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# ISOLATION AND CHARACTERIZATION OF α–L-RHAMNOSIDASE PRODUCING BACTERIUM, *Agrococcus* sp. BKD37, FROM A WAREHOUSE SOIL AND PARTIAL OPTIMIZATION OF ITS CULTURE CONDITIONS

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## ABSTRACT

**The present study was attempted to identify and partially optimize the culture conditions of a potential α-L-rhamnosidase producing bacterium isolated from warehouse soil of Dibrugarh, Assam (India). Twenty eight bacterial strains were isolated and the isolate BKD37 showed maximum rhamnosidase enzyme activity (10.01 ± 0.001 U $mL^{-1}$) in crude culture medium. The 16S rRNA gene sequencing and phylogenetic analysis of BKD37 strain showed maximum similarity with that of *Agrococcus* sp. The culture conditions of strain BKD37 for the production of extracellular rhamnosidase enzyme was partially optimized in a basal medium in shake flask. The maximum rhamnosidase production was achieved after 60 h incubation at 30°C and pH 6.0. The rhamnose and $NH_4NO_3$ were found to be the best carbon and nitrogen sources, respectively, in basal medium for α-L rhamnosidase enzyme production.**

**Keywords:** *Agrococcus* sp., α-L-rhamnosidase, bacterium, characterization, warehouse soil

## INTRODUCTION

Microbial enzymes are widely used in various industrial processes due to their diversity and ease in handling. The screening for useful microbial enzymes prevalent in untapped virgin soil provides ample scopes for bioprospecting of industrially potential microbes in the area (Lyngwi *et al*., 2016). On the other hand, the production of an enzyme from microbial sources is expected to curtail a good amount of resources and promote the development of new industries related to food, beverages and drugs (Chapman *et al.,* 2018). There has been a worldwide attempt to increase the production of novel α-L-rhamnosidase (EC 3.2.1.40) enzyme from various natural sources as well as through manipulation of the existing enzyme by using molecular tools to develop the desired properties.

α-L-rhamnosidase is one of the highly valued enzymes having a wide range of applications in food and pharmaceutical industries. The enzyme specifically cleaves terminal α-L-rhamnose in different natural substrates which include naringin, hesperidin, diosgene, rutin, quercitrin and terpenyl glycosides (Manzanares *et al.,* 2007; Cui *et al.,* 2007). α-L-rhamnosidase catalyzes the hydrolysis of naringin to releases prunin and L-rhamnose; whereas prunin undergoes further hydrolysis by β-D glucosidase enzyme to produce naringenin and glucose. Recently, the enzyme α-L-rhamnosidase has become biotechnologically important due to its role in debittering of citrus fruit juices, in production of rhamnose, prunin, biotransformation of antibiotics and in the enchantments of quality of alcoholic beverages (Orejas *et al.,* 1999; Feng *et al.,* 2005, Puri *et al.,* 2010).

The enzyme α-L-rhamnosidase has been isolated from various sources like animal tissues, plants, yeasts, fungi and bacteria (Yadav *et al.*, 2010). However, the microbial source of enzyme appears to be economically more viable and practicable. Therefore, isolation and identification of new potential rhamnosidase producing microbial strains have gained wide attention as they offer ease in production and molecular manipulation over the existing rhamnosidase isolated from other sources. Microbial α-L-rhamnosidase production has been reported from *Pseudomonas paucimobilis* (Miake *et al.*, 1995), Bacteroides JY-6 (Jang and Kim, 1996), *Sphingomonas* sp. (Hashimoto and Murata, 1998), *Clostridium stercorarium* (Zverlov *et al.*, 2000), *Fusobacterium* K-60 (Park *et al.*, 2005), *Ralstonia pickettii* (Orrillo *et al.*, 2007), *Lactobacillus plantarum* NCC245 (Avila *et al.*, 2009), *Staphylococcus xylosus* MK2 (Puri *et al.*, 2010), *Aspergillus niger* (Ni *et al.*, 2011), *A. terreus* (Soria *et al.*, 2012), *Streptomyces avermitilis* (Ichinose *et al.*, 2013), *Clavispora lusitaniae* (Singh *et al.*, 2015), *Novosphingobium* sp. PP1Y (De Lise *et al.*, 2016), *Alternaria alternata* (Zhang *et al.*, 2018), *Aspergillus niger* (Wang *et al.*, 2019). Further, several reports are available on the attempt to isolate and purify rhamnosidase enzymes from various sources.

After isolation of potential microbes with desired properties, the biochemical characterization of strain must be followed by molecular identification to overcome future taxonomic confusions and molecular manipulation (Petrosino *et al.*, 2009). Similarly, the physiological characterization enables to optimize the culture conditions for maximum production of desired enzyme (German *et al.*, 2011).

The North-East region of India is identified as part of the Indo-Burma Mega Biodiversity hot spot (Myers *et al.*, 2000) with a high probability of finding industrially important microbial strains with diverse genetic resources (Singh *et al.*, 2014). The screening of microbial strains from diverse ecological niches of this region was hypothesized to lead to the isolation of some novel rhamnosidase producing strains. Hence, the present study was aimed to screen rhamnosidase producing microbes from various soil samples collected from different ecological niches and to partially optimize the various culture parameters for maximum production of rhamnosidase enzyme.

## MATERIALS AND METHODS

### *Isolation of microorganisms*

The soil samples were collected aseptically as per standard protocols from various sampling sites of Dibrugarh district, Assam, India. The microorganisms were isolated from the soil samples separately by standard serial dilution technique using solid nutrient agar media containing naringin as an inducer of enzyme production. The inoculums were prepared by adding 1.0 g soil sample to 100 mL nutrient broth and incubated in Orbital shaking incubator (REMI) at 120 rpm for 24 h and 30°C. Then, 1.0 mL culture broth was serially diluted and inoculated into a selective medium having the composition ($L^{-1}$) 3.0 g $NH_4NO_3$, 0.01 g $FeSO_4 7H_2O$, 0.1 g KCl, 0.1 g $KH_2PO_4$, 0.01 g $MnSO_4$, 0.1 g $MgSO_4.7H_2O$, 0.01 g $ZnSO_4$, 1.0 g glucose, 2.0 g yeast extract, 1.0 g naringin, 15.0 g agar (Mukund *et al.*, 2014). The colonies were observed after 24 h of incubation at 37°C and the separate colonies were transferred to sterilized test tube slants containing maintenance medium. The plates were further incubated at 37°C for next 12 h to check for the late/slow-growing colonies. Colonies were marked by the abbreviated name of sample collection site and separately streaked on Petri-plates containing nutrient agar and PDA medium supplemented with inducer naringin (1%) to check the purity. The colonies which appeared on these plates after incubation were picked up and sub-cultured in a nutrient medium with 1% naringin and preserved in a 10% glycerol solution at -70°C for further uses.

### *Assay of α-L-rhamnosidase enzyme*

For screening the potential isolates, one loop-full of pure colonies of different isolates were inoculated into a 250 mL conical flask containing 25 mL basal medium with the composition ($L^{-1}$) 2.0 g $NaNO_3$,

0.2 g NaCl, 0.1 $MgSO_4$, 5 g sucrose, 2 g yeast extract and 3 g naringin for 24 h at 30°C. The rhamnosidase activity of the most potential isolate BKD37 was evaluated in above basal medium with initial pH ranging from 4.0 to 8.0 through a successive increment of 1 pH difference. A freshly grown isolated colony was inoculated to 20 mL basal medium and incubated at 30°C in a rotary shaker (150 rpm) for 24 h and used as inoculum. One mL of the above inoculum was transferred to 100 mL basal medium in a 250 mL conical flask by maintaining triplicate and incubated in an orbital shaker at 30°C and 150 rpm for 72 h. The cell-free supernatant obtained by centrifugation of liquid culture medium at 10,000 rpm for 15 min at 4°C was used as an enzyme source for determining the rhamnosidase activity. The α-L-rhamnosidase activity was determined using ρ-nitrophenyl-α-L-rhamnoside (p-NPR,Sigma Aldrich, USA) as a substrate. The rhamnosidase activity was measured according to Puri *et al.* (2010) with the help of a spectrophotometer (Shimadzu, Japan, DR3900). One unit (U) of enzyme activity was defined as the amount of enzyme required to release 1 µmol of ρ-nitrophenol $min^{-1}$. All the experiments were done in triplicate in a completely randomized design and the results were presented as mean values ± SE.

***Morphological and biochemical characterization***

The morphological tests *viz*., colony characters, Gram's staining reaction and spore formation were performed through microscopic examination of the most effective strain. Biochemical tests were performed using standard protocols of the *Bergey's Manual of Determinative Bacteriology* (Bergey, 2005).

***Molecular characterization***

**i. 16S rDNA sequence analysis**

The molecular identification of potential isolate BKD37 was carried out by 16S rDNA sequencing and homology. The extraction of DNA from the potential rhamnosidase producing strain was done by standard method (Weisburg *et al*., 1991) by using QIAGEN (CA, USA) DNA extraction kit, and its concentration was determined by measuring the optical density (OD) at λ 260/230 and λ 260/280 by UV-Vis spectrophotometer (NanoDrop 2000). The purity of extracted DNA was evaluated on 1% agarose gel where a single band of high molecular weight DNA was observed. The 16S rRNA region of the DNA fragment was amplified by polymerase chain reaction (PCR) using universal primers 27F (16SF-AGAGTTTGATCCTGGCTCAG) and 1492R (16SR-TACGGTTACC TTGTTACGACTT) (Liu *et al*., 1997). The PCR amplicon was purified by using QIAGEN PCR purification kit to remove contaminants and sequenced by using ABI 3730 capillary sequencer (16 capillary) with big-dye terminator reaction.

**ii. Phylogenetic tree analysis**

After amplification, the consensus sequence was generated from forward and reverse sequences, the sequences were assembled using Codon Code Aligner and subjected to Blast program against NCBI-nr/nt database. The taxonomic profiling was done based on the identity percentage (> 97%). Sequence alignment was performed using ClustalW. The evolutionary relationship was determined by the Neighbor-joining phylogenetic tree (Saitou and Nei, 1987). Base substitution was calculated based on Juke-cantor, one parameter model with 1000 bootstrap values in MEGA 6.0 (Kumar *et al.*, 2016). Identified sequences were compared with reference sequences from the NCBI nucleotide database.

**iii. Fatty acid profiling**

The fatty acid profile of selected bacterium BKD37 was done by fatty acid methyl ester (FAME) using Sherlock-Midi system (Buyer, 2002). For FAME analysis, the frozen bacterial isolate was streaked on solidified media, incubated at 28°C for 24 h, and re-checked for purity. The fatty acid profiling was carried out in Royal Life Sciences Pvt. Ltd., Telangana (India) as outsourcing services. Identification and comparison of fatty acids were made to the Aerobe (TSBA version 3.9) database of Sherlock Microbial Identification System. The dendrogram program of MIDI software package was used to produce un-weighted-pair matching based on fatty acid compositions.

***Partial optimization of culture condition for rhamnosidase production***

The favorable physicochemical and environmental parameters for maximum rhamnosidase production such as incubation period, temperature, pH, carbon sources and nitrogen sources were determined in shake flasks maintained at 150 rpm. To study the effect of carbon and nitrogen sources on rhamnosidase production, different carbon sources such as glucose, fructose, sucrose, starch, naringin and rhamnose at 1% (w/v) and nitrogen sources, namely, yeast extract, peptone, urea, $NH_4NO_3$, beef extract and soybean meal at 0.5% (w/v) were added to the basal medium in separate flasks. The fermenter components were autoclaved separately at 121°C for 20 min (Holman 1989). The effects of inoculum age (12-72 h), inoculum size (1-3%, v/v) were also studied. The effect of pH (4.0-9.0) and temperature (30-50°C) on the growth and rhamnosidase production were also studied. After every 12 h intervals 2 mL culture broth was withdrawn aseptically to measure the rhamnosidase activity and growth. The growth along the incubation period was determined spectrophotometrically by measuring the turbidity of culture broth at 600 nm (Mukherjee *et al.,* 2009).

***Statistical analysis***

The results were presented as arithmetic mean of three independent replicas ± SE. Determination of standard error of data obtained was carried out by the Statistical Analysis System (SAS) with the help of the Statistical Software Program 'SPSS version 16'.

## RESULTS AND DISCUSSION

***Isolation of rhamnosidase producing microbes***

Altogether 28 microbial colonies were isolated from 8 soil samples based on morphological distinctness. The quantitative assay of isolates for rhamnosidase activity was carried out in basal medium and the results presented as mean of unit activity $mL^{-1}$ (triplicates) ± SE [Table 1]. The isolate BKD37 showed maximum rhamnosidase activity (10.01 U $mL^{-1}$), followed by isolates KOS1 (8.4 U $mL^{-1}$) and DTS6 (6.66 U $mL^{-1}$). The rest isolates did not show any significant rhamnosidase activity. The minimum rhamnosidase activity was found in isolates TS14 (0.8 U $mL^{-1}$) and PFS5 (0.37 U $mL^{-1}$). Based on quantitative assay, the bacterial isolate BKD37 with highest rhamnosidase activity was selected for further study. This bacterial strain was isolated from the soil samples of a warehouse of Dibrugarh, Assam, India.

**Table 1**: **Rhamnosidase activity of bacterial isolates from various soil samples**

| S. No. | Isolate code | Enzyme activity (U $mL^{-1}$) | S. No. | Isolate code | Enzyme activity (U $mL^{-1}$) |
|---|---|---|---|---|---|
| 1 | DTS1 | 2.18 ± 0.020 | 15 | PFS1 | 2.07 ± 0.009 |
| 2 | DTS2 | 6.66 ± 0.006 | 16 | PFS2 | 5.33 ± 0.006 |
| 3 | DTS3 | 2.68 ± 0.004 | 17 | PFS3 | 3.57 ± 0.003 |
| 4 | DTS4 | 5.09 ± 0.004 | 18 | PFS4 | 0.37 ± 0.006 |
| 5 | KOS1 | 8.40 ± 0.003 | 19 | BKD30 | 4.99 ± 0.002 |
| 6 | KOS2 | 4.81 ± 0.003 | 20 | BKD35 | 2.09 ± 0.030 |
| 7 | KOS3 | 1.39 ± 0.002 | 21 | BKD37 | 10.01 ± 0.001 |
| 8 | CGS2 | 0.41 ± 0.003 | 22 | BKD40 | 1.50 ± 0.070 |
| 9 | CGS10 | 2.70 ± 0.003 | 23 | BKD | 4.95 ± 0.010 |
| 10 | CGS12 | 0.59 ± 0.001 | 24 | MS3 | 7.25 ± 0.080 |
| 11 | TG2 | 2.09 ± 0.024 | 25 | MS4 | 3.09 ± 0.002 |
| 12 | TG3 | 3.51 ± 0.004 | 26 | TS2 | 2.70 ± 0.006 |
| 13 | TG4 | 0.77 ± 0.005 | 27 | TS3 | 6.50 ± 0.004 |
| 14 | TGS5 | 1.05 ± 0.002 | 28 | TS4 | 0.80 ± 0.001 |

***Morphological and biochemical characteristics of strain BKD37***

The potential rhamnosidase producing bacterium isolate BKD37 which produced circular yellow-coloured colonies in nutrient agar medium and grew well in a temperature of 20-40°C (optimum 30°C). The bacterium was Gram positive and rod-shaped with cell length measuring 0.7 -1.0 µm and

cell width between 0.1- 0.5 µm. The bacterium was strictly aerobic, non-spore-forming and non-motile. The strain tested positive for catalase, oxidase, starch hydrolyzing and methyl red and Voges-Proskaur tests, but tested negative for casein and gelatin hydrolyzing activity, citrate utilization and indole production reaction. Acid production from glucose, fructose and sucrose was positive but negative from maltose and lactose. These physiological properties were partially similar to that of *Agrococcus carbonis* (Dhanjal *et al.,* 2011) and *Agrococcus baldri* (Zlamala *et al.,* 2002) Thus, the morphological and biochemical comparison of our bacterial strain BKD37 indicated its similarity with these strains of genus *Agrococcus*.

***Molecular characterization and phylogenetic analysis***

The 16S rDNA sequence of bacterial isolate BKD37 was submitted to the NCBI GenBank database with Accession No. MK447931.1. The 16S rDNA of BKD37 having 1409 base pairs was amplified by PCR. The phylogenetic tree was constructed using Neighbour-Joining method in Mega-X and compared with other bacterial gene sequences [Fig. 1]. Analysis of phylogenetic tree revealed that the sequence clustered with other *Agrococcus* spp. with high bootstrap value which indicated its significant phylogenetic relationship with *Agrococcus* spp.

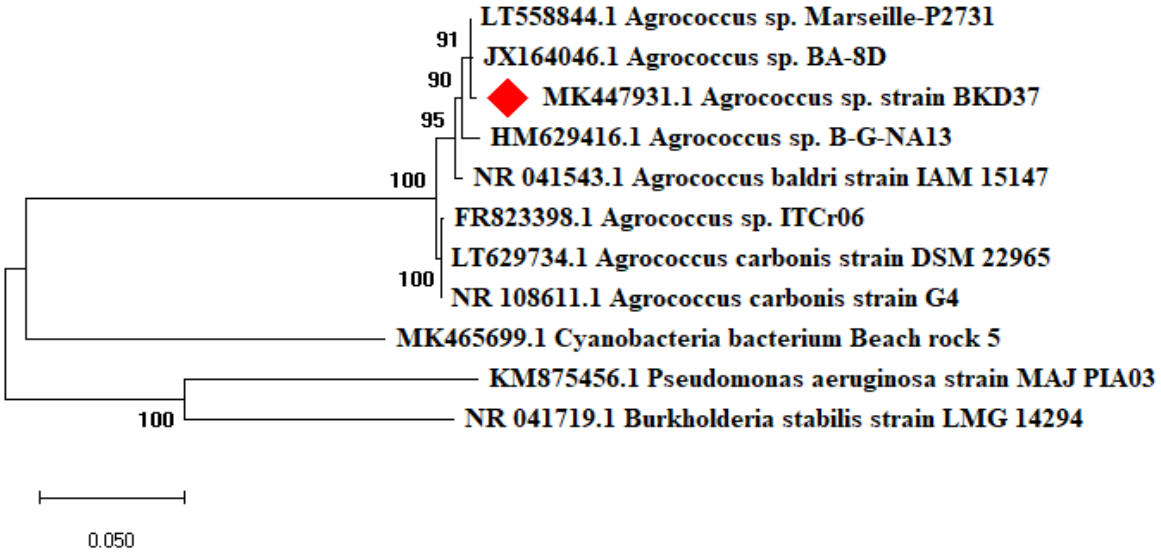


**Fig. 1: Neighbour-Joining tree constructed using16s rDNA sequence (partial) of *Agrococcus* sp. strain BKD37 with 16s rDNA sequences of allied species obtained from BLAST analysis**

***Fatty acid profile of Agrococcus strain BKD37***

A wide range of different fatty acids has been reported from microbial sources (O'Leary, 1962). The objectives of screening fatty acids in microorganisms have two dimensions. Firstly, the fatty acid profile represents the physiological responsiveness to the surrounding ecological niche it inhabits and the biochemical keys of taxonomical components (Groth *et al.,* 1996, Diamonde *et al.,* 2015). Secondly, in recent times, a microbial source has been viewed as ideal to explore and isolate commercially essential molecules including poly-unsaturated fatty acids (PUFA) (Tonato *et al.,* 2018). The result of fatty acid analysis of *Agrococcus* sp. BKD-37 strain is shown in Table 2. The data showed that the bacterial strain possessed 14 different fatty acids of varied chain length ranging from $C_{11}$ to $C_{20}$ dominated by branched saturated fatty acids. The concentration of branched saturated fatty acid C15:0 (32.24%) was highest, followed by C17:0 (26.43%), C15:1 (9.46%) and these were the major fatty acids found in the strain. The fatty acids C18:0 (2.99%) and C17:1 (2.18%) were also present in a considerable amount. In the list of fatty acids, 8 were present in less than 1%. Moreover, the majority of dominant fatty acids in the list of present analysis were branched fatty acid chains as

**Table 2: Major fatty acid composition of bacterial isolate BKD37 strain**

| Fatty acids | IUPAC/ Systematic name | Conc. (%) |
|---|---|---|
| 11:0 | 8-Methyldecanoic acid | 0.23 |
| 12:0 | Dodecanoic acid | 0.29 |
| 14:0 | 11-Methyltridecanoic acid | 0.31 |
| 14:1 | 12- Methyltridecanoic acid | 0.22 |
| 15:0 | 12- Methyltetradecanoic acid | 32.24 |
| 15:0Iso | 13-Methyltetradecanoic acid | 1.77 |
| 15:1 | 5(Z)-13-Methyl-5-tetradaecenoic acid | 9.46 |
| 16:0 | 14-Methylpentadecanoic acid | 0.49 |
| 17:0 | 15-Methyhexadecanoic acid | 26.43 |
| 17:1 | (8Z)-8-Hepatadecenoic acid | 2.18 |
| 18:0 | Octadecadienoic acid | 2.99 |
| 18:1 | (9Z)-9 Octadecadienoic acid | 0.89 |
| 19:0 | Nanadecanoic acid | 0.26 |
| 20:0 | (16Z)-16-Icosenoic acid | 0.26 |

indicated in a report on *Bacillus* sp. (Diamonde *et al.,* 2015). The branched-chain fatty acids are major components of cell membrane due to its lower melting temperature that increases membrane fluidity (Diamonde *et al.,* 2015). Several studies suggest that branched-chain fatty acids and polar fatty acid composition along with other unique molecules may be used as markers for the identification of bacterial strains (Wieser *et al.,*1999; Li and Cao, 2010). Similar studies also reported the fatty acids (C15: 0; 44.9-56.1% of total), (anteiso-C17:0; 26.9-30.0% of the total) (iso-C15:0; 8.2-11.6% of the total) for the species like *Agrococcus casei* (Bora *et al.,* 2007); Anteiso-C15: 0 (49.2%) and iso-C16: 0 (22.4 %) for species *Agrococcus terreus* - DNG5$^T$ (Zhang *et al.,* 2010) and anteiso15:0 (41.6%) and anteiso17:0 (32.8%) for *Agrococcus carbonis*-G4$^T$, (Dhanjal *et al.,* 2011). These chemotaxonomic characteristics concerning the fatty acid compositions are attributed to the genus *Agrococcus* (Groth *et al.* 1996; Zlamala *et al.,* 2002; Mayilraj *et al.,* 2006; Lee, 2008; Behrendt *et al.,* 2008). Therefore, owing to similar characteristics in fatty acid composition and other properties, our strain BKD37 appeared to be a species of genus *Agrococcus.* However, further studies may be needed to explain all the aspects of fatty acids present in the strain. The fatty acid profiling is a relatively new approach towards understanding the physiology and molecular identity of an organism. The present study was an attempt to have an insight into the fatty acid profile of the BKD37 strain isolated from soil samples of a warehouse where food grains are stored. This study alone may have little attribute toward the taxonomic identity of the strain, but provides important information about the molecular signature of the species and contributes to the understanding of the strain's physiological and environmental response to variable ecological niches (Logue *et al.,* 2015). Several reports suggest that the fatty acid pool of micro-organisms change in response to the surrounding environment and from strain to strain within a species (Ratledge, 2004). Similarly, the present study does not correlate fatty acid analysis with the rhamnosidase enzyme productivity.

***Effect of carbon sources on α-L-rhamnosidase enzyme production***

The physicochemical factors of culture medium play a critical role in the production of bio-molecules by microorganisms as a response to the surrounding environment. Thus, the rate and product of a particular fermentation using the desired microbe can be manipulated either by controlling the parameters or by gene cloning (Tonato, 2018). Differential response of the *Agrococcus* sp. BKD37 to various carbon (C) sources was recorded on the basis of rhamnosidase activity. The rhamnose was found to be the best carbon source for stimulating rhamnosidase production with enzyme activity of 13.43 U mL$^{-1}$ at 60 h along with a corresponding increase in the growth of culture (OD = 1.3) {Fig. 2A]. The other carbon sources like naringin (9.8 U mL$^{-1}$) and sucrose (5.23 U mL$^{-1}$) also enhanced rhamnosidase enzyme productivity, whereas minimum activity was observed when maltose and sucrose were supplemented as carbon source. The potential of carbon sources on growth and rhamnosidase production was in the order: rhamnose > naringin > sucrose > starch > lactose. Similar results have been reported by many workers on different microbes like *Aspergillus terreus* (Elinbaum *et al.,* 2002), *Aspergillus kawachii,* (Koseki *et al.,* 2008), *Clavispara lusitaniae* KF633446 (Singh *et al.,* 2018) while grown on rhamnose as the sole carbon source. Since both rhamnose and naringin are expensive to be used as carbon sources, the sucrose in combination

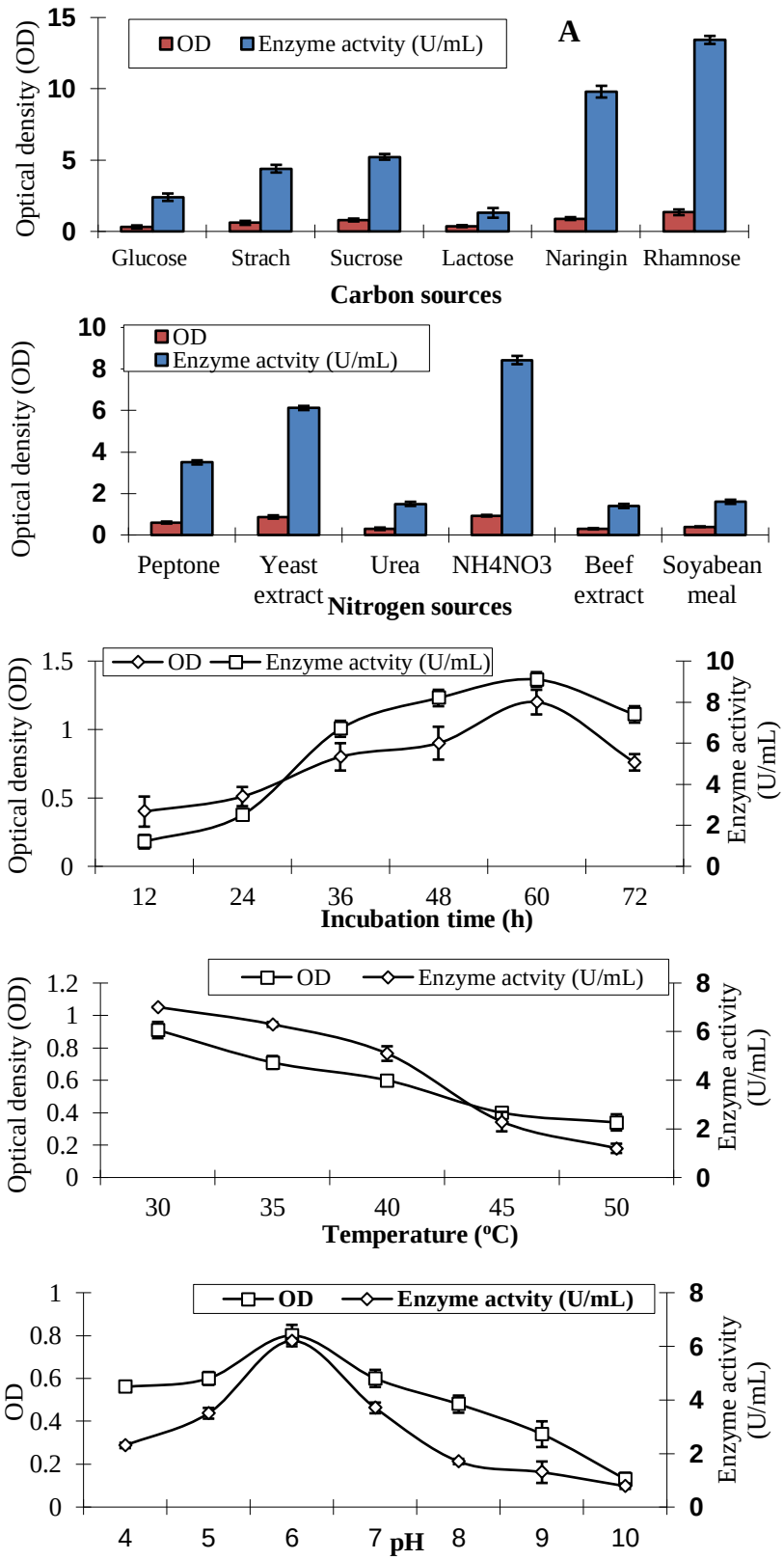


**Fig. 2: Effect of some production factors on enzyme activities**, A: carbon source; B: Nitrogen source; C: Incubation time; D: Temperature and D: pH

with a minimal amount of rhamnose/ naringin as an enzyme stimulator may be more effective in this respect. However, further screening may be needed to find a better alternative of rhamnose as carbon source from economically viable resources to scale up the enzyme production.

***Effect of nitrogen sources on rhamnosidase enzyme***

The effect of different nitrogen sources was tested for rhamnosidase production basal medium. Nitrogen is a secondary energy source which play a vital role in the growth and metabolic activity of bacteria. The nitrogen requirement for optimal rhamnosidase production is species dependent (Koseki *et al.*, 2008). The results regarding the effect of nitrogen sources on rhamnosidase production are presented in Fig. 2B. A significant amount of rhamnosidase activity was recorded when $NH_4NO_3$ was used as nitrogen source. The crude enzyme of culture medium containing $NH_4NO_3$ and yeast extract exhibited the highest rhamnosidase activity *viz.*, 8.43 U $mL^{-1}$, and 6.12 U $mL^{-1}$, respectively, after 60 h cultivation. Influence of nitrogen sources on rhamonosidase production was in the order of $NH_4NO_3$ > yeast extract > peptone > yeast extract > soybean meal > urea > beef extract. Thus, it is evident that both organic and inorganic

nitrogen sources are invariably used by strain BKD37 for the production of enzyme α-L-rhamnosidase. The lowest enzyme activity of 1.5 and 1.6 U $mL^{-1}$ was recorded in the crude enzyme-containing urea and soybean meal, respectively. A similar finding has been reported by studies where $NH_4NO_3$ and yeast extract have been depicted as an excellent nitrogen source for higher rhamnosidase productivity in an artificial medium (Thammawat *et al.,* 2008). Another study conducted by (Puri *et al.,* 2011) indicated citrus peel powder as an excellent nitrogen source for higher rhamnosidase production.

***Effect of incubation period on α-L-rhamnosidase enzyme production***

The duration of incubation time played an important role in the production of rhamnosidase. The optimum incubation period for higher growth and rhamnosidase enzyme productivity was determined. The culture broth were incubated upto 72 h and the growth as well as enzymatic activity was determined at intervals of 12 h incubation. After 60 h incubation, the maximum growth (1.2 U $mL^{-1}$) and high rhamnosidase activity (9.1 U $mL^{-1}$) was recorded [Fig. 2C]. Maximum rhamnosidase activity was recorded just after the isolate BKD37 reached stationary growth phase and remained almost constant upto 72 h after which the enzyme activity declined. The reduced production time is an important factor as it decreases the fermentation costs as well as the risk of contamination by opportunistic microorganisms during the fermentation process (Rajal *et al.,* 2009). The BKD37 strain produced maximum growth and enzyme production at 60 h of cultivation, and thereafter showed gradual decrease in growth and enzyme activity. However, Singh *et al.* (2015**)** reported that *Clavispora lusitaniae*-84, a yeast strain, produced α-L-rhamnosidase in just 48 h incubation period in selective medium.

***Effect of incubation temperature on α-L-rhamnosidase production***

The incubation temperature required for maximum production of rhamnosidase enzyme was studied at temperatures ranging from 30 to 50°C. The maximum growth (OD = 0.91) and optimum enzyme activity (7.01 U $mL^{-1}$) for BKD37 strain was found at incubation temperature of 30°C, followed by 35°C (6.3 U $mL^{-1}$), 40°C (5.1 U $mL^{-1}$) and 45°C (2.3 U $mL^{-1}$) [Fig. 2D]. The minimum growth and activity were noticed at 50°C (1.2 U $mL^{-1}$). In present study, an exponential growth pattern was observed in temperature range of 10 to 30°C, while growth ceased at temperature < 10°C and > 50°C. Reports reveal that the optimum temperature for maximum rhamnosidase activity in *Staphylococcus xylosus* MAK2 and *Clavispora lusitaniae* was 30°C (Puri *et al*., 2010; Singh *et al*., 2015). Thus, it appears that 30°C is the optimum temperature for rhamnosidase enzyme activity. The optimum temperature for growth and rhamnosidase production varies in different species (Yadav *et al.,* 2010). Several fungal species reportedly have a temperature range of 40-65°C for rhamnosidase production (Orejas *et al.,* 1999; Manzanares *et al.,* 2001; Ge *et al.,* 2017). The optimum temperature for enzyme production by different bacterial species was found to be 50°C in case of *Bacillus* sp. GL1 (Itoh *et al.,* 2004), *Lactobacillus plantarum* NCC 245 (Avila *et al.,* 2009) and *Pediococcus acidilactici* (Michlmayr *et al.,* 2011). However, the optimum temperatures of 30°C with a wide range of incubation periods are favourable for cost-effective enzyme yield (Arcus *et al.,* 2016).

***Effect of pH on α-L-rhamnosidase enzyme production***

The pH of medium plays crucial role in growth rate and enzyme productivity of microbes (Booth, 1985). The effect of pH on the growth and rhamnosidase production is shown in Fig. 2E. The study showed a significant effect of pH on growth and rhamnosidase productivity of strain BKD37. The highest rhamnosidase enzyme activity (6.2 U $mL^{-1}$) and bacterial growth (0.8) was observed at pH 6.0 and lowest at pH 10.0. When the pH of culture medium was raised from initial value 4.0, there was a gradual increase in growth and enzyme production until it reached pH 6.0 which was followed by gradual decline upto pH 8.0 [Fig. 2E]. The result indicated a broad pH range in which bacterial strain BKD37 could grow and produce rhamnosidase enzyme. In present study, rhamnosidase activity was at par in slightly acidic pH like 4 (2.3 U $mL^{-1}$) and 5.0 (3.5U $mL^{-1}$) as well as a neutral pH 7.0 (3.7 U $mL^{-1}$). Similar observations have been recorded in rhamnosidase enzymes in different microbes like *Pichia angusta (*Yanai and Sato, 2000*), Pseudoalteromonas species* (Orrillo *et al.,*

2007) and *Lactobacillus acidophilus* (Beekwilder *et al.*, 2009) showing optimum activity at pH 6. Shanmugam and Yadav (1995) reported pH 6.5 as optimum for rhamnosidase enzyme from *Rhizopus nigricans* and beyond this pH, there was a significant decrease in rhamnosidase productivity.

Since the enzyme activity was determined using crude culture medium as an enzyme source, the calculated catalytic values may be a combined effect of growth and that of the catalytic properties of the enzyme. Thus, further studies are required for molecular characterization of enzyme α-L-rhamnosidase, derived from strain *Agrococcus* sp. BKD37. Based on present study of optimal growth and enzyme production under different culture parameters, an optimal culture medium may be suggested for strain BKD37 by adding sucrose with a minimal amount of rhamnose as carbon source, $NH_4NO_3$ as nitrogen source, temperature and pH set at 30$^oC$ and 6.0, respectively. As mentioned earlier the optimum time for the harvest of the enzyme is 60 h of cultivation.

**Conclusion:** Based on the studies on colony morphology, physiological properties supported by phylogenetic analysis of 16S rDNA sequence and fatty acid analysis, the bacterial isolate BKD37 was found to be *Agrococcus* sp. This paper is the first report of significant rhamnosidase enzyme production by genus *Agrococcus.* The genus is a relatively less explored group of bacteria which on further screening may provide opportunities for finding economically viable enzymes having industrial applications, especially in food and pharmaceutical industries. Further studies on extraction and purification of enzyme from bacterium *Agrococcus* sp. BKD37 is in progress in our laboratory.

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