoculum , cell disruption methods were optimized by OVAT (One Variable At a Time) approach. The isolate showed highest enzyme activity at pH 8 after 72 hours of incubation at 41°C. Optimum age and volume of the inoculum was found to be 72 hr old and 1%(v/v) respectively. Beta-Galactosidase production was highest with Whey and Casein as Carbon and Nitrogen sources respectively. Mg^{++} at 5mM was found to be enhancing the enzyme production. β -Galactosidase yield was highest when cells were permeabilized with SDS-chloroform followed by Lysozyme. By employing 16s rDNA sequencing and phylogenetic analysis the isolate was identified as ***Streptomyces thermocarboxydus strain NBRC 16323***.Study was validated by comparing β -galactosidase production of basal (unoptimized) medium to that of OVAT optimized media and it showed 2x fold increase.

Keywords: Isolation and Screening, Actinomycetes, β -Galactosidase assay , OVAT approach, Process variables, Validation

Introduction

β-galactosidase (EC3.2.1.23) is the enzyme that catalyzes the hydrolysis of β-1,4-D-galactosidic linkage in lactose releasing D-glucose and D-galactose as end products. The enzyme mainly is used in Food , Dairy and Pharma industries to manufacture syrup sweeteners, lactose-hydrolyzed products for lactose-intolerant people [1, 2]. The β-galactosidase is used to synthesize galacto-oligosaccharides (GOS) by transferring galactosyl residues of lactose to saccharide acceptors [3, 4]. GOSs are the vital prebiotics to improve gut health by enhancing probiotic intestinal bacterial population [5, 6]. The enzyme also offers a promising potential biosensor application with various chromogenic or fluorogenic substrates yielding a colored product, thus making the amperometric detection more easy. Such successful attempts were made to evaluate glucose and lactose in food products and also urine lactose in the diagnosis of mastitis in cows [7, 8].

Dairy whey which comprises 85% of the milk used for manufacturing cheese and paneer (a dairy product in India that tastes similar to fresh mozzarella in unsalted form) is usually disposed into surrounding land environment affecting the physical and chemical texture of soil and gradually decreasing yield of crop. When released as industrial affluent in commercial scale into water bodies, whey reduces dissolved oxygen of water thus posing a threat to aquatic life [9, 10]. Whey mainly constitutes 55% of milk nutrients namely lactose, soluble proteins and lipids with considerable proportion of minerals as well. Hence discarding whey is not only a significant loss of potential bionutrients but also serious pollution to environment and soil. Though industrial wastewater treatment methods and policies offer safer disposal of whey with specified parameters they turn expensive and increase cost of production in course of time. To achieve sustainability in economical way ,Whey can be used as a potent raw material for the growth of β -galactosidase producing microbes and also can be subjected to further downstream processes by which value added products such as lactose hydrolyzed whey, GOS can be produced thus bringing down cost of disposal in whole or partially.

Microbial production of the enzymes offers merits like ease of fermentation, higher productivity and cost reduction using agro residues and affluents. Although *Escherichia coli* is the predominant source of β -galactosidase, crude isolates of enzyme from these coliforms may not be preferred in food processes owing to possible unsafe factors associated with them [11]. Hence there is a need to isolate the enzyme from microbes with GRAS status (generally recognized as safe).

Actinomycetes is one of the largest and diverse microbial groups in both terrestrial and marine and environments. Exploring Actinomycetes as a source of bioproducts started early in 19th century . Many active metabolites against viruses, bacteria, fungi, nematodes as well insects have been isolated and reported in numerous studies all over the world. Some members are potential sources of industrially important enzymes such as amylases, cellulases, proteases and chitinases [12, 13]. But this group of microbes has not been explored so far for β-galactosidase production and utilization. Hence the present study was aimed to isolate β-galactosidase producing Actinomycetes and optimize the growth conditions by OVAT (one variable at a time) method for highest enzyme production and identification of that isolate by phylogenetic tree analysis.

Materials And Methods

Sampling Dairy soil samples were collected from local cattle sheds in Aurangabad and **s**erial dilutions of the samples were plated on Starch Casein Agar (SCA) and incubated for 48-72 hrs at 30°C.

Primary Screening

Well isolated colonies from each medium were re-inoculated onto SCA supplemented with 0.1% X-Gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside) and incubated for 48-72hrs at 30°C. Blue colonies on X-Gal plates indicated positive for β-galactosidase activity.

Secondary screening by Enzyme Assay

Positive isolates from primary screening were tested for β-galactosidase assay by Miller method [25]. Cell pellets of the selected isolates grown in Starch Casein Broth were harvested by taking small aliquots (2ml) of each culture and centrifuging at 8000rpm for 10mins at 4°C. Supernatant was discarded and the cell pellet was resuspended in the same volume of chilled Z buffer. Cells were

permeabilized by adding 100 µl chloroform and 50 µl 0.1% SDS (sodium dodecyl sulfate). 0.2 mL of substrate ONPG, *o*-nitrophenyl-β-D-galactoside was added to the crude enzyme extract and incubated at 37°C for 30-45mins till yellow color developed. The reaction was stopped by adding 0.5 mL of 1MNa_2CO_3 and centrifuged for 5 mins at 10000rpm to remove cell debris and chloroform. OD of the ONP(o-nitro phenol)released from ONPG was recorded at 420 nm .

Enzyme activity was calculated from the formula below [14].

EA = $\frac{OD_{420} \text{ x RV}}{0.0045 \text{ x EV x T}}$ where

EA -- Enzyme Activity , expressed in units of µmoles /min/ml of lysate

OD_{420} -- Optical density of sample at 420nm

RV -- Reaction volume (total of lysate, buffer, substrate, stop solution) in ml

0.0045 -- Optical density of 1µmole/ml solution of ONP at pH 7

EV --Extract (lysate)volume in ml

T -- Reaction time in mins

The isolate showing highest β-galactosidase activity selected for further optimization studies and identification of the species.

Identification Of β-Galactosidase Producing Isolates

Gram's Staining &16S rDNA Sequencing Gram's staining was performed on the isolates that showed highest enzyme activity. Selected isolate was identified at genomic level by gene Ombio Technologies Pvt Ltd , Pune, by 16S rDNA sequencing by PCR amplification with forward and reverse primers .

Growth Studies

Optimization of process parameters by OVAT (one variable at a time) approach

Role of incubation time, incubation temperature, pH of the medium , C & N sources , Metal ions, Age & size of inoculum, Cell Disruption methods were studied to evaluate their effect on β-galactosidase production by the Actinomycetes isolate.

Incubation temperature Flasks with 30 ml of culture medium Starch Casein Broth (SCB) supplemented with 1% Lactose were inoculated with loopful of culture and incubated at temperatures <10ºC, RT

(Room Temperature), 30ºC, 37ºC,and 41ºC for 2days. After incubation, β-galactosidase was assayed by Miller method above.

Incubation time Flasks containing 30 ml of SCB with 1% Lactose were inoculated and incubated for various time intervals 24, 48, 72, 96 and 120hrs at the optimized temperature .After incubation, β-galactosidase was assayed.

pH SCB was prepared at different pH levels such as 6, 6.5, 7,7.5, and 8 by NaOH and HCl separately, inoculated and incubated at optimized temperature and time .Activity of β-galactosidase was determined at the end of incubation period.

Carbon sources Different carbon sources namely Glucose, Lactose, Acid Whey, Starch and Galactose were added to SCB at 1% concentration in each of the flask, inoculated and incubated at other optimized parameters . Enzyme activity was assayed.

Nitrogen sources Nitrogen was added to SCB in the form of Peptone, Casein, Egg albumin, Sodium Nitrate and Urea at a concentration of 1% in each flask. All the flasks were inoculated, incubated at previuosly optimized parameters and enzyme was assayed after incubation period .

Metal ions Metal ions Mg^{++}, Zn^{++}, Cu^{++}, Mn^{++}, Fe^{++}, K^{+} were added as their respective salts separately to each of the flasks containing optimized SCB at a concentration of 5mM. All the flasks were inoculated with the isolate , incubated and assayed after the incubation period.

Age of Inoculum of the Isolate 1-10day old inoculum is prepared, inoculated into the optimized medium each day and incubated. Enzyme activity was assayed after the incubation period of each inoculum addition.

Volume of Inoculum of Isolate An inoculum volume of 0.01% - 1.33% of the isolate was added to each of the flasks and incubated. The enzyme was assayed after incubation period .

Effect of different Cell Disruption Methods Chemical cell permeabilization was brought about by adding SDS (200 µl/ml) to organic solvents Chloroform,Acetone,Toulene (400µl/ml each in separate disruption mixtures).Physical cell lysis was brought about by UltraSonication(15sec on/off cycle for 5mins) and Enzymatic cell lysis was done with Lysozyme (in TE buffer at 150 µl/ml of lysate, incubated for 30 mins).The supernatants obtained after each of the permeabilization treatments were assayed for enzyme activity

Results and Discussion

In this study well defined individual colonies from 10^{-5} and 10^{-6} serial dilutions from dairy soil sources on Starch Casein Agar plates were primarily screened using X-gal supplemented media and the 8 isolates that showed blue colonies were selected for secondary screening by β-galactosidase assay as shown in Fig-1. Enzyme assay of the 8 isolates with ONPG as substrate resulted in one highest enzyme producing isolate Acti-3 as seen in Fig-2. A Similar study was reported by Favier etal.,(1996) using an enriched Columbia agar medium added with X-gal to isolate blue colonies that indicated the presence of β-galactosidase activity [15].

Figure-1 X-gal Plates

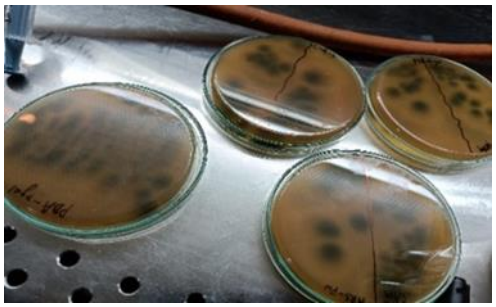
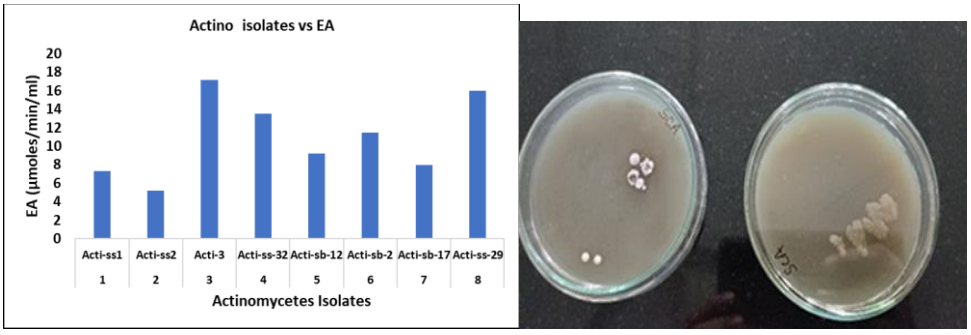
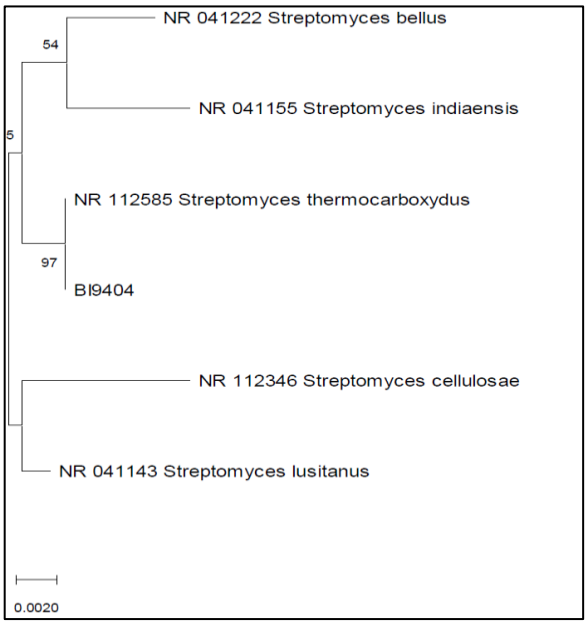


Figure-2 β-galactosidase assay of Actinomycetes isolates and selected isolate



Identification of the isolate The isolate Acti-3 was found to be Gram positive and further identified by rDNA sequencing. PCR amplification was done from the genomic DNA by using forward and reverse primers and the translated nucleotide sequence was analyzed for similarities by BLASTN tool and identified as ***Streptomyces thermocarboxydus strain NBRC 16323*** (courtesy-Gene Ombio Technologies Pvt ltd, Pune) as shown in Fig-3.

Figure-3 Phylogenetic relationship with first ten BLAST results from NCBI GenBank Database



(courtesy -geneOmbioTechnologies)

The novel isolate *Streptomyces thermocarboxydus NBRC 16323* was studied for optimization of growth medium by OVAT(one variable at a time) approach .

Effect of various growth parameters on β-galactosidase activity β-galactosidase enzyme production by *Streptomyces thermocarboxydus* was found to be maximum at pH 8 and temperature 41°C with an incubation time of 72h. As seen in Fig-4, enzyme production by the isolate was found to be highest at 41° but was lower when incubated at lower temperatures and also the enzyme production peaked after 3days of incubation and started to decrease later, indicating lesser nutrient availability to the multiplying cells. In case of pH, β-galactosidase production increased from pH 6.5 onwards and displayed highest activity when grown at pH 8, as represented by Fig-5. Similar study was reported by Sumit Sharma & Priyanka Singh on isolated blue colonies of *Lactobacillus delbrueckii* on X-Gal-MRS agar inoculated in different culture conditions (Carbon & Nitrogen sources, metal ions, pH, Temperature and natural substrates) showed maximum production of enzyme in starch added medium at pH6 and 45ºC temperature [16]. Another study on β-galactosidase from 12 different yeasts by Das Prapty, Sarma Hridip K, Barman Pranjan etal also reported highest enzyme activity at 30°C with 72h of incubation period at pH 9 [17].

Lactose acts as the inducer of β-galactosidase synthesis. With respect to the effect of Carbon sources on the growth, *Streptomyces thermocarboxydus* showed highest β-galactosidase production with 1% Whey (natural lactose rich substrate) followed by Lactose as seen in Fig-6 . Less enzyme activity with other C sources Starch, Dextrose and Galactose indicates that the enzyme production was not induced to the

fullest. In case of Nitrogen sources the isolate showed highest enzyme production when the culture medium was suppplemented with 1% Casein followed by $NaNO_3$ as shown in Fig-6 while egg albumin and Urea found to inhibit enzyme production.

With respect to inoculum age and size, the Actinomycetes isolate showed maximum enzyme production with a 3day old inoculum at 1% volume (v/v) as seen in Fig-7. The decrease in enzyme production at larger inoculum size can be attributed to nutrient depletion by the increased number of active cells. Similar observation was explained by Das Prapty, Sarma Hridip K, Barman Pranjan etal with Maltose and Ammonium sulphate as C & Nsources and Rice as natural substrate [17]. Heena & Niveditha also reported that the optimal enzyme production for *Lactobacillus sps* using MRS medium was on 2nd day at pH 7 & at 30ºC with inoculum size of 4%, Lactose & Peptone as C and N sources at 3% substrate (lactose) concentration, Mg^{2+} as divalent ions [18]. In another study reported by Oparaji E H, Okwuenu P C etal, β-galactosidase production by *Lactobacillus acidophilus* isolated from dairy industrial waste water was highest at pH 6 after 12 days of incubation at an inoculum size of 2% and with Lactose and Peptone as optimum carbon and nitrogen sources [19]. Petar Kolev, Diana Rocha-Mendoza, etal also explained in their study that 18 strains showed higher β-galactosidase activity when grown in acid whey supplemented mediun when compared to MRS medium and *Lactobacillus helveticus* strain OSU-PECh-4A yielded 5 times higher activity than average [20].

Metal ions accept or donate electrons and efficiently stabilize enzyme -substrate conformation. The isolate *Streptomyces thermocarboxydus* showed maximum enzyme production with 5mM of Mg^{2+} ions (added as $MgSO_4$) indicating metallo-active enzyme, as seen in Fig- 8. Inhibition of enzyme production was maximum when the medium was supplemented with Fe^{++}. Maximum β-galactosidase production was reported in the presence of 0.1% MgSO4 by Lactobacillus sp. KLSA 22 by Ahmed etal in 2016 [21] and by Lactobacillus casei MB2 isolated from traditional dairy product of Himachal Pradesh by Heena & Niveditha in 2020 [18].

Cell permeabilization treatments of *Streptomyces thermocarboxydus* with SDS-Chloroform gave highest enzyme yield (100:200 µl/ml) followed by Lysozyme (150µl/ml) as shown in Fig-9. Decline in enzyme activity with Acetone, Toulene treatments indicate inefficient cell lysis as compared to that of SDS-Chloroform. Similar studies of cell disruption for release of the enzyme with various extraction methods namely- sonication, SDS-Chloroform, Lysozyme ,Acetone-Toulene were reported by Prasad etal., (2013) and sonication method was high yielding for *B. animalis* ssp.lactis Bb12 whereas lysozyme-EDTA treatment was found to be effective for *L. delbrueckii* ssp. bulgaricus ATCC 11842 [22]. Shrushti Makwana, Subrota Hati, etal in 2013, also evaluated various permeabilization treatments on 3 Lactobacillus cultures and reported that a sonication period of 5 min (pulse 15 sec off / 30 sec on at 55% amplitudes gave highest yield of the enzyme [23].

Figure-4 Effect of Incubation Temp and Incubation Time on Enzyme production

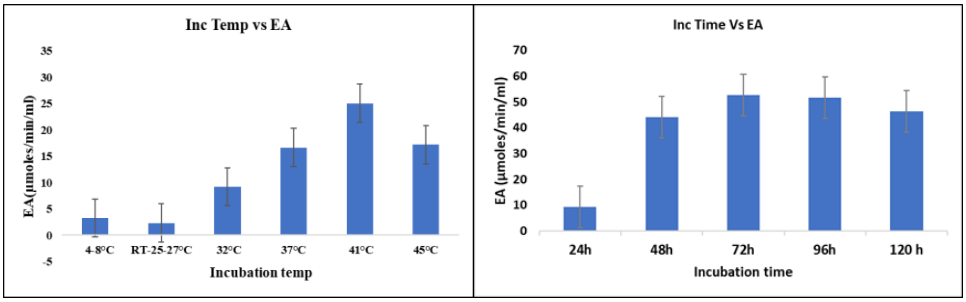


Figure-5 Effect of pH on Enzyme Production

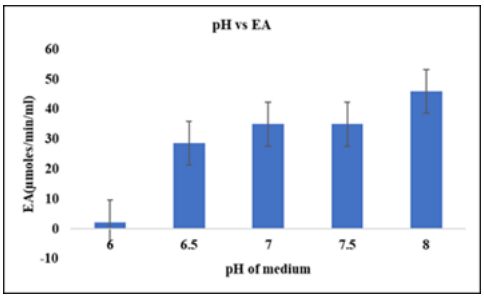


Figure-6 Effect of Carbon and Nitrogen sources on Enzyme production

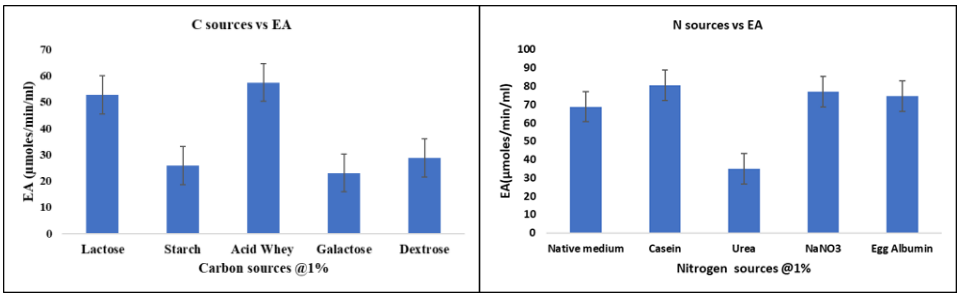


Figure-7 Effect of Inoculum Age and Size on Enzyme Production

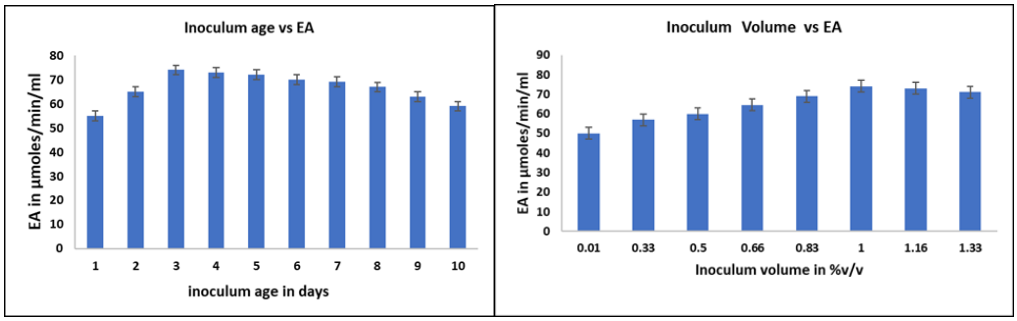


Figure-8 Effect of Metal ions on Enzyme production

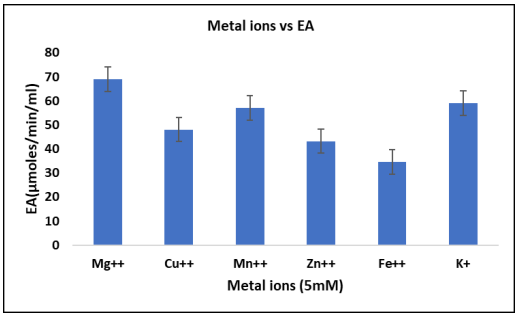


Figure-9 Effect of Cell Disruption Methods on Enzyme yield

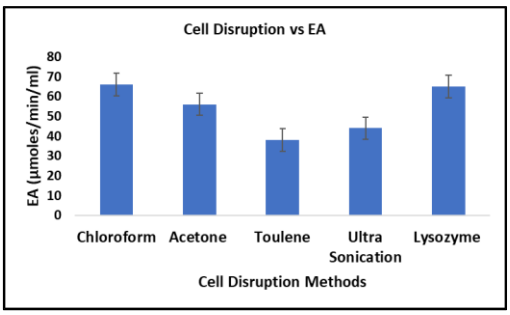


Table 1 depicts the summary of investigated process variables and optimized levels as observed for the isolate

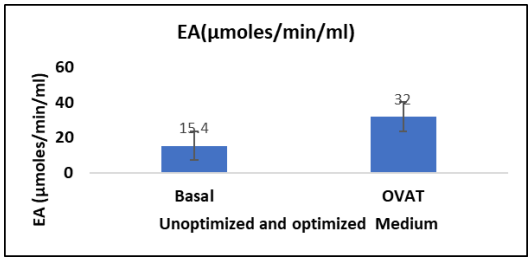
Table-1 Process variables and optimized levels

Variables	Levels of Variables	Optimized Levels
Incubation period	24, 48, 72, 96 (hrs)	72h
Incubation temperature	4-8, 20, 30, 37,41(°C)	41°C
pH	6.0, 6.5, 7.0, 7.5, 8	8
C sources @1%	Lac, Dex, Gal,Wh, St	Wh
N sources @1%	Urea, Casein, NaNo3, EAb	Casein
Inoculum age	01-10days	3 day old
Inoculum volume (%ml)	0.01%-1.33%	1%
Metal ions(5mM)	Mg^{++},Cu^{++},Mn^{++}, Fe^{++},K^{+}, Zn^{++}	Mg^{++}
Cell Permeabilization	SDS :Chl, Ace &Tou, USon, Lys	SDS:Chl

Lac-Lactose, Dex-Dextrose, Gal-Galactose, Wh-Whey, St-Starch, EAb- Egg Albumin, SDS-Sodium Dodecyl Sulphate, Chl-Chloroform, Ace-Acetone, Tou-Toulene, USon-Ultra Sonication, Lys- Lysozyme

Validation The present research findings were validated by comparing the β-galactosidase production by the isolate *Streptomyces thermocarboxydus* in basal unoptimized Starch Casein Broth and OVAT optimized medium and a 2x fold increase in the enzyme production was achieved as seen from Fig-10.

Figure-10 β-galactosidase production in unoptimized & optimized media



Conclusion

Utilization of dairy affluent Whey for β-galactosidase production offers huge potential in not only reducing costs of production but also to reduce the environmental damage caused by acid whey disposal into streams and soil. (Mayta-Apaza et al,2021) [24]. The present study offers the isolation of a new Actinomyctes strain *Streptomyces thermocarboxydus strain NBRC 16323* that has the potential to

grow on whey. The newly optimized medium with whey holds promising prospects for utilization of whey thus reducing the cost of production as the enzyme yield was twice that of the basal Starch casein Broth.

Statistical Optimization of the media with Plackett-Burman Model and Central Composite Design holds the future potential of this study to achieve more viable and economical output.

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