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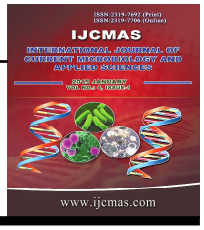

Original Research Article

Isolation, Purification and Characterization of β-Glucosidase from leaves of *Hordeum vulgare*

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Keywords

Hordeum vulgare, β-Glucosidase, Purification.

A B S T R A C T

β-glucosidase (EC 3.2.1.21) was extracted from leaves of *Hordeum vulgare* and was purified using ammonium sulphate fractional precipitation, acetone precipitation and Sephadex G-75 chromatography. The molecular weight of enzyme was found to be 58kd .The enzyme β-glucosidase has optimum pH of 5.0 and the optimum temperature was found at 60ºC. The enzyme activity was also characterized in different carbon, nitrogen, metal ions and inhibitors. Bioethanol was produced from leaves of *Hordeum vulgare.*

Introduction

β-Glucosidases are ubiquitous and can be found in bacteria, fungi, plants and animals. These are produced intracellular and extracellular in various organisms depending on the cultivation conditions. These enzymes can be classified on the basis of substrate activity and nucleotide sequence identity (Bhatia *et al.,* 2002; Henrissat and Davies, 1997). Based on their substrate specificity, they are divided into three classes; class I which contain aryl β–glucosidases, class II contains true cellobiases and class III contains β–glucosidases having broad substrate specificity. Most of the β–glucosidases belongs to class III which can cleave β 1,4; β 1,6; β 1,2 and α 1,3; α 1,4; α 1,6 glycosidic bonds (Bhatia *et al.,* 2002; Riou *et al.,* 1998). Henrissat and Bairoch (1996) stated that β–glucosidases are in family 1 and 3 from the 88 glycosyl hydrolase families. Family 1 contains β–glucosidases which are found in archeabacteria, plants and mammals and also exhibit β–glactosidase activity. Family 3 contains β–glucosidases which are produced from fungi, bacteria and plants having a characteristics two-domain structure (Varghese *et al.,* 1997).

β-Glucosidases are mostly used in cellulose conversion process but also have broad applications such as activation of phytohormones and defense against pathogens in plants (Esen, 1993), cellular signaling and oncogenesis (Bhatia *et al.,* 2002), release of aroma from wine grapes and hydrolysis of bitter compounds during juice extraction, formation of alkyl- and aryl-glycosides by trans-glycosylation from

natural polysaccharides or their derivatives and alcohols and also used in pharmaceutical, cosmetic, and detergent industries (Bhat, 2000; Gargouri *et al.*, 2004). Cellulose, which constitutes the highest proportion of municipal and plant wastes, represents a major source of renewable energy and raw materials. Therefore, the utilization of cellulosic wastes to produce energy is potentially of great importance. Cellulases bring about the hydrolysis of cellulose, a homo-polymer of β-1,4 linked glucose units that comprises of amorphous and crystalline regions, by synergistic action of its constituent enzymes.

These enzymes include; a) β-1,4-endoglucanase (1,4-β-Dglucan 4-glucanohydrolase; EC 3.2.1.4, cellulase), which cleaves internal β-1,4-glycosidic bonds, b) cellobiohydrolase (1,4-β-D-glucancellobiohydrolase; EC 3.2.1.91, cellulase 1,4-β-cellobiosidase), an exo-acting enzyme which releases cellobiose from reducing and nonreducing ends of cellulose and c) β-glucosidase (β-D-glucosideglucohydrolase; EC 3.2.1.21, cellulase 1,4-β-glucosidase) that hydrolyzes cellobiose to glucose (Bhat and Bhat, 1997).

β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi *et al.* 2002). Thus, β-glucosidase not only produces glucose from cellobiose, but also reduces cellobiose inhibition, allowing endoglucanase and exoglucanase enzymes to function more efficiently.

Materials and Methods

Collection of seed sample

The leaves of *Hordeum vulgare* was collected for the isolation of β-glycosidase enzyme. The leaves were ground to fine powder in a chilled mortar and pestle using liquid nitrogen and extraction buffer (1 ml buffer/1g tissue) which was mixed with it. After that the extract was centrifuged at 12000 rpm 4^{o}C/30 minutes. Then the supernatant was ultra-filtered to obtain crude enzyme solution.

Precipitation of Enzyme

Precipitation by ammonium sulphate: The crude extract was precipitated by adding ammonium sulfate at different saturation levels (30%, 50% and 70%) and kept overnight in refrigerator. Then centrifugation was done at 12000 rpm for 10 min at 4□C. Thereafter the pellet was collected and dissolved in minimum volume of citrate buffer for enzyme activity determination.

Precipitation by acetone: Chilled acetone (-20□C) at different saturation (40%, 60%, 80% and 100%) was added to the crude extract 50% (V/V), and the mixture was stirred and incubated overnight at 4□C. The precipitate obtained from the crude extract by centrifugation at 10000 rpm for 10 min. Thereafter, the pellet was collected and dissolved in minimum volume of citrate buffer for enzyme activity determination.

Determination of β-glycosidase activity: The β-glycosidase activity was determined against p-nitrophenyl β-D-glucopyranoside (p-NPG) as its substrate in citrate buffer at room temperature and

the activity was estimated using double beam spectrophotometer at wavelength 405 nm.

Partial purification and SDS-PAGE

The isolated enzyme was partially purified by gel filtration chromatography viz., sephadex G-75 and the molecular weight was determined through SDS-PAGE technique.

Optimization of purified β-glucosidase

The effect of different pH (3. 5, 7, 9 & 11), temperatures (20^{o}C, 40^{o}C, 60^{o}C, 80^{o}C & 100^{o}C), metal ions ($CaCl_2$, NaCl, KCl, $ZnSO_4.7H_2O$ & $MgSO_4.7H_2O$), carbon source (mannitol, maltose, sucrose, lactose & glucose), nitrogen source (ammonium sulphate, ammonium nitrate, ammonium chloride, sodium carbonate & hydroxylamine hydrochloride) and inhibitors (copper sulphate, SDS, mercaptoethanol, ferrous sulphate & EDTA) was studied on the activity of β-glucosidase enzyme.

Effect of different concentration of substrate β- glucosidase production

Different concentration of p-nitro phenyl β-D- glucopyranoside at the range of 2–10mM was added and incubated with enzyme under optimum condition. After incubation at 37^{o}C for 24 hours, the samples were withdrawn and assayed for β- glucosidase activity.

Characterization of β- glucosidase

The characterization of enzyme was done by the study effect of inhibitors on β- glucosidase production. To study the effect of inhibitors on protease production yield, different inhibitors used were silver nitrate, antimony trichloride, magnesium chloride, D-gluconolactone and copper sulphate.

Effect of immobilization on enzyme activity

Effect of immobilized enzyme activity was studied using 4% sodium alginate and 1.4% calcium chloride at different pH range (3, 5, 7, 9 & 11).

Bioethanol production from *Hordeum vulgare* leaves

Leaves of *Hordeum vulgare* were used for the bioethanol production. The bioethanol produced was poured into a Petri dish and lighten with matchstick. After that its ethanol content was compared to the lab grade ethanol.

Results and Discussion

Isolation and activity determination of β-glucosidase

The enzyme was isolated from the leaves of *Hordeum vulgare,* precipitated by ammonium sulphate and acetone to get the partially purified extract of enzyme (without cell debries). After precipitation the enzyme's activity was determined using p-nitrophenyl β-D-glucopyranoside as substrate at 404nm which was found to be 0.055u/ml. The purity of β-glucosidase was studied by purification table and found to be 26.1% as shown in Table 1.

Characterization of purified β-glucosidase activity

Effect of pH

The β- glucosidase extracted from barley leaves was optimized at different pH (3, 5, 7, 9 and 11) range using sodium

hydrogen phosphate-citrate buffer. The maximum activity was observed at pH 5 (0.444U/ml±0.02) (Graph 1). The optimum pH for catalyzing β-glucosidase action of different plants ranged as strawberry– 4.0 (Bothast and Saha, 1997), *Lodgepole* Pine - 5 to 6 in variety of substrate (Dharmawardhana *et al.,* 1995).

Effect of temperature

The β- glucosidase extracted from barley leaves was optimized at different temperature (20^{o}C, 40^{o}C, 60^{o}C, 80^{o}C and 100^{o}C) range using sodium hydrogen phosphate-citrate buffer. The maximum activity was observed at 60^{o}C (1.132U/ml±0.17) (Graph 2).The optimum temperatures in different plants were as Strawberry 60ºC (Poulton and Li, 1994), *Leuconostocmesenteroides* 50ºC (Cicek and Esen, 1999).

Effect of carbon source

The β- glucosidase extracted from barley leaves was optimized at different carbon (mannitol, maltose, sucrose, lactose and glucose) source using sodium hydrogen phosphate-citrate buffer. The maximum activity was observed in maltose (0.918U/ml±0.02) (Graph 3).

Effect of nitrogen source

The β- glucosidase extracted from barley leaves was optimized at different nitrogen (ammonium sulphate, ammonium nitrate, ammonium chloride, sodium carbonate and hydroxylamine hydrochloride) source using sodium hydrogen phosphate-citrate buffer. The maximum activity was observed in Ammonium sulphate (0.841U/ml±0.04) (Graph 4).

Effect of metal ions

The β- glucosidase extracted from barley leaves was optimized at different metal ions (calcium chloride, sodium chloride, potassium chloride, zinc sulphate pentahydrate and magnesium sulphate pentahydrate) using sodium hydrogen phosphate-citrate buffer. The maximum activity was observed in Calcium chloride (1.255U/ml±0.21) (Graph 5).Similarly the presence of monovalent and divalent metal ionsNa^{+}, K^{+},Ca^{2+}, Mg^{2+} and Zn^{2+} also positively influenced the activityof β-glucosidase (Kaur *et al.*, 2007).

Effect of inhibitors

The β- glucosidase extracted from barley leaves was characterized at different inhibitors (silver nitrate, Antimony trichloride, Magnesium chloride, D-gluconolactone and Copper sulphate) using sodium hydrogen phosphate-citrate buffer. The maximum inhibition was observed in Antimony trichloride (0.22U/ml±0.05) (Graph 6).Similarly in an effort to investigate marine microbial extracts for β-glucosidase inhibitors, we developed a new protocol, using esculin as substrate inan agar plate based assay, to screen a large number of microbial extracts in a short span of time (Sony Pandey *et al.*, 2013).

Effect of substrate concentration

The effect of different concentration of substrate was studied on production of β-glucosidase from Barley leaves. The maximum production was observed at 0.5mg/ml and 2mg/ml (Graph 7).The effect of mono/disaccharides (1 mg/ml) on β-glucosidaseactivity was studied using pNPG as a substrate (Kaur *et al.*, 2007).

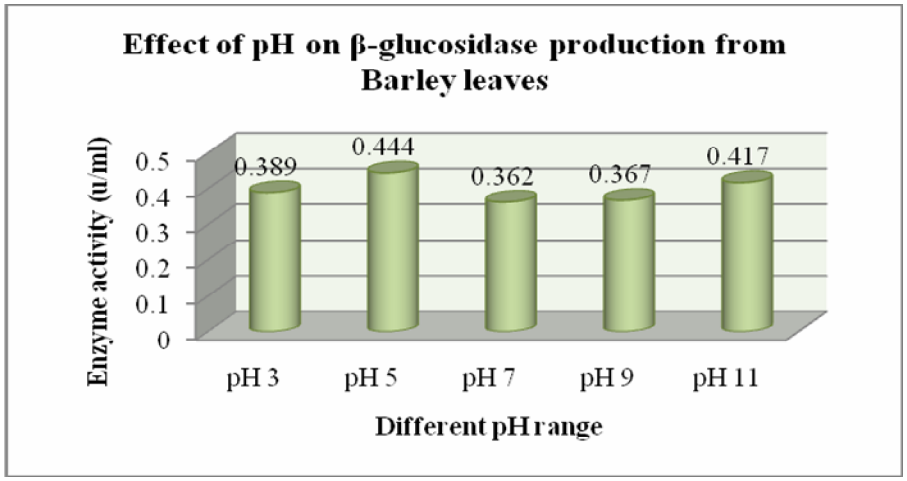
Table.1 Fold Purification table of β-glucosidase via Barley leaves

Volume (cm^3)	Concentration	Total protein	Activity	Total activity	Specific activity	Purification fold	Overall yield
$15cm^3$	10.64	159.6	0.059	0.88	0.0064	1.00	100%
$7cm^3$	9.53	66.71	0.057	0.39	0.0052	0.83	44.3%
$7cm^3$	10.69	74.83	0.068	0.47	0.0077	1.16	53.4%
$4cm^3$	9.82	39.28	0.059	0.23	0.0063	1.00	26.1%

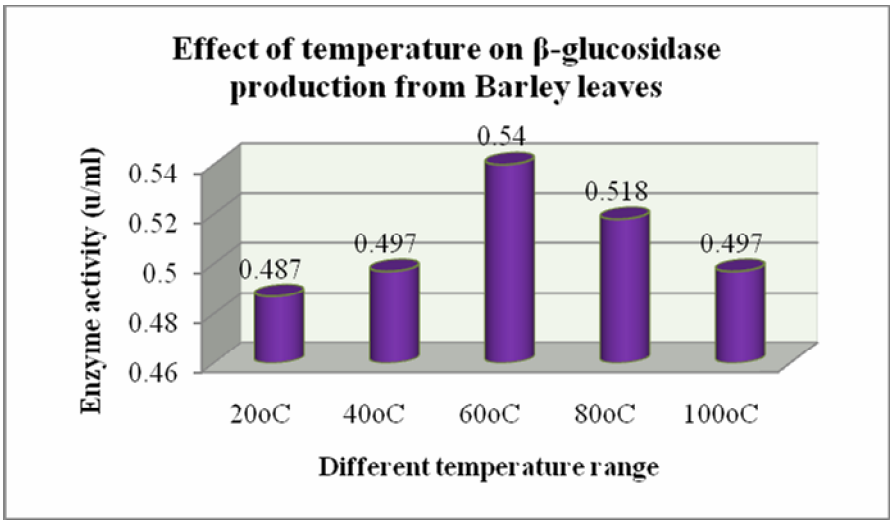
Table.2 Molecular weight determination from SDS (Coomassie staining) gel of Barley leaves

S. No.	M.W. of marker (kDa)	Distance of marker (cm)	Distance of protein band (cm)	M.W. of protein band (kDa)
1.	175kd	0.8cm	0.5cm	183kd
2.	100kd	1.9cm	1.5cm	165kd
3.	75kd	2.9cm	2.5cm	85kd
4.	45kd	3.9cm	4.6cm	41kd
5.	37kd	5.0cm	5.1cm	25kd
6.	20kd	6.0cm	6.0cm	20kd

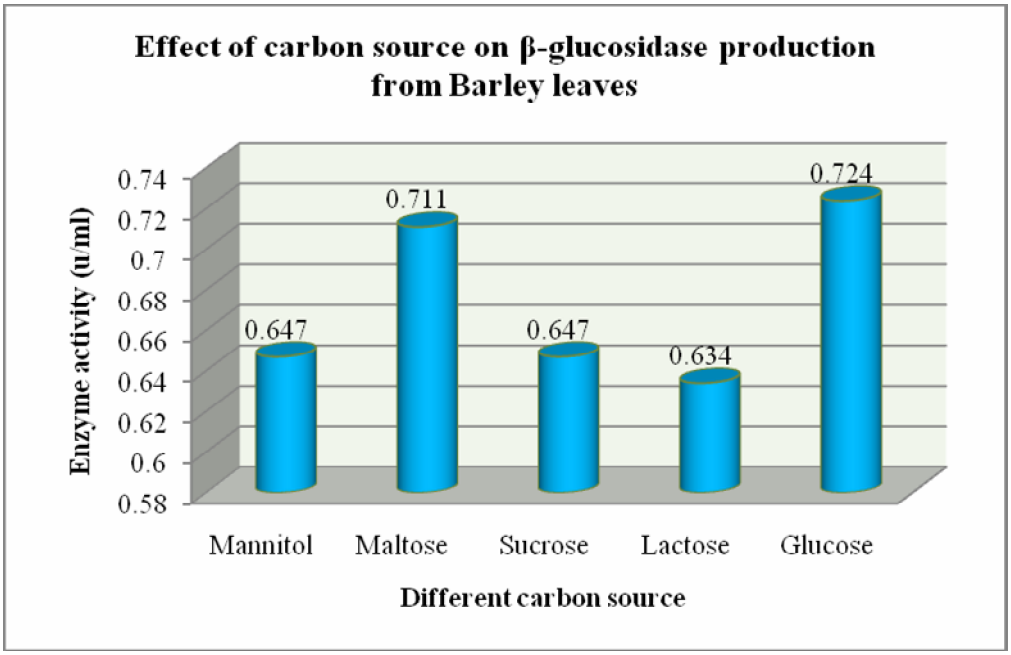
Graph.1 Effect of pH on β- glucosidase activity from Barley leaves (a) Maximum activity was observed at pH 5 (b) Minimum activity was observed at pH 7



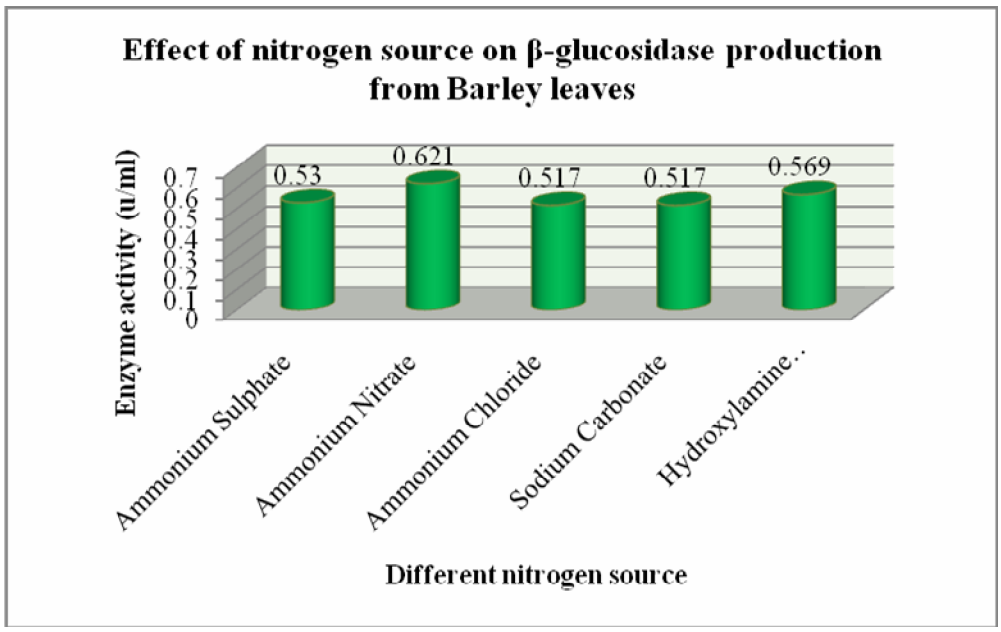
Graph.2 Effect of temperature on β- glucosidase activity from Barley leaves (a) Maximum activity was observed at 60°C (b) Minimum activity was observed at 20°C



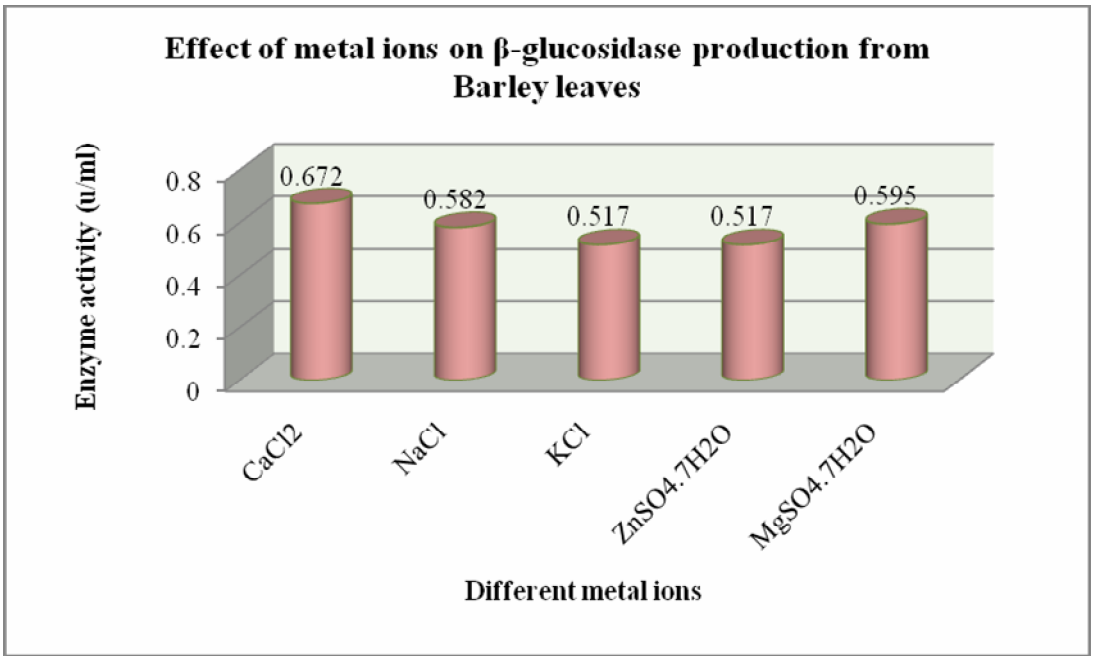
Graph.3 Effect of carbon source on β- glucosidase activity from Barley leaves (a) Maximum activity was observed in maltose (b) Minimum activity was observed in lactose



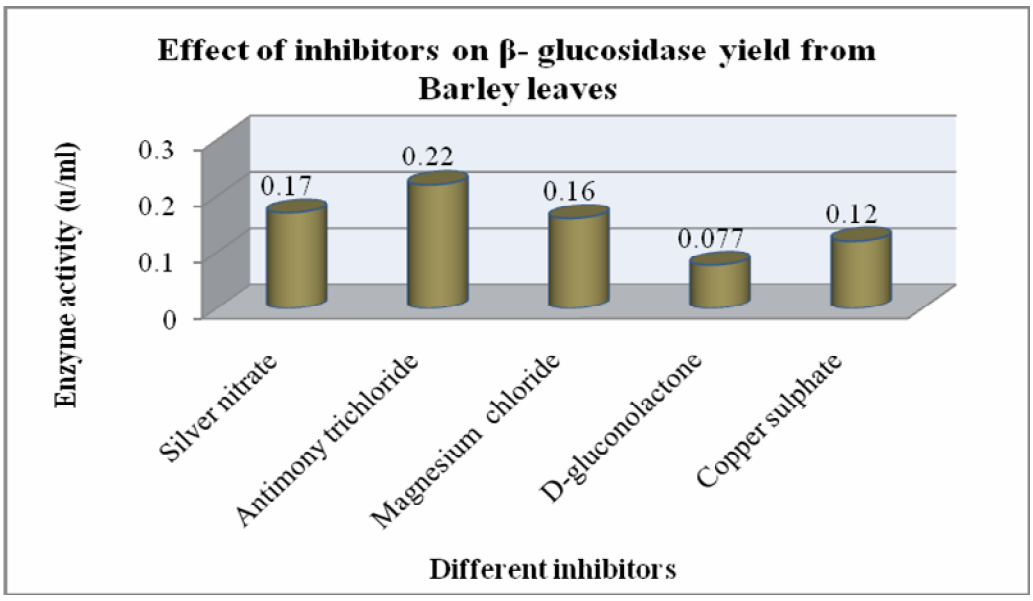
Graph.4 Effect of nitrogen source on β- glucosidase activity from Barley leaves (a) Maximum activity was observed in ammonium nitrate (b) Minimum activity was observed in ammonium sulphate and sodium carbonate



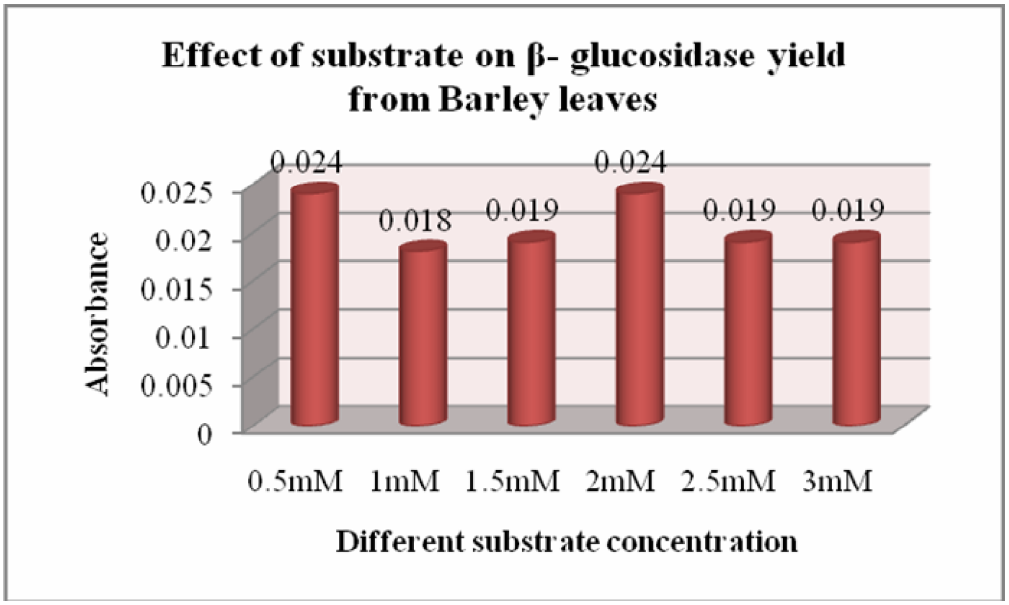
Graph.5 Effect of metal ions on β- glucosidase activity from Barley leaves (a) Maximum activity was observed in calcium chloride (b) Minimum activity was observed in potassium chloride and zinc sulphate



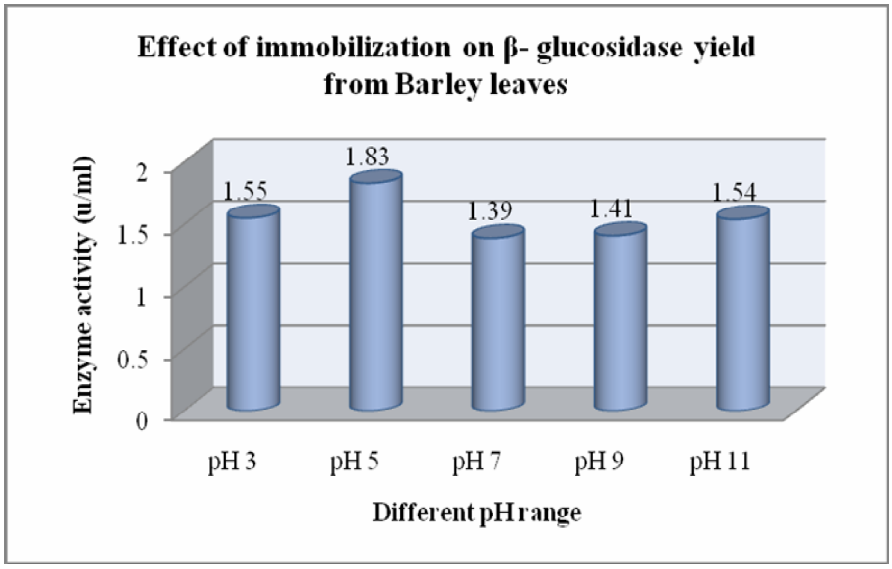
Graph.6 Effect of inhibitors on β-glucosidase activity from Barley leaves (a) maximum inhibition was observed in antimony trichloride (b) minimum inhibition was observed in D-gluconolactone



Graph.7 Effect of substrate concentration on β- glucosidase activity from Barley leaves (a) Maximum activity was observed in 0.5mM & 2mM (b) Minimum activity was observed in 1mM



Graph.8 Effect of substrate concentration on β- glucosidase activity from Barley leaves (a) Maximum activity was observed at pH 5 (b) Minimum activity was observed at pH 7



Graph.9 Determining the molecular weight of protein by SDS-PAGE

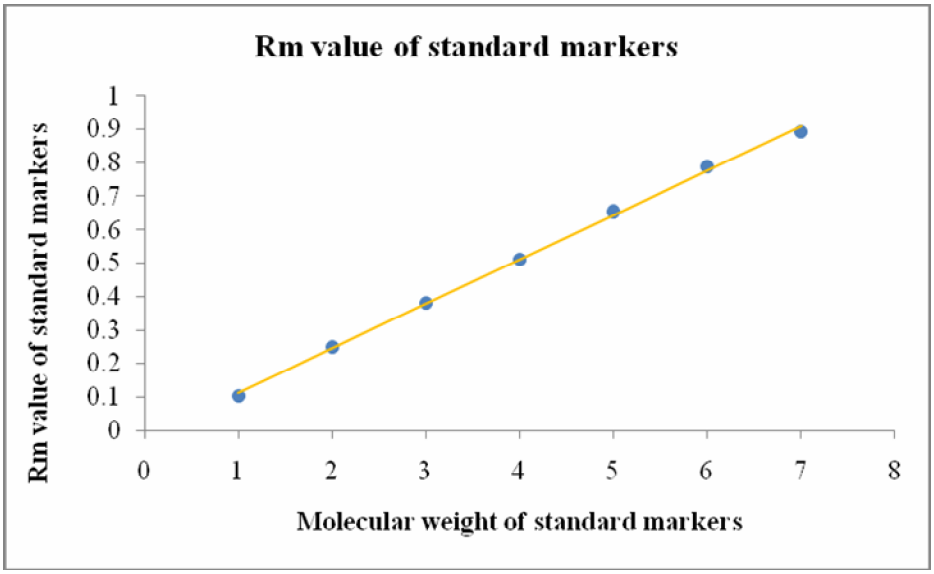


Figure.1 Bands of β- glucosidase isolated from *Barley leaves*(a) Lane 2-4 shows bands of β- glucosidase (b) Lane 1 shows ladder from 10kDa to 100kDa

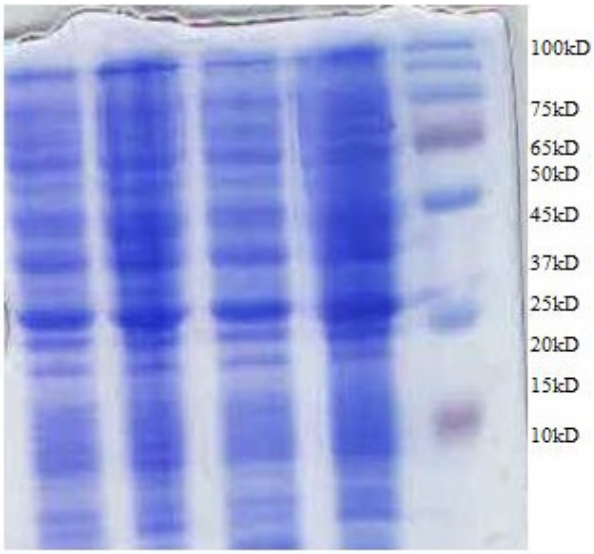


Figure.2 Bioethanol extracted after distillation from *Hordeum vulgare* leaves

Effect of immobilization on β-glucosidase activity from Barley leaves

The β- glucosidase extracted from barley leaves was optimized at different pH range (3, 5, 7, 9 and 11) in immobilized condition using sodium hydrogen phosphate-citrate buffer. The maximum activity was observed at pH5 (1.83U/ml±0.35) (Graph 8).β-Glucosidase was immobilized in calcium alginate as described by Kawaguti *et al.*, 2006.

Molecular weight determination of enzyme

The molecular weight of enzyme isolated from barley leaves was determined by SDS-PAGE using specific markers of known molecular weight. The molecular weight of alkaline protease from Barley leaves was found to be 58kDa (Table 2) (Graph 9) (Figure 1).The molecular weight of different bands in Rauvolfia serpentine was found in the range of 19.48 KDa to 92.257 KDa (Verma *et al.,* 2011).

Bioethanol production from *Hordeumvulgare*leaves

Leaves of *Hordeumvulgare* were used for the bioethanol production. By comparing the alcohol content of bioethanol produced from the leaves of *Hordeum vulgare* was found to be higher than absolute alcohol (Figure 2).

β-glucosidase (EC 3.2.1.21) was extracted from leaves of *Hordeum vulgare* and was purified using ammonium sulphate fractional precipitation, Acetone precipitation and Sephadex G-75 chromatography. The molecular weight of enzyme was found to be 58kd .The enzyme β-glucosidase has optimum pH 5.0 and the optimum temperature was found at 60ºC. The enzyme activity was also characterized in different carbon, nitrogen, metal ions and inhibitors. Bioethanol was produced from leaves of *Hordeum vulgare.*

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