International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 2 (2014) pp. 154-167**
http://www.ijcmas.com

Original Research Article

Optimization of fermentation parameters and enzyme immobilization of alpha-galactosidase isolated from different bacteria

H.S.Anil Kumar[1]*, Vadlapudi Kumar[2] and N.Vidyavathi [1]

[1]Department of Biotechnology, NMAM Institute of Technology, Nitte-574 110 Karnataka, India.
[2]Department of Biochemistry, Kuvempu University-PG Centre, Davanagere-577 002, Karnataka, India
*****Corresponding author*

Keywords

α-Galactosidase; Bacteria; Fermentation medium; Carbon source; Metal source.

A B S T R A C T

α-Galactosidase (α–D-galactoside galactohydrolase, EC. 3.2.1.22), is an important enzyme in the processing of galactooligosaccharides that hydrolyzes galactooligosaccharides especially raffinose and stachyose found in most of the leguminous foods, as these sugar are responsible for intestinal discomfort and flatulence. α-galactosidase producing bacteria are isolated. Optimization of parameters like pH, temperature and agitation speed for the fermentation process found 7 – 8, 36 – 38^{o}C and 170 rpm respectively. In the nutritional parameter optimization raffinose found to be effective carbon source from many bacterial isolates for alpha-galactosidase production. Peptone preferred to be best source of nitrogen for fermentation medium of galactosidase after NM_4NO_3 for isolated bacteria. Effect of K2HPO4 and MgSO4.7H_2O. as a source of mineral found to enhancer of enzyme production in the medium.

Introduction

A - D - Galactose - galactohydrolase (E.C.3.2.1.22), commonly referred to as α-galactosidase, catalyzes the hydrolysis of α-galactosidic linkages in oligo-saccharide such as raffinose, melibiose, stachyose and verbascose, polysaccharides like galactomannans, and glycoconjugates such as glycoproteins and glycolipids. Ceramide trihexosides, its higher homologues and derivatives are also cleaved by the action of α-galactosidase. (Bau *et al.*, 1961; and Fischer *et al.*, 1895), have isolated for the first time, α-galactosidase from bottom yeast. Because of its action on melibiose it was named as melibiase.

α-Galactosidase is an exoglycosidase that catalytically removes α-linked terminal nonreducing galactose residues from small oligosaccharides such as melibiose and raffinose and larger galactopoly saccharides and galactolipids. It is widely distributed in biological systems (Dey and

Pridham, 1972) and the genes encoding α-galactosidases have been isolated from microorganisms, plants and animals. α-galactosidases occur widely in microorganisms. However, despite the wide occurrence of this enzyme, only a small number have been purified and extensively studied. As might be expected, research in this area has centred on sources of α- galactosidase that offer the greatest economic potential.

Microbial enzymes are routinely used in many environmentally friendly and economic industrial sectors. There is increasing demand to replace traditional chemical processes with biotechnological processes involving micro-organisms and enzymes such as pectinases (Bajpai 1999; Bruhlmann *et al*., 2000). xylanases (Beg *et al.,* 2000). cellulases (Bajpai 1999), mannanase (Montiel *et al.,* 2002), a-galactosidase (Clarke *et al.,* 2000), and raccases and ligninases (Bajpai 1999; Onysko 1993), which not only provide an economically viable alternative but are also more environmentally friendly (Viikari *et al*., 2001).

The glycosidic bond is one of the most stable bonds in nature. With an estimated half life of around 5 million years (Wolfenden *et al.,* 1998), the enzymes which catalyze the hydrolysis of this bond may be considered among the most powerful enzyme catalysts known. These enzymes play central roles in diseases such as diabetes, and industrial processes like food technology. This ensures that the glycosyl hydrolases are a medically relevant, industrially important and scientifically interesting group of enzymes.

The economic feasibility of the industrial enzymes depends on its production costs. The cost of the enzyme primarily depends on the fermentation process applied to isolate high yielding stable strains and fermentation media. Hence high yielding and stable strains should be isolated by using a media containing inducers of the products and devoid of repressors. In general, no defined medium was established for the best production of any metabolite because the genetic diversity present in different microbial sources causes each organism or strain to have its own special conditions for maximum product production. Therefore, it is essential to have a detailed investigation on growth and metabolite production pattern of newly isolated microbial strain under different environmental conditions to achieve maximum production benefit (Prakasham *et al.,* 2005). Microbial alpha-galactosidases are produced by both submerged and solid state fermentation. Most of industrial α-galactosidases are produced by submerged fermentation compared to other fermentation methods.

Materials and Methods

Screening of α- galactosidases producing bacteria

Legumes were used as a source of bacterial culture, moistened legumes which infected by bacteria was inoculated on the screening medium contained 1% tryptone, 0.5% yeast extract , 1% NaCl and 0.01% pNPG (para Nitrophenyl α- D galactopyranoside). Inoculated plates were incubated at 37^{o}C for 24 hrs in broth culturing on a rotary shekar (150rpm/min) of bacterial isolates after screening for α-galactosidases production

Extraction

Cells were harvested by centrifugation at 10,000 rpm for 5 min at 4^{o}C then washed twice with McIlvaine buffer (Na_2HPO_4 –

Citric acid pH 5.8) and suspended in same buffer. Cells were disrupted with acid washed sand upon shaking at maximum speed on a vertex mixer. Cellular debris was removed by centrifugation at 10,000 rpm for 10 min at 4^{o}C and α - Galactosidase activity in supernatant was determined.

Enzyme Assay

α-Galactosidase activity was assayed by following the hydrolysis of p-nitrophenyl-α -D-galactopyranoside (Sigma). A 200-µL volume containing the sample and 1 mm substrate in 0.2M McIlvaine buffer (pH 5.8) was incubated at 37 ^{0}C for 20 min. The reaction was terminated by the addition of 500 µL 0.25M $NaCO_3$ and the absorbance recorded at 405 nm. One unit of α - galactosidase activity is defined as the amount of enzyme releasing 1 µmol of the *p*-nitrophenol per min.

Morphological analysis Biochemical tests of bacterial isolates

In natural habitats, bacteria usually grow together in populations containing a number of species. In order to adequately study and characterize an individual bacterial species, one needs a pure culture. The spread plate technique is an easy, direct way of achieving this result. In this technique, a small volume of dilute bacterial mixture is transferred to the centre of an agar plate and is spread evenly over the surface with a sterile, L-shaped glass rod. The glass rod is normally sterilized by dipping in alcohol and flamed to burn off the alcohol. After incubation, some of the dispersed cells develop into isolated colonies. A colony is a large number of bacterial cells on solid medium, which is visible to the naked eye as a discrete entity. In this procedure, one assumes that a colony is derived from one cell and therefore represents a clone of a pure culture. After incubation, the general form of the colony and the shape of the edge or margin can be determined by looking down at the top of the colony. The nature of the colony elevation is apparent when viewed from the side as the plate is held at eye level. After a well-isolated colony has been identified, it can then be picked up and streaked onto a fresh medium to obtain a pure culture.

Morphological characterizations of the isolates were done by observing the colony morphology and log phase culture under the light microscope.

Biochemical tests

Indole Test

Indole test is done to determine the production of indole in presence of enzyme tryptophanase. Isolates were incubated in a sterile peptone broth for 24 hours at 36°C. Following incubation, few drops of kovac's reagent containing ρ-dimethylaminobenzaldehyde, isoamyl alcohol and concentrated hydrochloride is added to the broth. Formation of pink to red colour ring on the surface layer of the aldehyde broth indicates positive whereas yellow colour solution indicates negative.

Methyl Red (MR) and Voges-Proskauer (VP) test

MR and VP tests are conducted to detect end products of glucose metabolism. In microorganisms, glucose is either metabolized to neutral compounds like 2,3-butanediol, acetoin etc using butylenes glycol pathway or converted to acidic end products like acetic acid, lactic acid etc., using mixed acid pathway. Methyl red test is used to identify organisms using mixed acid pathway whereas Voges-Proskauer

test is used to identify butylene glycol pathway utilizing microorganisms.

Isolates were inoculated into two tubes of MR-VP broth and was incubated for 24 hours at 36°C. After incubation, few drops of methyl red a pH indicator was added to one of the tubes labelled MR tube where as 5-6 drops of Barritt's reagent A and B were added to VP labelled tubes. If red colour is observed in MR tubes, it indicates positive while no colour change is indicated as negative. In case of VP tubes, if a red colour is developed within 15 to 20 minutes addition of reagents is read as positive where as a copper colour indicates negative

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Citrate Utilization test

Some microorganisms utilize citrate as the sole carbon source leading to the production of alkaline by-products like ammonia. Bromothymol blue indicator is used to detect alkaline pH of the medium (blue colour above pH 7.6 and yellow colour below pH 6.0). Isolates were streaked on the Simmon's citrate agar slants and was incubated for 24 hours at 36°C. If a blue colour is developed within 24 hours of incubations is read as positive whereas yellow colour is negative.

Catalase test

Some microorganisms produce enzyme catalase that catalysis the breakdown of hydrogen peroxide, a toxic end product into water and oxygen. In bacteriology, catalase test is mainly used to differentiate one genus from other. Colonies of isolates were smeared on the glass side. One drop of H_2O_2 was added to the smear. Immediate formation of effervescence or oxygen bubble is read as positive while delayed or no bubble formation is indicated as negative.

Nitrate reduction test

This test is used to identify microorganisms that convert nitrates (NO_3) to nitrite (NO_2) or some other nitrogenous compound, such as molecular nitrogen (N_2), using the enzyme nitrate reductase. This process is the result of either anaerobic respiration or denitrification. The nitrite production is detected by the addition of sulfanilic acid (Solution A) and α-naphthylamine (Solution B) into nitrate broth. Isolates were inoculated into the nitrate broth containing potassium nitrate whereas un-inoculated tube was kept as control. After incubation, a few drops of reagents, Solution A and Solution B were added to the test tubes. Formation of red colour is read as positive.

Identification of α-galactosidase producing isolate

The selected bacterial isolates showing α-galactosidase activity were labelled as A1, A2, A3 and A4, herewith they will be referred with mentioned names. The cultural, morphological and biochemical characteristics of the isolates were identified using Bergey's Manual of Determinative Bacteriology, 8th edition (Buchanan RE and Gibbons N E, 1974).
The bacterial isolates were further identified by 16S rDNA analysis.

16S ribosomal DNA (rDNA) sequencing for further characterization of Bacterial isolates

DNA isolation from bacteria culture using Bioserve Bacterial Genomic DNA isolation Kit (Catalogue No 2022), Amplification of the 16SrDNA region using primers designed in the conserved region, the primers used were FD1 and

RP2. Gel Elution of PCR Product using Bioserve Gel Elution Kit (Catalogue No 2021).

Sequencing of the ~1.5kb region using internal sequencing primers. Sequencing was performed with 4 different primers designed in the conserved regions on 16SrDNA, the primer are 16SEQ2R, 16SEQ3F, INS16SREV, 16SEQ4F, 16SEQ4R

Fermentation parameters optimization:

Initially all the medium parameters were optimized for enhanced α-galactosidase production by using classical approach. Different physical and nutritional parameters were optimized to enhance the enzyme production. The basal medium used for preliminary studies contained sucrose 10g/L, yeast extract 5g/L, K_2HPO_4 5g/L, $MgSO_4.7H_2O$ 2g/L and $MnSO_4.7H_2O$ 2g/L (pH 6.0). The organism was grown at 36°C in a shaking incubator at an agitation speed of 150rpm. All the experiments were performed in 250ml flask containing 100ml medium.

Optimization of physical parameters

Three different physical parameters such as temperature, pH, and agitation speed were chosen for optimization to enhance the α-galactosidase production of bacterial isolates. Parameters and their conditions chosen were given in Table 2.

Optimization of nutritional parameters

In case of enzyme α-galactosidase, carbon source can be either inducer or inhibitor of enzyme production. Hence, various carbon sources, nitrogen sources, and minerals were chosen to optimize α-galactosidase production and it is summerized in Table 3.

Based on preliminary screening studies of various media components, a fermentation medium comprising raffinose 10g/L, Yeast extract 3g/L, K_2HPO_4 1g/L, $MgSO_4.7H_2O$ 0.49g/L and $FeSO_4.7H_2O$ 1g/L (pH 6.0) was formulated and further used in later studies. Maximum enzyme activity was obtained when cultures were grown at 36°C, pH 6.0 and agitation speed of 150 rpm for 24 hours.

Results and Discussion

Bacteria producing α-galactosidase would act on pNPG and releases p-Nitrophenol as product which diffuses into the medium with yellow colour. Four colonies from different samples were selected and inoculated into screening medium, pH 7.0. After 24 hours of incubation, the cells were harvested and disrupted by sonication. The supernatant obtained on centrifugation at 10,000 rpm, 10 minutes, 4°C was assayed for intracellular α-galactosidase enzyme activity. The extracted crude enzymes from different isolates were screened for α-galactosidase activity using pNPG substrate.

Morphological and Biochemical Characterization

The morphological and biochemical characteristics of isolates A1, A2, A3 and A4 were given in the Table 1. The above results shows A1 and A2 positive for Indole test which indicates tryptophan is metabolized by tryptophanase leading to formation of Indole. Whereas A3 and A4 showed negative for Indole formation.

Methyle Red test showed positive by A2 and A4 which indicates these two bacteria undergo carbohydrate metabolism leading to formation of acedic end products like organic acid. A1 and A3 showed negative for methyl red test indicating the

carbohydrate metabolism these organisms leading to formation of neutral compounds like acetoin or butanediol, at this condition methyl red turns yellow in colour.

Voges-Proskauer test showed positive by A3 indicates identifies bacteria that ferment glucose, leading to 2,3-butanediol accumulation in the medium. The addition of Barritt's reagent will detect the presence of acetoin - a precursor in the synthesis of 2,3-butanediol.In the presence of the reagents and acetoin, a cherry-red color develops. A1, A2 and A4 showed negative for this test.

Citrate utilization test showed positive by A1, A3 and A4 isolates indicating they oxidize citrate, they remove it from the medium and liberate CO_2. CO_2 combines with sodium (supplied by sodium citrate) and water to form sodium carbonate an alkaline product. This raises the pH, turns the pH indicator to a blue colour.

Catalase test was given positive for all four bacterial isolates. Some bacteria contain flavoproteins that reduce O_2, resulting in the production of hydrogen peroxide (H_2O_2) or superoxide (O_2^-). These are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly. A bacterium must be able to protect itself against such O_2 products or it will be killed.Many bacteria possess enzymes that afford protection against toxic O_2 products. Obligate aerobes and facultative anaerobes usually contain the enzymes superoxide dismutase, which catalyzes the destruction of superoxide, and either catalase or peroxidase, which catalyze the destruction of hydrogen peroxide into water and oxygen. When we add few drops of hydrogen peroxide on bacterial colony bubbles of oxygen represent a positive catalase test.

Nitrate reduction test was shown positive by A3 and A4, where as A1 and A2 showed negative for the test. Chemolitho-autotrophic bacteria (bacteria that obtain energy through chemical oxidation; they use inorganic compounds as electron donors and CO_2 as their primary source) and many chemoorgano-heterotrophs (bacteria that require organic compounds for growth; the organic compounds serve as sources of carbon and energy) can use nitrate (NO_3^-) as a terminal electron acceptor during anaerobic respiration. In this process, nitrate is reduced to nitrite (NO_2^-) by nitrate reductase.

A comparison of A, A2, A3 and A4 16s rDNA sequence with other bacterial 16s rDNA was done for 100% similarity and maximum score. With this analysis it is confirmed that A1 as E*nteroobacter* sp. A2 as *Bacillus amyloliquefaciens* strain H102. A3 as *Bacillus* sp. and A4 as *Bacillus thuringiensis* strain 2PR56.

Fermentation parameters optimization

Fermentation medium plays a pivotal role in enzyme production. Several researchers reported an increase in microbial enzyme production under optimized medium conditions (Adinaryana *et al*. 2003; Prakasham *et al*. 2005a). Hence, medium formulation is an important prerequisite to enhance enzyme production. Preliminary studies were done using classical approach to develop fermentation medium to

Table.l Results of Biochemical tests and Morphological features

Biochemical Test	A1	A2	A3	A4
Indole Test:	+	+	-	-
Methyl Red (MR) test	-	+	-	+
Voges-Proskauer (VP) test:	-	-	+	-
Citrate Utilization test	+	-	+	+
Catalase test	+	+	+	+
Nitrate reduction test	-	-	+	+
Morphological feature				
Shape	Rod	Rod	Rod	Rod
Colour	White transparent	Yellow	White	Cream
Colony margin	Round	Rhizoid	Round	Irregular

'-' = Negative result '+' = Positive result

Table.2 Physical parameters for fermentation optimization

Physical parameters	Growth conditions
Temperature (^{o}C)	28, 30, 32, 34, 36, 38, 40
pH	5.0, 6.0, 7.0, 8.0, 9.0
Agitation speed (rpm)	110, 130, 150, 170, 190, 210

Table.3 List of Nutritional parameters

Carbon source	Nitrogen source	Minerals
Glucose	Urea	KH_2PO_4
Fructose	Peptone	$MgSO_4$
Sucrose	NH_4NO_3	$FeSO_4$
Lactose	$(NH_4)_2SO_4$	$MnCl_2$
Raffinose	$NaNO_3$	NaCl

Table.4 Effect of different carbon source (sugar)

Bacterial isolate	Glucose	Fructose	Sucrose	Lactose	Raffinose
***Enterobacter* sp.**	8.1	8.3	6.7	5.2	10.3
***Bacillus amyloliquefaciens* strain H102**	12.1	11.8	10.4	8.3	14.2
***Bacillus* sp.**	26.9	21.5	17.9	20.0	25.3
Bacillus thuringiensis strain 2PR56	18.4	17.1	13.5	16.7	19.0

*Result expressed in gal activity in U/ml

16S ribosomal DNA (rDNA) sequencing for further characterization of Bacterial isolates

16S rDNA sequencing was performed to identify bacterial isolates. 16S rDNA sequence (~1.5kb in length) of the isolates 1ist given below-

Bacterial isolate A1

5'acggctaccttgttacgacttcaccccagtcatgaatcacaaagtggtaagcgccctcccgaaggttaagctacctacttcttttgcaa
cccactcccatggtgtgacgggcggtgtgtacaaggcccgggaacgtattcaccgtagcattctgatctacgattactagcgattccga
cttcatggagtcgagttgcagactccaatccggactacgacgcactttatgaggtccgcttgctctcgcgaggtcgcttctctttgtatgc
gccattgtagcacgtgtgtagccctactcgtaagggccatgatgacttgacgtcatccccaccttcctccagtttatcactggcagtctcc
tttgagttcccggccgaaccgctggcaacaaaggataagggttgcgctcgttgcgggacttaacccaacatttcacaacacgagctga
cgacagccatgcagcacctgtctcagagttcccgaaggcaccaatccatctctggaaagttctctggatgtcaagagtaggtaaggttc
ttcgcgttgcatcgaattaaaccacatgctccaccgcttgtgcgggcccccgtcaattcatttgagttttaaccttgcggccgtactcccc
aggcggtcgacttaacgcgttagctccggaagccacgcctcaagggcacaacctccaagtcgacatcgtttacggcgtggactacca
gggtatctaatcctgtttgctccccacgctttcgcacctgagcgtcagtctttgtccagggggccgccttcgccaccggtattcctccaga
tctctacgcatttcaccgctacacctggaattctacccccctctacaagactctagcctgccagtttcgaatgcagttcccaggttgagcc
cggggatttcacatccgacttgacagaccgcctgcgtgcgctttacgcccagtaattccgattaacgcttgcaccctccgtattaccgcg
gctgctggcacggagttagccggtgcttcttctgcgggtaacgtcaatcgacaaggttattaaccttatcgccttcctccccgctgaaag
tactttacaacccgaaggccttcttcatacacgcggcatggctgcatcaggcttgcgcccattgtgcaatattccccactgctgcctccc
gtaggagtctggaccgtgtctcagttccagtgtggctggtcatcctctcagaccagctagggatcgtcgcctaggtgagccgttacccc
acctactagctaatcccatctgggcacatctgatggcaagaggcccgaaggtccccctctttggtcttgcgacgttatgcggtattagct
accgtttccagtagttatccccctccatcaggcagtttcccagacattactcacccgtccgccgctcgtcacccgagagcaagctctctg
tgctaccgctcgacttgcatgtgttaggcctgccgccagcgttcaatctgagcccaatctcactct3'

Bacterial isolate A2 (Q5)

5'acggctaccttgttacgacttcaccccaatcatctgtcccaccttcggcggctggctccataaaggttacctcaccgacttcgggtgtt
acaaactctcgtggtgtgacgggcggtgtgtacaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattcc
agcttcacgcagtcgagttgcagactgcgatccgaactgagaacagatttgtgggattggcttaacctcgcggtttcgctgccctttgttc
tgtccattgtagcacgtgtgtagcccaggtcataaggggcatgatgatttgacgtcatccccaccttcctccggtttgtcaccggcagtc
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aagctcccatctgtccgctcgacttgcatgtattaggcacgccgccagcgttcgtcctgagccatgatcaaactct3'

Bacterial isolate A3

5'agagtttgatcctggctcaggatgaacgctggcggcgtgcctaatacatgcaagtcgagcgaactgattagaagcttgcttctatgac
gttagcggcggacgggtgagtaacacgtgggcaacctgcctgtaagactgggataacttcgggaaaccgaagctaataccggatag
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caggattagataccctggtagtccacgccgtaaacgatgagtgctaagtgttagagggtttccgcccctttagtgctgcagctaacgcatt
aagcactccgcctggggagtacggtcgcaagactgaaactcaaaggaattgacgggggcccgcacaagcggtggagcatgtggtt
taattcgaagcaacgcgaagaaccttaccaggtcttgacatcctctgacaactctagagatagagcgttcccccttcgggggacagagt
gacaggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttgatcttagttgccag
catttagttgggcactctaaggtgactgccggtgacaaaccggaggaaggtggggatgacgtcaaatcatcatgccccttatgacctg
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gtaggctgcaactcgcctacatgaagctggaatcgctagtaatcgcggatcagcatgccgcggtgaatacgttcccgggccttgtaca
caccgcccgtcacaccacgagagtttgtaacacccgaagtcggtggagtaaccgtaaggagctagccgcctaaggtgggacagatg
attggggtgaagtcgtaacaaggtagccgt3'

Bacterial isolate A4

5'cccccatcatttgttcccccttaggcggccgggttcaaaaaggttaccccaccgacttcgggtgttacaaactctcgtggtgtgacgg
gcggtgtgtacaaggcccgggaacgtattcaccgcggcatgctgatccgcgattactagcgattccagcttcatgtaggcgagttgca
gcctacaatccgaactgagaacggttttatgagattagctccacctcgcggtcttgcagctctttgtaccgtccattgtagcacgtgtgta
gcccaggtcataaggggcatgatgatttgacgtcatccccaccttcctccggtttgtcaccggcagtcaccttagagtgcccaacttaat
gatggcaactaagatcaagggttgcgctcgttgcgggacttaacccaacatctcacgacacgagctgacgacaaccatgcaccacct
gtcactctgctcccgaaggagaagccctatctctagggttgtcagaggatgtcaagacctggtaaggttcttcgcgttgcttcgaattaa
accacatgctccaccgcttgtgcgggcccccgtcaattcctttgagtttcagccttgcggccgtactccccaggcggagtgcttaatgc
gttaacttcagcactaaagggcggaaaccctctaacacttagcactcatcgtttacggcgtggactaccagggtatctaatcctgtttgct
ccccacgctttcgcgcctcagtgtcagttacagaccagaaagtcgccttcgccactggtgttcctccatatctctacgcatttcaccgcta
cacatggaattccactttcctcttctgcactcaagtctcccagtttccaatgaccctccacggttgagccgtgggctttcacatcagactta
agaaaccacctgcgcgcgctttacgcccaataattccggataacgcttgccacctacgtattaccgcggctgctggcacgtagttagcc
gtggctttctggttaggtaccgtcaaggtgccagcttattcaactagcacttgttcttccctaacaacagagttttacgacccgaaagcctt
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agtcccagtgtggccgatcaccctctcaggtcggctacgcatcgttgccttggtgagccgttacctcaccaactagctaatgcgacgcg
ggtccatccataagtgacagccgaagccgcctttcaatttcgaaccatgcggttcaaaatgttatccggtattagccccggtttcccgga
gttatcccagtcttatgggcaggttacccacgtgttactcacccgtccgccgctaacttcataagagcaagctcttaatccattcgctcga
cttgcatgtattaggcacgccgccagcgttc3'

enhance α-galactosidase production from bacterial isolates by studying various medium parameters.

Effect of temperature on growth

The bacterial isolates were grown at temperature range of 28 to 40°C. The pH of the medium was maintained at 7.0. A graphical presentation in Figure 1. shows optimum temperature for α-galactosidase activity and growth. The isolate has shown growth at broad temperature in the range of 32 to 38°C.

Effect of pH on growth

The bacterial isolates were cultured at varying pH range of 5-9 at 36°C for 12 hours at 150 rpm in a shaking incubator. Growth rate of organisms were determined. From figure 2. it was observed that maximum growth of the isolate was observed at optimum pH range of 7.0 to 8.0. In general, it was reported that bacterial α-galactosidases have neutral optimum pH of 6 to 7.5 whereas yeast and fungal α-galactosidases have acidic optimum of pH of 2-3 (Ulezlo *et al*, 1982).

Effect of agitation speed (rpm)

Agitation plays a pivotal role in growth of the microorganism and enzyme production. Experiments were carried out by growing the isolates at varying rpm range of 110 to 210 at 36°C and pH 7.0. A-galactosidase activity improved with an increase in agitation speed upto 150 rpm and further increase revealed no noticeable improvement as shown in Figure 3. Maximum activity at 170 rpm was reported in *Acinetobacter sps.* CBT01 (Sirisha *et al* 2010)

Nutrition parameters

Effect of carbon sources

The effect of different carbon sources ie sugars on α-galactosidase enzyme production was summarized in Table 4. Raffinose showed maximum activity. Other sugars, galactose, lactose and sucrose showed substantial activity. Glucose and Raffinose were found to be good carbon source for *Bacillus amyloliquifaciens* strain H102 *Bacillus sp.* and *Bacillus thuringiensis* strain 2PR56, while Raffinose is found to be a good carbon source for *Enterobacter* sp. bacterial isolate. The results are in correlation with previous reports indicating α-galactosidase production is either induced or enhanced in presence of sugars: galactose, raffinose and stachyose (Marisa *et al*.1996; Jin *et al*. 2001; Kotwal *et al*. 1995) whereas lactose completely inhibit or had a very little influence on enzyme production. In some cases Glucose inhibits α-galactosidase production. Glucose inhibition of α-galactosidase production might be due to catabolite repression (Delente *et al.* 1974).

Effect of various nitrogen sources

Both organic and inorganic compounds were chosen as nitrogen Sources the results shown in Table 5. Peptone was confirmed to be the best source of nitrogen for α-D-galactosidase production through fermentation. Next to peptone it was NH_4NO_3 for *Bacillus* sp., and *Bacillus thuringiensis* strain 2PR56, whereas urea for *Enterobacter* sp. and *Bacillus amyloliquifaciens* strain H102. Many researchers reported use of tryptone as nitrogen source in the growth medium for α-galactosidase production (Delente *et al*. 1974; Marisa *et al*.1996).

Table.5 Effect of different Nitrogen sources

Bacterial isolate	Urea	Peptone	NH_4NO_3	$(NH_4)_2SO_4$	$NaNO_3$
Enterobacter sp.	9.2	11.2	8.4	7.1	7.6
Bacillus amyloliquefaciens strain H102	12.8	14.1	7.3	8.5	8.3
Bacillus sp.	19.5	26.4	20.5	17.8	18.5
Bacillus thuringiensis strain 2PR56	15.1	21.7	17.4	15.8	12.5

*Result expressed in gal activity in U/ml

Table.6 Effect of metal sources

Bacterial isolate	KH_2PO_4	$MgSO_4$	$FeSO_4$	$MnCl_2$	NaCl
Enterobacter sp.	10.2	9.5	7.4	6.3	4.6
Bacillus amyloliquefaciens strain H102	12.7	11.4	8.4.	7.2.	3.3
Bacillus sp.	20.1	19.1	16.5	15.2	5.5
Bacillus thuringiensis strain 2PR56	18.1	17.2	15.4	14.8	5.7

*Result expressed in gal activity in U/ml

Figure.1 Effect of Temperature on growth and enzyme activity

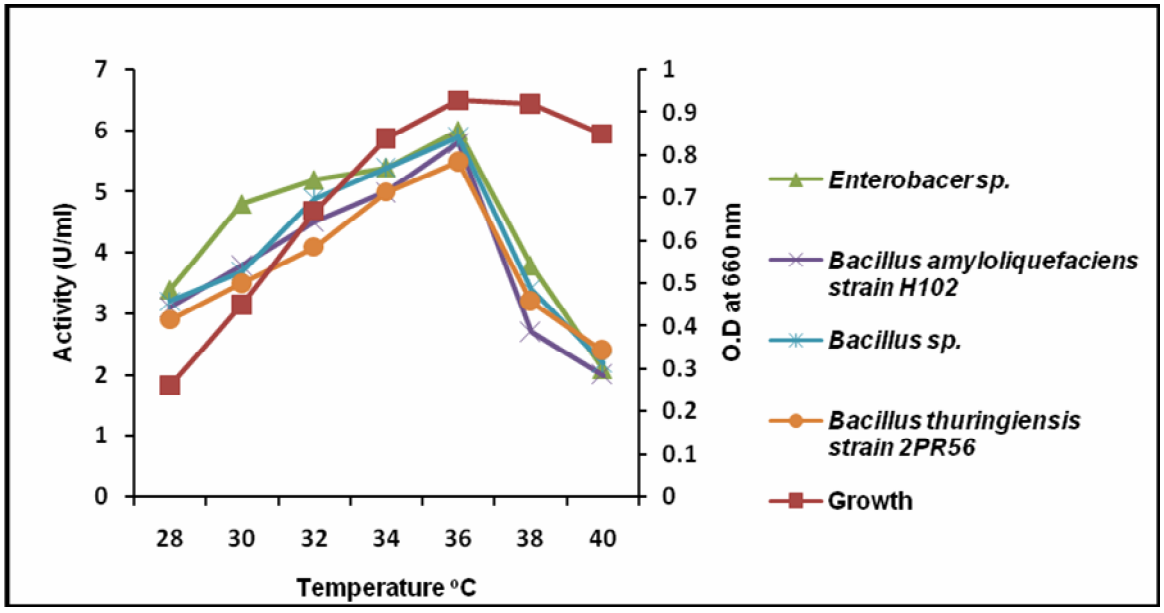


Figure.2 Effect of pH on growth and enzyme activity

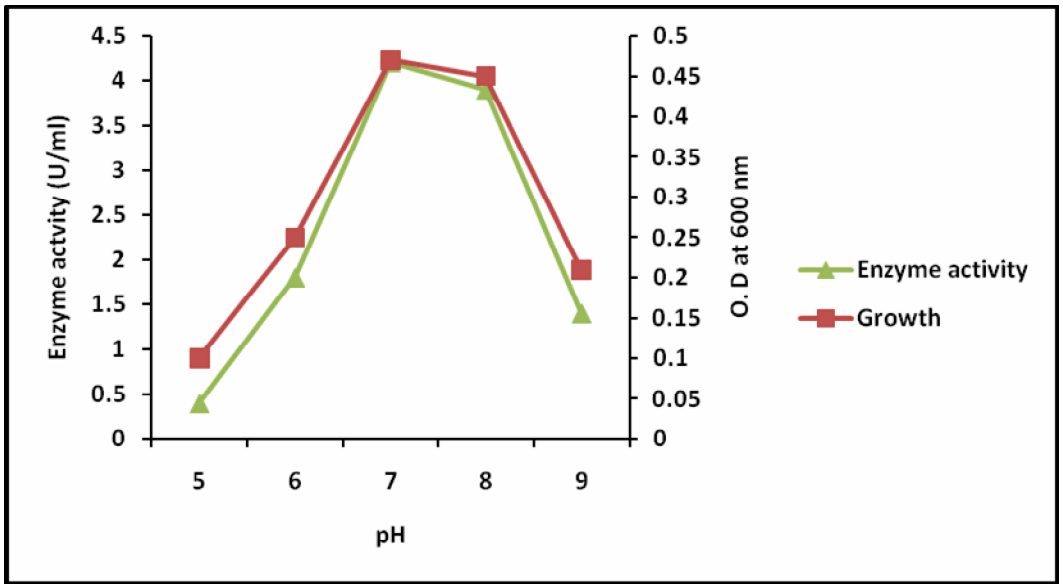
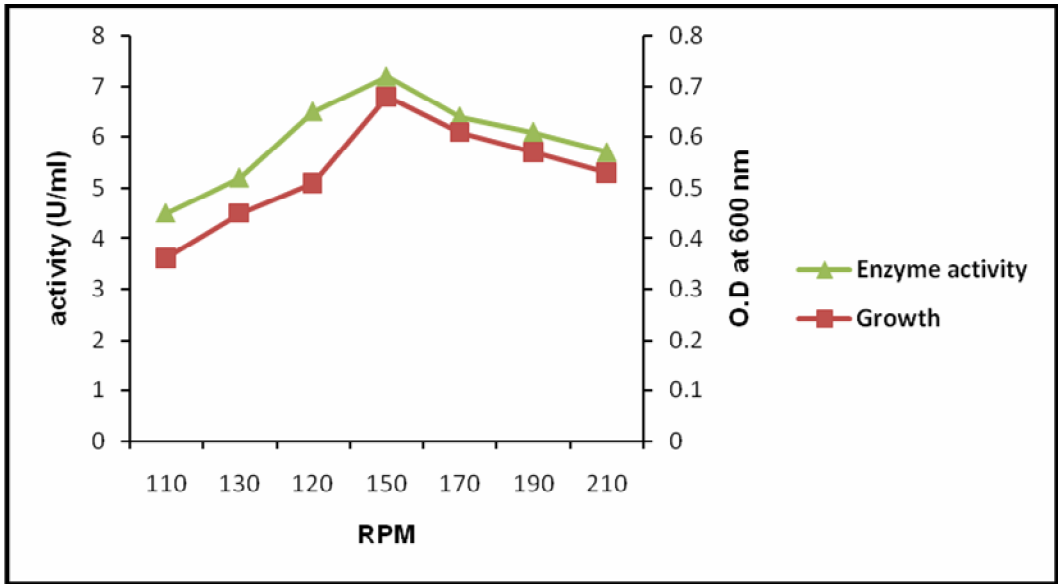


Figure.3 Effect of agitation speed (rpm) on growth and enzyme activity



Effect of minerals sources

Various mineral sources were chosen for enzyme production. Maximum activity was observed in presence of K_2HPO_4 and $MgSO_4.7H_2O$. Medium activity was observed in presence of $FeSO_4$ and $MnSO_4$, very low activity in presence of NaCl. The results are summarized in Table 6.

This work demonstrated basic optimum parameters need to be set for the production of α-galactosidase from different bacterial isolates which helps setting of fermentation process in large scale for industrial level. It is worthwhile to study optimizing parameters of enzyme production as microbial enzymes have

progressively replaced enzymes from other sources and might constitute 90% of the total enzyme market. α-galactosidase possess wide range of application in the field of therapeutics and food processing, the above investigations allow further standardization of industrial processing to demand of present market.

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