Original Article

Purification and Characterization of α-L-rhamnosidase from *Bacillus amyloliquefaciens* –D1

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ABSTRACT

Rhamnosidase enzyme important role in debittering technology of citrus fruit juices. The other industrial sector considered a potential user of wine enhancement, antibiotic preparation, preparation of rhamnose, prunin and hydrolysis of glycoside. The enzyme rhamnosidase enzyme isolated from in animals, plants, and microbial sources. The present study was attempted to purification and characterization of rhamnosidase enzyme from *Bacillus amyloliquefaciens*-D1.α-L- rhamnosidase enzyme which was extracted from the fermented broth of *Bacillus amyloliquefaciens*-D1 was purified about 3.08-fold with yield 35.77 by ammonium sulfate precipitation followed by Sephadex G-100 column chromatography. The purity of the enzyme was confirmed by High-performance chromatography and 12% SDS-PAGE indicate a single peak showed and molecular weight found 67kDa. The purified enzyme optimum pH and temperature were 6.0 and 40°C respectively. The effects of mental activity found showed that Fe^{2+} (131.4%) and NaCl (129.37%) were strong activators, while KCL and Cu^{2+} was a strong inhibitor of the rhamnosidase enzyme production. The enzyme kinetic constants K_m and V_{max} were 15.09 (mg/ml) and 2.22 (mg/ml/min), respectively. Further exploitation of the strain in large scale field application will help in citrus processing and bioprocess industries.

Key words: Ammonium sulfate, *Bacillus amyloliquefaciens,* Column chromatography, HPLC, rhamnosidase, SDS-PAGE.

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INTRODUCTION

An α-L-rhamnosidase is one of the highly valued enzymes having a wide range of applications in the 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.[1] The 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.[2] Recently, the enzyme α-L-rhamnosidase has become biotechnologically important due to its role in debittering citrus fruit juices, in the production of rhamnose, prunin, biotransformation of antibiotics, enchantments of quality of alcoholic beverages, and hydrolysis of rutin.[3-5] The enzyme α-L- rhamnosidase has been isolated from various sources like animal tissues, plants, yeasts, fungi, and bacteria.[6-8] However, the microbial source of enzymes 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 easier production and molecular manipulation over the existing rhamnosidase isolated from other sources. The enzyme a-L-rhamnosidase (E.C. 3.2.1.40), which has been found thus far in plants[9] and animal tissues[10] while many microbial α-L-rhamnosidase productions

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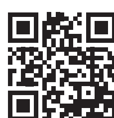

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has been reported from *Pseudomonas paucimobilis,*[11] Bacteroides JY6,[12] *Sphingomonas* sp,[13] *Fusobacterium* K-60,[14] *Ralstonia pickettii,*[15] *Lactobacillus plantarum* NCC245,[16] *Clostridium stercorarium*,[17] *Aspergillus niger*,[18] *Aspergillus terreus,*[19] *Streptomyces avermitilis,*[20] *Aspergillus terreus,*[21] *Clavispora lusitaniae,*[22] *Novosphingobium* sp. PP1Y,[23] *Aspergillus oryzae,*[24] *Alternaria alternata*,[25] *Bacteroides thetaiotaomicron*,[26] *Aspergillus niger*,[27] *Aspergillus niger,*,[28] *Agrococcus* sp.[29] and *Aspergillus niger* JMU-TS528.[30] Further, several reports are available on the attempt to isolate and purify rhamnosidase enzymes from various sources. The present study attempts the purification and characterization of rhamnosidase enzyme from *Bacillus amyloliquefaciens*-D1

MATERIALS AND METHODS

Chemicals

p-Nitrophenyl-a-L-rhamnopyranoside, Spehadex G-100, naringin, L-rhamnose, were purchased from Sigma Chemical Company St. Louis. Protein marker was procured from Sigma. A dialysis tube was procured from Fisher Scientific Company, USA. All other chemicals were of analytical or high purity grade available commercially.

Micro-organisms

Bacillus amyloliquefaciens-D1 isolated from citrus fruits garden in Upper Assam. GenBank Accession number: MK334656.1

Growth and culture condition

A freshly grown isolated colony was inoculated to 100 mL production medium (g L^{-1}: glucose- 5.0, Na_2HPO_4- 6.0, KH_2PO_4- 3.0, NH_4Cl- 1.0, NaCl- 0.5, $MgSO_4$- 0.12, $CaCl_2$- 0.1, naringin- 2 and pH- 6) was used for growth and enzyme production. 50 ml of the resultant medium in Erlenmeyer flask (100 mL) was aerobically cultured at 30°C for 1-4 d on a rotary shaker at 150 rpm. 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.

Assay of α-L-rhamnosidase

α-L-rhamnosidase activity was determined using p-nitrophenyl-α-L-rhamnosidase (p-NPR, Sigma Aldrich, USA) as a substrate. The rhamnosidase activity was measured according to[31] 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 p-nitrophenol min^{-1}. All the experiments were done in triplicate in a completely randomized design and the results were presented as mean values ± SE.

Purification of rhamnosidase enzyme

The cell-free supernatant was precipitated by adding ammonium sulphate at different saturation levels (30 to 90%). After each addition, the enzyme solution was stirred for 1houre at 4°C. The precipitation protein was collected by centrifugation at 8000 × g for 15 minutes at 4°C and re-suspended in a 0.05M citrate buffer (pH 4.5) to obtain the concentrated enzyme suspension. After that, the enzyme suspension was dialyzed in a bag (Sigma 10-100kDa) with same buffer using 4 to 6 time change fresh buffer. Dialyzed fraction was then subjected to gel filtration by Sephadex G-100 column (300 × 10mm). Fraction (5ml/tube) were collected at a flow rate of 15ml/h with fraction collector. The fraction showing absorbance at 280nm was analyzed for protein content and rhamnosidase activity. Protein was measured by method of Lowry[32] with bovine serum albumin as standard. The active fraction lyophilized for further study.

Molecular Mas Determination

SDS-PAGE was performed according to the method followed by[33] using slab gel apparatus (Biotech India). The denatured protein along with different molecular markers (Pharmacia) used Phosphorlyase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0kDa), carbonic anhydrase (30.0 kDa), Soyabean trypsin inhibitor (20.1 kDa), and α-lactabumin (14.4kDa). Developed gels were stained with Coomassie Blue R-250 or silver staining[34] according to the manufacturer's instructions.

HPLC analysis of rhamnosidase

A C_{-18} column (4.6x10 mm) for high performance liquid chromatography (HPLC) (Agilent 1100 Series) was used to test the enzyme purity. The 5uL sample volume was injected and separated using a solvent system of acetonitrile-water (80:20) at a flow rate of 1.0ml/min. A highly sensitive MWD UV detector was used to read the absorbance. p-Nitrophenyl-α-L-rhamnopyranoside in samples was detected at 280nm and identified by comparison of retention times with standard.

Characterization of purified rhamnosidase enzyme

Effect of temperature

The influence of temperature on the activity of rhamnosidase enzymes was measured in the range of 20-90°C. The reaction mixture of enzymes was kept for 30 min at pH 6.0 and the relative activity was determined.

Effect of pH

The effect of pH on the activity of rhamnosidase was measured in the 3.0-10.0 range, using the different buffers at a concentration of 10 mM (3, 4 citrate buffer; 5-acetate buffer; 6-8 phosphate buffer; 9-10, glycine-NaOH) with an incubation period of 30 min at 40°C after that the relative activity was determined.

Effect of different metals ions activity of rhamnosidase

Enzyme assays were performed in presence of different metal ions, at 10 mM concentration. The chloride salts of Zn^{2+}, $Ca^{2+,}$ KCL, Mn^2+, Cu^2+, $Fe^{2+,}$ and NaCl were used. The purified enzyme was pre-incubated with different above different metal ions at 40°C for 30 min and then relative activity was calculated. The activity of the enzyme without any metal ions was considered 100%.

Determination of *Km* and *Vmax*

K_m and V_{max} of the rhamnosidase were determined by measuring enzyme activity with different concentration of p-Nitrophenyl-α-L-rhamnopyranoside as a substrate. Kinetic constants were calculated using Lineweaver–Burk plot.

Statistical Analysis

The arithmetic mean of three independent replications was calculated and tested for standard deviation. Determination of standard error of resulted data was carried out by the Statistical Analysis System (SAS) with the help of the statistical software program 'SPSS version 16'.

RESULTS

Purification of rhamnosidase enzyme

The rhamnosidase enzyme was produced from *Bacillus amyloliquefaciens*-D1 by submerged fermentation at 35°C for a 48h fermentation periods. Result was summarized (Table 1) by purification steps of the rhamnosidase enzyme. The crude enzyme solution was fractionated by ammonium sulphate precipitation. After 60% ammonium sulphate precipitation enzyme suspension was dialyzed using citrate buffer (pH 5.5) for 72h at 4°C. After 60% precipitation was maximum enzyme activity (602.5 IU) compared to other precipitation. The dialyzed enzyme solution was loaded onto a Sphadex G-100 column. The elution profile of the enzyme solution shown in (Figure 1). The fraction numbers 31 to 35 shown higher specific activities 1488.61 (U/mg). From these steps, enzyme-specific activity improved from 482.70 (U/mg) to 1488.61 (U/mg), indicting purification of the enzyme by G-100 chromatography. Thus, these fractions were lyophilized for further study.

Table 1: Purification steps of rhamnosidase enzyme.

Volume (ml)	Purification Steps	Total activity (IU)	Total protein (mg/ml)	Specific activity U/mg	Purification fold	Purification yield (%)
100	Crude extract	1535.23	3.18	482.70	1.00	100
20	Ammonium sulphate precipitation	802.5	1.36	590.07	1.22	52.27
10	Sephadex G-100	549.3	0.369	1488.61	3.08	35.77

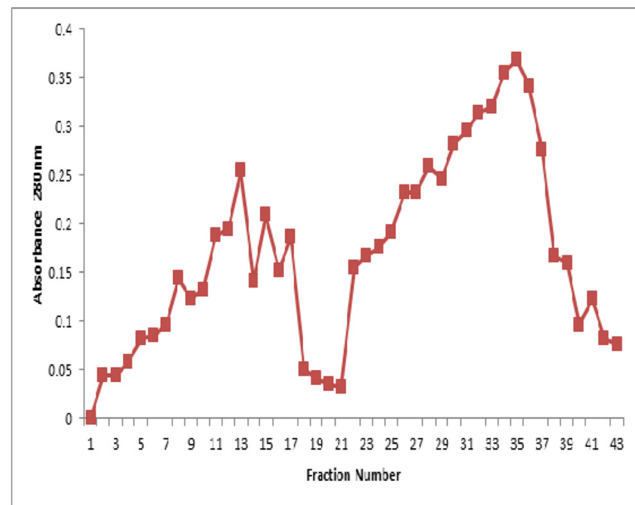

Figure 1: Fraction collection from Sephadex G-100.

The molecular mass of purified rhamnosidase enzyme

The enzyme solution obtained from Sephadex G-100 column chromatography was converting into powder from lyophilization. The result (Figure 2) of SDS-PAGE analysis showed a single protein band with the molecular mass was calculated and found to be approximately 67kDa using relative mobility of the different standard markers. Similar microbial strain *Bacillus amyloliquefaciens*

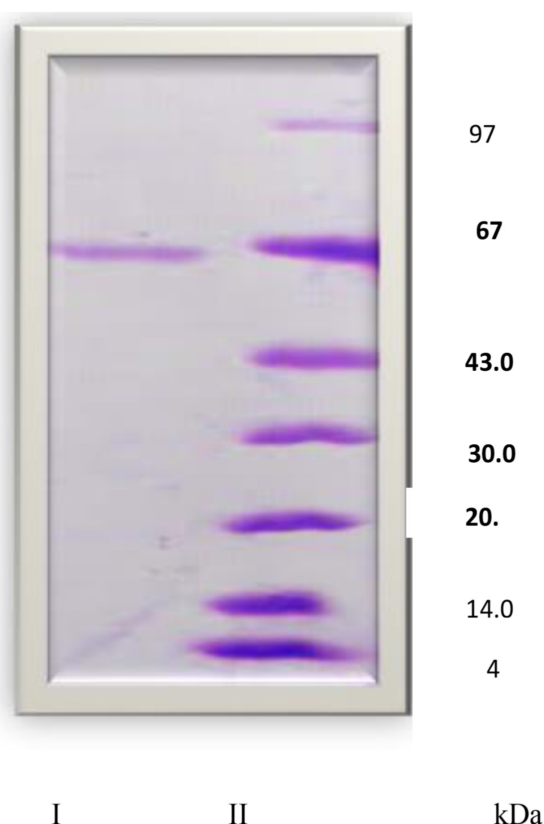

Figure 2: Results of SDS-PAGE analysis of the purified enzyme lane (I) (Purified enzyme)lane (II) phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), Soyabean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4kDa).

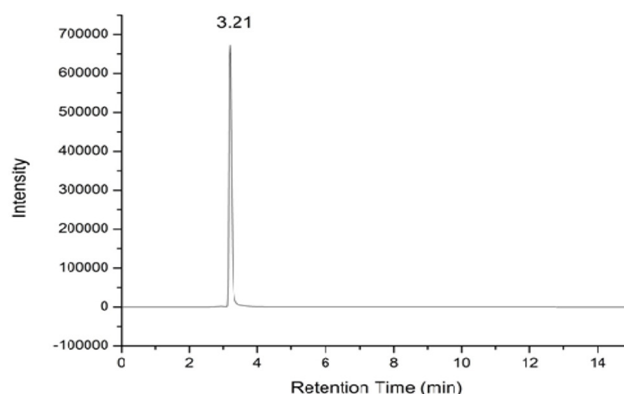

Figure 3(a): Standard curve of p-Nitrophenyl-a-L-rhamno-pyranoside.

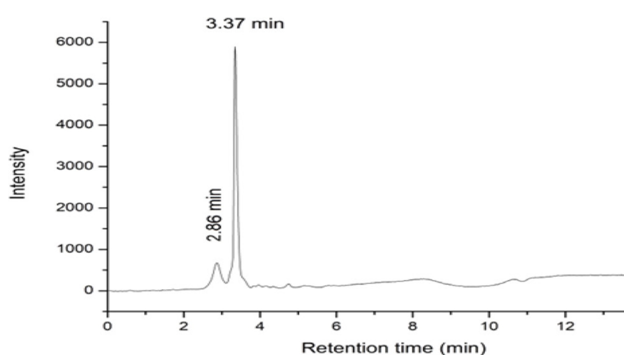

Figure 3b: Sample of purified rhamnosidase enzyme chromatogram curve in HPLC.

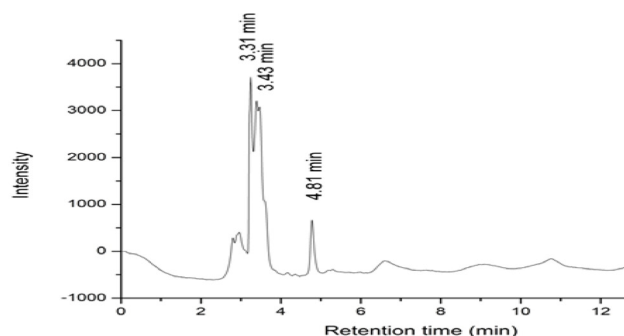

Figure 3c: Crude extract chromatogram in HPLC.

11568 isolated from soil was purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified enzyme gave a single protein band corresponding to a molecular mass of 32 kDa reported by.[35] The molecular mass of rhamnosidase has been reported to range from 40 to 240 kDa.[36] The molecular mass of the rhamnosidase enzyme was found, in different microbes are *Aspergillus terreus 75* kDa,[24] *Penicillium corylopholum* MTCC-2011 67.kDa,[37] *Pichia angusta* 90 kDa.[38] The similar kind of molecular weight found *Penicillium corylopholum* MTCC-2011 67kDa,[39] *Aspergillus niger* JMU-TS528 90kDa,[40] *Aspergillus kawachii* 90kDa.[41] To check the further purity of rhamnosidase enzyme, the enzyme solution loaded on to an HPLC. Result (Figure 3a, 3b, and 3c) enzyme showed a single peak at a retention time 3.37min, confirming that the enzyme solution was pure.

Characterization of Purified Rhamnosidase Enzyme

Effect of pH

The pH of the medium plays a crucial role in enzymes productivity of microbes.[42] The effect of pH on the rhamnosidase production is shown in (Figure 4). The study showed a significant effect of pH rhamnosidase productivity of strain D1. The highest rhamnosidase enzyme activity was observed at pH 6.0 and lowest in the pH 10.0. While the pH of the culture medium was raised from its initial value of 4.0. There was a gradual increase in the growth and enzyme production until it reached pH 6.0 which was followed by a gradual decline up to pH 8.0. The result also indicated a broad pH range in which bacterial strain D1could produce

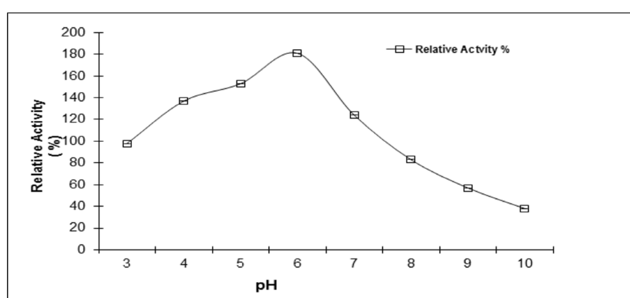

Figure 4: Effect of pH.

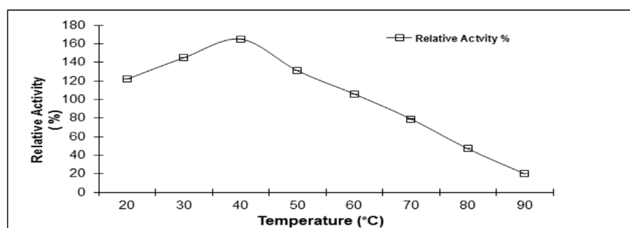

Figure 5: Effect of temperature.

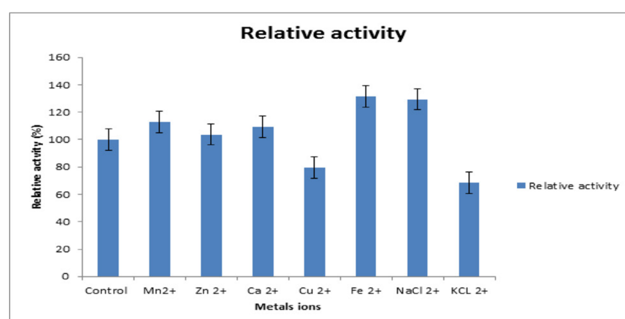

Figure 6: Effect of metal ions.

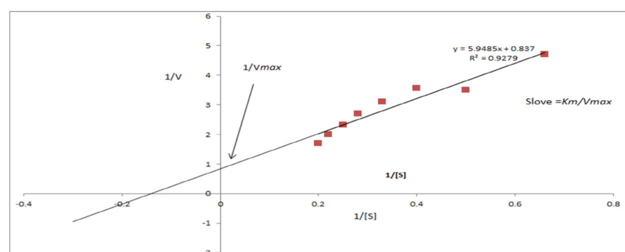

Figure 7: Lineweaver –Burk plots of *Bacillus amyloliquefaciens* -D1strain.

rhamnosidase enzyme. The present study, rhamnosidase activity was found at par in slightly acidic pH like 4 and 5.0 as well as a neutral pH 7.0. Similar observations have been recorded in rhamnosidase enzymes in different microbes like *Pichia angusta,*[38] *Pseudoalteromonas* sp.,[15] *Lactobacillus acidophilus*[43] showing optimum activity at pH 6.

Effect of temperature

The rhamnosidase activities were assayed at different temperatures (20°C to 90°C) to find the optimum temperature. The result (Figure 5) indicated that enzyme activity increase with an increase in temperature until peak activity was observed at 40°C. After that, as the temperature was increased from 40°C to 90°C, a sharp decline in enzyme activity was observed. Rising the temperature to 90°C caused denaturation of the enzyme. The optimum temperature for purified rhamnosidase others microbes was noted at temperature are *Aspergillus kawachii* 50°C,[41] *Acrostalagmus luteoalbus* (55°C),[44] *Pediococcus acidilactici* 50°C,[45] *Aspergillus niger* JMU-TS528 60°C.[30]

Effect of metals ions

To obtain the effect of metal ions (10mol/L), the enzyme was treated with different metal ions for 30 min at 40°C. After that, the enzyme activity was determined using a standard procedure. The result (Figure 6) shows that Fe^{2+} (131.4%) and NaCl (129.37%) were strong activators, while KCL and Cu^{2+} was a strong inhibitor of the rhamnosidase enzyme (Figure 6). The stimulation and inhibition of the enzyme activity depend on the active site of the enzyme. These findings were mostly in following with the other studies.

Enzyme kinetics

The enzyme kinetic parameters K_m and V_{max} of the rhamnosidase enzyme were estimated by Lineweaver–Burk plot using various concentrations of p-Nitrophenyl-a-L-rhamnopyranoside as a substrate. The main purpose of estimating enzyme kinetics is to obtain the catalytic efficiency of proteins. The result (Figure 7) revealed that the K_m and the V_{max} of rhamnosidase 15.09mg/ml and 2.22 mg/ml/min, respectively. Different microbial strains showed different K_m and V_{max} value.[6] rhamnosidase from *Alternaria alternata* SK37.001 the enzyme kinetic parameters of K_m and V_{max} were 4.84 mM and 53.1 µmol mg/min respectively.[25]

DISCUSSION

Purification and characterization are important steps to understand enzymes for their further study and applications. With the increasing industrial demands for biocatalysts to scale up with the industrial process conditions, continuous efforts are being made for the search of the useful enzymes from natural sources. Even though, the fact that large numbers of the different enzymes have been identified and many are being used

in the different industrial sectors. The available enzymatic selection is still not sufficient to meet the ever increasing demand. Microbial enzymes are preferred to those from both plant and animal sources because they are inexpensive to produce, and their enzyme contents are more predictable, controllable and reliable. *Bacillus* has been recognized for its biotechnological applications on a large scale. Current endeavors have indicated the capability of the potential of the *Bacillus* genus which includes industrial production of enzymes with great interest in detergent and food sectors.[46] They are reported for the production of primary metabolites such as vitamins and ribonucleosides and secondary metabolites including bacteriocins and biosurfactants and of plant growth-promoting formulations, generate biofuels (hydrogen), biopolymers (polyhydroxyalkanoates), and bioactive molecules.[47] In this context, purification of intracellular microbial enzymes rhamnosidase assumed significance and it will help in understanding the cellular metabolism and regulatory pathway. It is also significant for the commercial production of some of industrial sectors and pharmaceutically important enzymes. Microbial enzyme metabolism has been studied frequently, but very little work related to this has been included in studies with modified chemically defined media. Downstream processing is fundamental for any fermentation process and it involves isolation and purification sequences to obtain a pure and homogenous product like enzymes.[48] Strain D1 was *Bacillus* is generally considered a dynamic microorganism that can survive in antagonistic environmental conditions and grow easily to very high densities. The *Bacillus amyloliquefaciens* in the food processing industry such as debittering citrus fruit juice and improved the bioavailability of polyphenols.[35] *Bacillus amyloliquefaciens*-LN is a non-hemolytic, non-enterotoxin producing, and showed probiotic characteristics collectively with acidic tolerance, bile salt tolerance, and anti-pathogenic activities.[49] Hence, the strains of *Bacillus amyloliquefaciens* have got possibilities to be used as a feed additive to reduce the concentrations of ZEN in feedstuffs. B. *amyloliquefaciens* D1 produces a significant amount of extracellular naringinase in the medium.

CONCLUSION

The rhamnosidase enzyme has potential application of in diverse industrial sectors, such as pharmaceutical, food and agriculture industry. Many microbial rhamnosidase obtained from different sources have been extensively purified and characterization in terms of molecular weight, pH, temperature and different metal ions. Present study shown that rhamnosidase which was extracted from the fermented broth of *Bacillus amyloliquefaciens* –D1 was purified about 3.08-fold with yield 35.77 by ammonium sulphate precipitation followed by Sephadex G-100 column chromatography. The purity of the enzyme was confirmed by High-performance chromatography and 12% SDS-PAGE indicate a single peak has shown and molecular weight found 68kDa. The purified enzyme optimum pH and temperature were 6.0 and 40°C respectively. An effect of metal activity found showed that Fe^{2+} (131.4%) and NaCl (129.37%) were strong activators, while KCL and Cu^{2+} was a strong inhibitor of the rhamnosidase enzyme production. The enzyme kinetic constants K_m and V_{max} were the K_m and the V_{max} of naringinase 15.09mg/ml and 2.22 mg/ml/min, respectively. The bacteria as enzyme sources have many advantages that, the rhamnosidase enzymes produced are normally extracellular, making easier production for the downstream process. The development of economically feasible technologies for rhamnosidase production and the enzymatic hydrolysis of rhamnosidase materials will enable to utilize the large quantities of the substrate such as the residues of both food industries and agriculture. Based on the findings, it can be concluded that due to the potential rhamnosidase activity of the strain *Bacillus amyloliquefaciens* D1, further exploration of the strain both at biochemical and molecular levels along with large scale field application can be exploited in citrus processing and bioprocess industries.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

SAS: Statistical Analysis System; **SDS:** Sodium dodecyl sulfate; **p-NPR:** p-nitrophenyl-α-L-rhamnosidase; **kDa:** Kilodaltons.

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