World Journal of Pharmaceutical Sciences
ISSN (Print): 2321-3310; ISSN (Online): 2321-3086
Published by Atom and Cell Publishers
Available online at: http://www.wjpsonline.org/
Original Article

Purification and characterization of β-galactosidase from leaves of *Zizyphus oenoplia*

Ashwin Butle and M. B. Patil*

Department of Biochemistry, RTM Nagpur University, Nagpur - 440033, India

Received: 17-05-2015 / Revised: 23-06-2015 / Accepted: 24-06-2015

ABSTRACT

A novel β-galactosidase was purified to homogeneity, from *Zizyphus oenoplia* leaves, using chilled acetone precipitation followed by ammonium sulphate precipitation and affinity chromatography on cross linked g uar-gum. The enzyme was a monomeric with a molecular weight of about 23 kDa on SDS-PAGE. It was active between pH 4 - 7, with an optimum activity at pH 5.5, and was fairly stable from pH 4.5 to 8.0. The enzyme showed optimum activity at 37° and was stable up to 80 °. The presence of metal ions such as Mg++ and Mn++ positively influenced the activity of β-galactosidase but the activity was inhibited in the presence of Cd++, Hg++ and Pb++. The enzyme showed maximum activity towards β – oNPGal, the substrate α – pNPGal.

Keywords: Zizyphus , Enzyme, β- galactosidase, Affinity chromatography, Protein

INTRODUCTION

β-D-Galactosidase (EC 3.2.1.23, β-D galactoside, galactohydrolase, lactase) catalysis the non-reducing β-D-galactosyl residues from polysaccharides. They are widely distributed in plants, microorganisms and animals [1, 2]. This enzyme has many industrial and medicinal applications like cleavage of blood group A and B glycotopes, biosensors for lactose determination and enzymatic hydrolysis of lactose in whey and milk products [3]. Especially enzymatic hydrolysis of lactose has many advantages in food industry. Lactose hydrolyzed products decrease the lactose intolerance problems [4]. Lactase hydrolyses lactose to glucose and galactose and nutrition value of milk treated by lactase, does not decrease, because the hydrolysis products of lactose as glucose and galactose are not removed from the system [4, 5]. Furthermore, compared to lactose, addition of released glucose and galactose adds more sweet taste to the products [5–7]. β-galactosidase has been purified from different plants like kiwifruit, mango, kidney beans, pea and also chick pea [8–12]. *Ziziphus oenoplia* M., belonging to family Rhamnaceae (vernacular name: Siakul) is a shrub, distributed in tropical and subtropical India in dry climates. The *Zizyphus* genus has been reported to have many medicinal properties [13, 14]. There have been a number of reports about purification and recovery of enzymes such as β-galactosidase from *Piper bettle* leaves [15, 16]. Isoforms of β-galactosidases have been reported in mung bean seedlings [17], papaya *(Carica papaya)* seeds [18], and from *Nasturtium (Tropueolum mujus* L.) cotyledons [19]. In the present research paper, characterization of β-galactosidase isolated from leaves of *Zizyphus oenoplia* has been reported. According to the literature survey this appears to be the first report on isolation of β-galactosidase enzyme from leaves of *Zizyphus oenoplia*. The enzyme has been purified by using unique techniques and characterized by following all standard protocols. Homogeneity of purified enzyme was checked by native elcectrophoresis and SDS–PAGE. Molecular weight of purified enzyme was determined by SDS–PAGE using standard protein markers.

MATERIALS AND METHODS

Materials: Leaves were collected from single identified tree, allowed to shade dry and stored in vacuum tight container. The plant was taxonomically identified in the Herbarium of the Department of Botany of the RTM Nagpur University. Coomassie Brillant Blue R-250 and substrate o-nitrophenyl-β-D galactopyranoside (oNPGal), p-nitrophenyl-α-D galactopyranoside (pNPGal) were purchased from Sigma (St. Louis, MO, USA). Molecular weight markers were obtained from Merck (Eurolab GmbH Darmstadt, Germany). Acetone, acrylamide, guar-gum, various

***Corresponding Author Address: Dr. M. B. Patil**, Professor, University Department of Biochemistry, RTM Nagpur University, Nagpur Mob. No.: 9860018333, email: mbpatil@hotmail.com*

carbohydrates, methylene bisacrylamide and all other reagents were of analytical grade.

Methods:

Extraction: Dried leaves from *Zizyphus oenoplia* were powdered by blender. Ten g powdered leaves were homogenized in a blender with 100ml of 0.2M sodium phosphate buffer, pH 7 (PBS), kept on the shaker at 4 º for 1h, filtered through folds of cheese cloth, centrifuged at 10,000 rpm for 10 m (Remi C24). The resultant supernatant, designated as crude extract was used for further purification of enzyme.

Protein precipitation:

Acetone precipitation: Four fold extra acetone (-20 °) was added in each tube having the crude extract, vortexed and incubated for 60 min at -20 °. The pellet, obtained after centrifugation for 10 m at 12000 rpm (Remi C24), was dissolved in the small volume of extraction solution and dialyzed against the same. This fraction was designated as acetone precipitated sample (APS) [20].

Ammonium sulphate precipitation: The proteins in APS were fractionated and precipitated by adding ammonium sulphate at 4º. The precipitate obtained between 20 to 60% saturation was collected by centrifugation at 12000 rpm for 30 m. The precipitate was dissolved in sodium phosphate buffer pH 7 and dialyzed against the same till the solution was free from carbohydrates and designated as ammonium sulphate fraction (ASF) [21].

Affinity Chromatography: The ASF was subjected to affinity chromatography on cross-linked guar-gum column previously equilibrated with PBS [22].The unbound proteins were washed thoroughly with PBS till the elute showed no readings at 280nm. The bound proteins were eluted from the column by extraction solution containing 0.1M galactose. The flow rate was adjusted to 5ml/10min using fraction collector and peristaltic pump (L. K. B. Pharmacia). Fractions of 5mL each were collected and checked for protein at 280nm and subjected to dialysis against PBS till galactose was completely removed. All the fractions were checked for β-galactosidase activity [23]. The proteins in the fractions showing β- galactosidase activity were subjected to electrophoresis.

Polyacrylamide Gel Electrophoresis: Homogeneity of the affinity purified fractions was checked by simple PAGE and 10% SDS PAGE. The molecular weight of the purified β- galactosidase was determined by the method of Weber and Osborn, (1969) on a Biorad Mini Protean electrophoresis unit using Lysozyme – 14 kD, Carbonic anhydrase – 29 kD, Ovalbumin – 44 kD, Bovine Albumin – 67 kD, Phosphorylase-b – 97 kD as standard protein markers [24]. After electrophoresis the gels were stained with coomassie brilliant blue (R-250) then destained with 40% methanol and 10% acetic acid for 2 h. Purified homogeneous fraction was designated as ZOGE (*Zizyphus oenoplia* β – galactosidase Enzyme*).*

α - and β – galactosidase activity: The substrates used in the study were 3mM α – pNPGal and 3mM β – oNPGal. α and β-galactosidase assay of ZOGE was carried out by the method of Murray *et al*.,(1983) [23]. The assay mixture contained 20 to 100µl of enzyme solution and 3mM substrate prepared in 0.1M sodium acetate buffer pH 4.7. The reaction mixture was incubated at 37ºC for 40 m and the reaction was stopped by adding 2ml, 0.2M sodium carbonate. The liberated p-nitrophenol was measured at 400 nm. One unit of galactosidase activity was considered as the enzyme liberating 1 µ mole of p-nitrophenol per min under experimental conditions.

Protein concentration: Protein concentration was done by the method of Lowry *et al*., (1951) [25], using BSA as standard protein

Estimation of Carbohydrate content: Total carbohydrate content of the purified enzyme was estimated by phenol sulphuric acid method using α -D- glucose as standard [26].

pH Stability: Effect of pH on the enzyme activity was determined by the method of Patil and Butle (2014) using different buffers at different pH values ranging from pH 1 to 13 [27]. 100 µl purified enzyme solution and 100 µl buffer solution was incubated for 1 h at ambient temperature. Aliquots were withdrawn and assayed for α & β galactosidase activity as described earlier, using suitable controls.

Effect of temperature and thermal inactivation: To study the effect of temperature 30 µl purified enzyme solution was added to 1 ml 0.006 M sodium phosphate buffer pH-7 and incubated at 20, 40, 60, 80 º for 1 h and β-galactosidase assay was carried out at 37 º after cooling. The effect of thermal inactivation was tested by heating 250 µl purified enzyme solution in 250 µl, 25 mM sodium phosphate buffer pH-7 at 37 º in multiblock heater. Aliquots were withdrawn and estimated for enzyme activity after 20, 40, 60 80 and 100 m [21].

Effect of metal ions: The method of Kawagishi *et al*. (1990) [28] was used to check the effect of metal ions on enzyme activity. 100 µl EDTA treated enzyme was mixed with 100 µl 0.1 M metal ion solution and incubated at 37 º for 1h. β-

galactosidase assay was carried out as described earlier using suitable controls.

Determination enzymes constants: V max and Km of β galctosidase for o-NPGal was determined by the method adopted by Ramteke and Patil (2010) [29]

RESULTS AND DISCUSSION

β -galactosidase enzyme has been widely used for industrial as well as medical application. In dairy industries β -galactosidase has been used to prevent crystallization of lactose, to improve sweetness and to increase the solubility of milk products [30]. One of the major applications of β-galactosidase in industry is the preparation of lactose-hydrolyzed milk and whey. Especially in the cheese industry lactose is a big trouble because, it has uncertain solubility and it is associated with the high biochemical and chemical oxygen demand. [31, 32] Lactose hydrolysis can be achieved by hard conventional method i.e. acid treatment at higher temperature, but enzymatic catalysis of lactose is normally carried out mild operating conditions, thereby preventing loss of the end products [33]. Results show that β -galactosidase was purified with good yield on cross linked guar-gum by affinity chromatography (Table 1, Figure 1).

***Estimation of Carbohydrate content*:** Purified β -galactosidase shows presence of carbohydrates when tested by phenol sulphuric acid method.

Molecular Weight: The purified β -galactosidase exhibited little low molecular weight of 23 kD respectively on SDS-PAGE as shown in Figure 2. Lane 1 represents purified β-Galactosidase while Lane 2 shows molecular weight marker 14 to 97 kD Merck (Eurolab GmbH Darmstadt, Germany). *T. procumbans calyx protein* was also isolated and purified by Ramteke and Patil [34] in which the protein has been reported to have molecular weight of 23kD.

Galactosidase Activity: The purified enzyme exhibited both α and β – galactosidase activities with α – pNPGal and β – oNPGal. β -galactosidase activity was predominant than the α – galactosidase activity. Therefore, the enzyme with β -galactosidase activity was subjected to further purification. Likewise *Vigna mungo* protein exhibited both α and β galactosidase properties [35]. Protein from *mung beans* [36], and Vicia *faba* seeds [37], also showed both types of galactosidase activities.

pH Stability: Generally plant β-galactosidases show pH optima in acidic range [36]. In this study optimal pH value of β -galactosidase was found to be 5.5 (Figure 3). The enzyme activity was lost at pH below 4 and above 8 (Figure 2). This value agrees well with literature reports [12, 36]. β-galactosidases isolated from muskmelon, kiwifruit and papaya also seemed to be optimally active at acidic pH range [39 - 41].

Thermal stability and thermal inactivation: The hydrolyzing activity of β -galactosidase was monitored at the range of 20 to 80 º. The optimum temperature of ZOBG was found to be at 37 º (Figure 4). Although optimal temperature value of other β-galactosidase was reported as 60 º [11, 12, 36], it was found to be 37 º in the present study. The enzyme was optimally active in the temperature range of 30-40 º (Fig. 4). Thermal stability of enzyme is critically important because of industrial application. Similar results were obtained by different researchers [11, 12, 36].

Effect of metal ions: Result presented in figure 5 show the effect of metal ions on enzyme activity. The enzyme was unable to show activity in the presence of Cd^{2+}, Hg^{2+} and Pb^{2+}ions. ZOBG also shows similar effects found in thermostable glycoprotein of *Tridax procumbance Linn* [21].

Determination of kinetic constant: The Line weaver–Burk plot is shown in Figure 6. The Michaelis– Menten constant Km for the oNPGal substrate was estimated to be 1.09 mM, while its maximum velocity, Vmax was 0.90 U/ml/m. Km value of chick pea β-galactosidase was observed before as 1.73 mM while substrate was oNPGal [36]. Previously it has been reported that purification leads to improving Km values of enzyme [42]. X-ray studies of proteinase K showed that, increased flexibility of protein caused increased access to active site of enzyme [43]. Thus, decrease of Km value of enzyme is not surprising.

CONCLUSION

β-Galactosidase was purified from the *Zizyphus oenoplia* by using standard methods of purification. The recovery of the enzyme appears to be good at the end of purification steps as compared to other purified plant β-galactosidase. Purified ZOGE was stable for several days at +4 º. The purified β -galactosidase having wide pH and temperature stability makes ZOGE of suitable for many industrial applications.

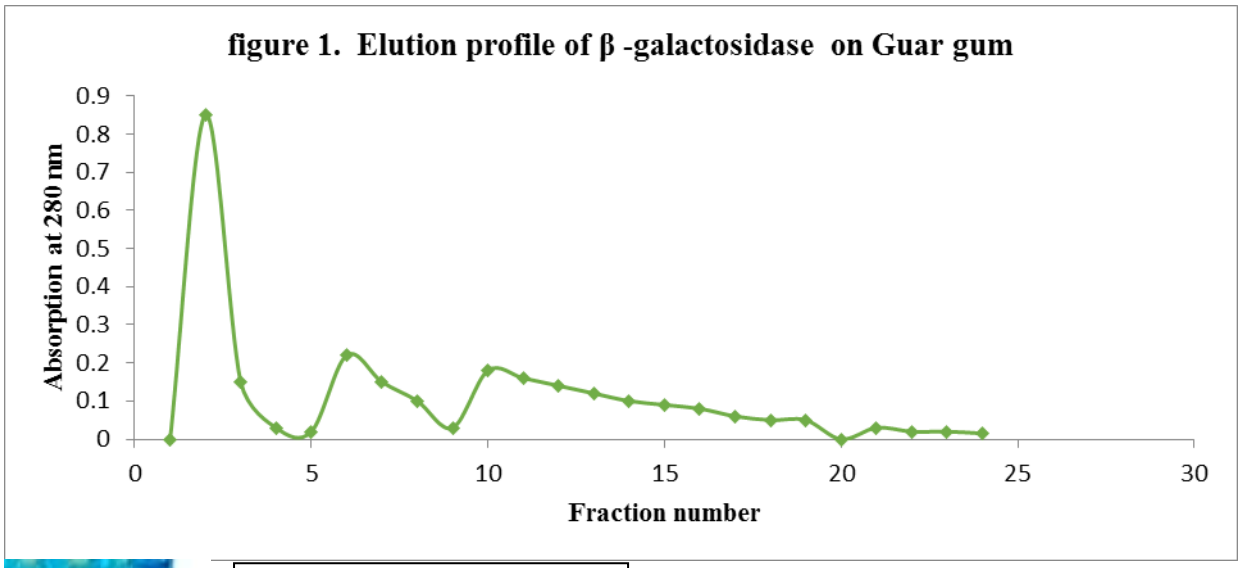
Acknowledgement

The present work was carried out with the financial support of the UGC New Delhi, India with project number 2012/1262. Authors thank Head, Department of Biochemistry RTM Nagpur University, Nagpur.

Table 1. Summary of purification of β –galactosidase of *Z. oenoplia.*

Purification step	Volume (ml)	Proteins (mg/ml)	Activity U/ml	Total Units	SA[b]	Purification fold	Yield (%)
Crude extract	100	7.6	5120	512000	673.68	1	100
Amm. sulphate fraction	27	1.14	1280	34560	1122.8	1.66	67.5
Affinity chromatography Fraction	10	0.04	800	8000	20000	29.71	16

SA- Specific Activity



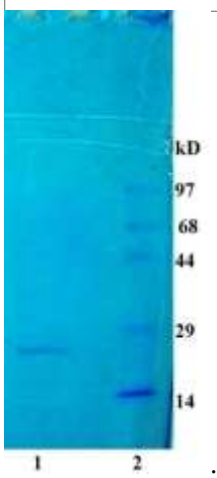
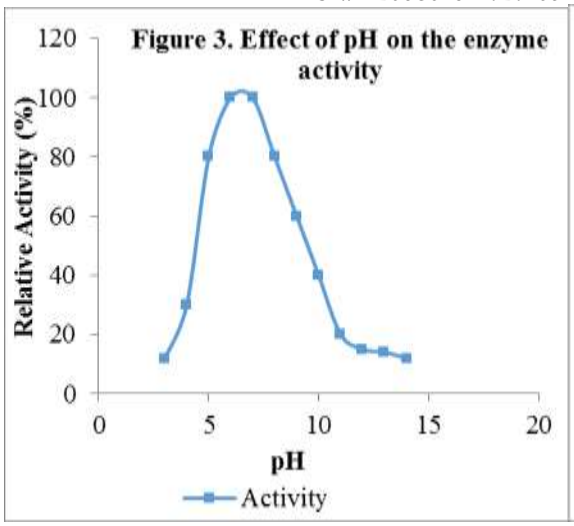


Figure2. The SDS-PAGE pattern of β -galactosidase reveals a single band, corresponding to the molecular weight to be 23 kD



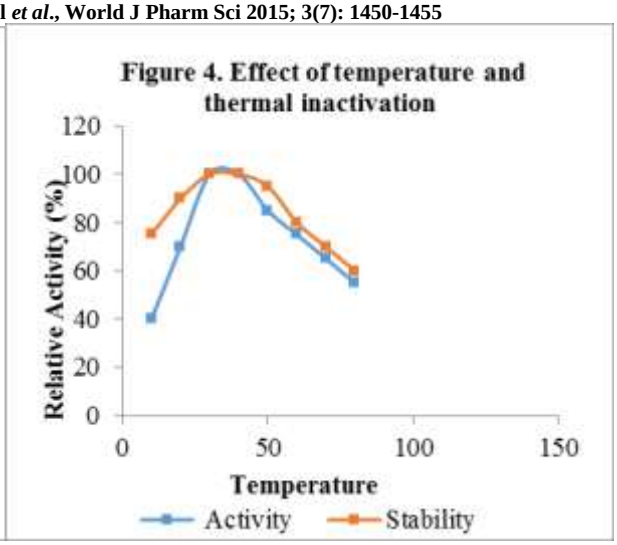
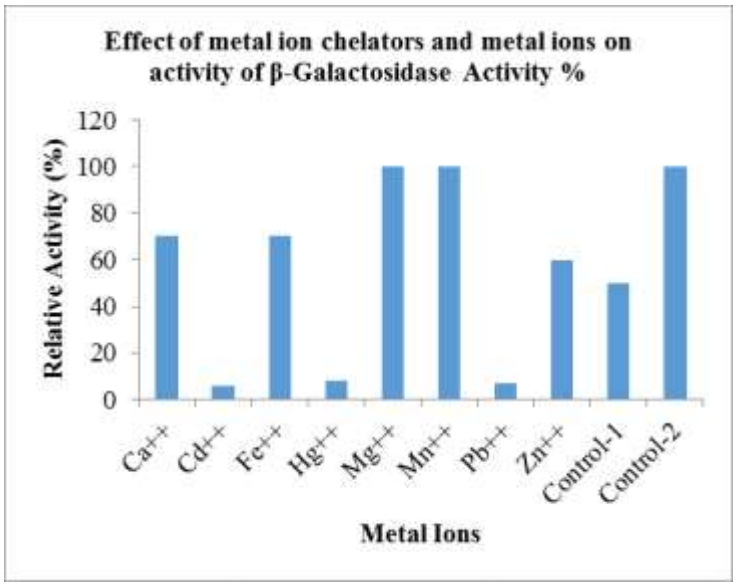


Figure5. Effect of metal ions (0.1M) on activity of β-Galactosidase



Control 1: Chelator treated control, 2: Untreated/original activity

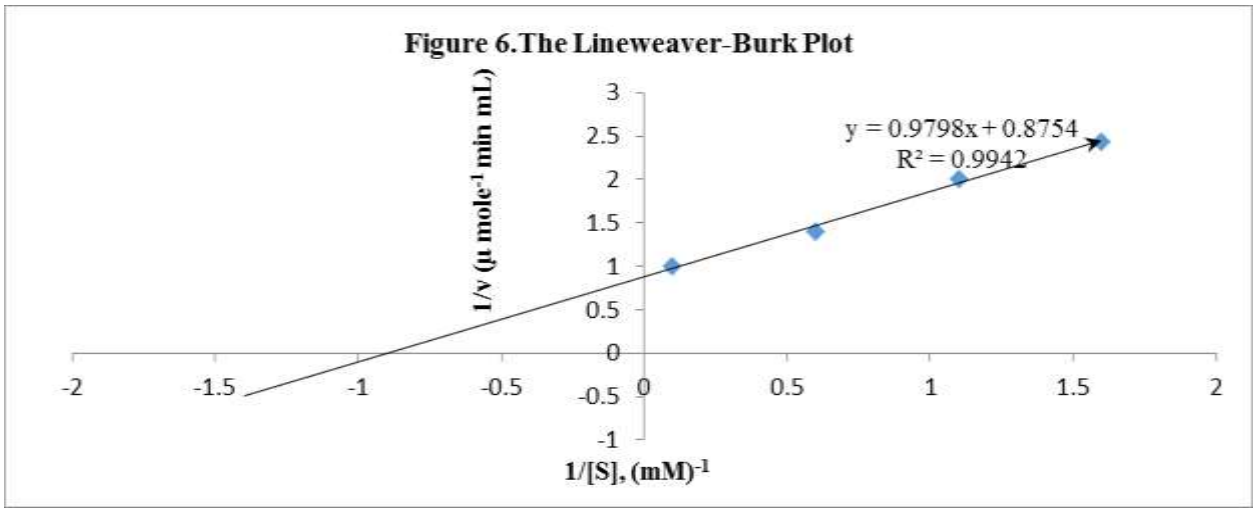


Figure 6. The Lineweaver–Burk plot. Aliquots of 450 µl of the desired concentrations of o-NPGal were incubated with 50 µL of β-galactosidase. The assay was carried out according to the standard assay procedure. The values of Km and Vmax were estimated from the double reciprocal plot.

REFERENCES

[1] Puri M et al. Cell disruption optimization and covalent immobilization of β-D-Galactosidase from Kluyveromyces marxianus YW-1 for Lactose Hydrolysis in Milk. Appl Biohem Biotech 2010; 160: 98–108.
[2] Panesar PS et al. Microbial production, immobilization and applications of β-D-galactosidase, J Chem Technol Biot 2006;81: 530– 543.
[3] Sheik AS, Gunasekaran P. Current trends of ß-galactosidase research and application, Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology. 2010; 2: pp 880–889.
[4] Mlichová Z, Rosenberg M. Current trends of β-galactosidase application in food technology. Food Nutr Res2006; 45 (2): 47–54.
[5] Ladero M et al. Hydrolysis of lactose by free and immobilized β-galactosidase from Thermus sp. strain T2. Biotechnol Bioeng 2003; 81: 241–252.
[6] Shah NP et al. Use of dry whey and lactose hydrolysis in yoghurt bases, Milk Sci Int 1993; 48: 494–498.
[7] Kosseva MR et al.Use of Immobilised bioatalyst in the proessing of Cheese Whey. Int J Biol Macromol 2009; 45: 437–447.
[8] Ross GS et al. Kiwifruit β-galactosidase – isolation and activity against specific fruit cell-wall polysaccharides. Planta 1993; 189: 499–506.
[9] Ali ZM et al. β-galactosidase and its significance in ripening mango fruit. Phytohem1995; 38: 1109–1114.
[10] Biswas S et al. Purification and characterization of a thermostable β-galactosidase from kidney beans (*Phaseolus vulgaris L*.) cv. PDR14, Plant Phys. 2003;160: 327–337.
[11] Dwevedi A, Kayastha AM. A β-galactosidase from pea seeds (PsBGAL): purification, stabilization, catalytic energetics, conformational heterogeneity, and its significance. J Agric Food Chem 2009;57: 7086–7096.
[12] Kishore D et al. Immobilization of β-galactosidase onto functionalized graphene nano-sheets using response surface methodology and its analytical applications. PLoS One 2012; 7: 7.
[13]National Institute of Science Communication, The Wealth of India. A Dictionary of Indian Raw Materials & Industrial Products Erstwhile Publication & Information Directorate, CSIR, New Delhi.1998; pp 122.
[14] Ramalingam R et al. In-vitro anti-denaturation and antibacterial activities of *Zizyphus oenoplia*. Scholars Research Library, Der Pharmacia Lettre, 2010; 2 (1): 87-93
[15] Shovon MS et al. Purification, Characterization and effect of physico-chemical agents on the stability of β-Galactosidase from Betel leaves. J bio-sci2010; 18: 108-115.
[16] Said AS et al. Characterization of partially purified β-galactosidase from *Bacillus subtilis*. J Appl Sci Res 2012; 8(4): 2379-85.
[17] Li SC et al. Purification and characterization of isoforms of β-galactosidases in mung bean seedlings. Phytohem 2001; 57: 349–359.
[18] Ohtani K, Misaki A. Purification and characterization of β -D-Galactosidase and α-D-Mannosidase from Papaya *(Carica papaya)* Seeds. Agric Bio Chem1983; 47 (11): 2441-2451.
[19] Edwards M et al. A β-D-galactosidase from nasturtium *(T ropueolum mujus* L.) cotyledons. The Americ Soc Biohem Biol Chem 1988; 263 (9): 4333-4337
[20] Ye-Yun LI et al. Purification and partial characterization of β-Glucosidase from fresh leaves of tea plants (*Camellia sinensis* (L.) O. Kuntze) Acta Biohim Biophy Sini 2005; 37(6): 363–370.
[21] Ashwin Butle, Patil MB. Studies on thermostable glycoprotein of *Tridax Proumbans Linn*. J Glob Biosci 2014; 3(2): 529-535.
[22] Ramteke AP, Patil MB. Purification and characterization of *Tridax proumbans* calyx lectins. Biosci Biotech Res Asia2005; 3(1): 103 – 110.
[23] Murray DR. Acid Glycosidase isoenzyme in developing buds and seeds of *Pisum sativurm* L. P flanzaphyol 1983; 110: 7 – 15.
[24] Weber K Osborn M. The reliability of molecular weight determination by Sodium dodecyl sulphate polyacrylamide gel electrophoresis. J biohem 1969; 244: 4406-4412.
[25] Lowry OH. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193: 265-275.
[26] Dubois, M et al. Colorimetric method for determination of sugar and related substances. Anal Biohem1956;28: 350-356.
[27] Patil MB, Butle A. Characterization of fructosyltransferase produced by *Syncephalastrum racemosum Cohn.* Pharm Innov J 2014; 3(6): 87-91.
[28] Kawagishi H et al. Isolation and characterization of lectin from *Grifola frondosa* fruiting bodies. Biohem Biophys Acta 1990; 1034: 245-252.
[29] Ramteke A, Patil MB. Studies of lectin on some wild medicinal plants. PhD Thesis, RTM Nagpur University: Nagpur, 2010.
[30] Kara F. Release and characterization of beta galactosidase from Lactobacillus plantarum. M.Sc. Thesis, Middle East Technical University: Turkey 2004.
[31] Grosova Z et al. Perspectives and applications of immobilised β-galactosidase in food industry – a review. Czech J Food Sci 2008; 26 (1): 1–14.
[32] Guimaraes WV et al. Fermentation of sweet whey by ethanologenic Escherichia coli, Biotechnol Bioeng 1992; 40: 41–45.
[33] Gekas V, Lopez-Leiva MM. Hydrolysis of lactose: a literature review. Proess Biohem 1985; 20: 2–12.
[34] Ramteke AP, Patil MB. Purification and Characterization of *Tridax procumbans* calyx lectins. Biosci Biotech Res Asia 2005; 3(1): 103 – 110.
[35] Suseelan KN et al. Purification and characterization of two lectin from *Vigna mungo* (black gram). J Biol Sci 1997; 22(4): 439 – 455.
[36] Hankins CN, Shannon LM. The physical and enzymatic properties of a phytohem -agglutinin from *Mung beans.* J Biol Chem 1978; 253: 7791-7797.
[37] Day PM et al. Multiple forms of *Vicia faba* a galactosidase and their relationship, Phytohemistry 1982; 21 : 2195-2199.
[38] Kishore D, Kayastha AM. A β-galactosidase from chick pea (*Cicer arietinum*) seeds: its purification, biohemical properties and industrial applications. Food Chem 2012; 134: 1113–1122.
[39] Balasubramaniam S et al. Purification and properties of a β-galactosidase from carambola fruit with significant activity towards cell wall polysaccharides. Phytohemistry 2005;66: 153– 163.
[40] Ranwala AP et al. The role of β-galactosidase in the modification of cell wall components during muskmelon fruit ripening. Plant Physiol 1992; 100: 1318–1325.
[41] Ali ZM et al. Isolation, characterization and significance of papaya β -galactanases to cell wall modification and fruit softening during ripening. Physiol Plantarum1998; 104: 105–115.
[42] Dennison C, Lovrein R. Three phase partitioning: concentration and purification of proteins. Protein Exp Purif 1997; 11:149–161.
[43] Singh RK et al. Enhancement of enzyme activity through three-phase partitioning: crystal structure of a modified serine proteinase at 1.5 Å resolution. Protein Eng 2001; 14: 307–313.