



Original Research Article

Nutritional Optimizations for Improved Exo-Inulinase Production from *Aspergillus oryzae* for High-Fructose Syrup Preparations

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ABSTRACT

Keywords

Inulo-oligosaccharide, Submerged fermentation, Vogel's medium, Exo- inulinase, Intermediate pellets

Inulinase finds industrial applications for the production of high fructose syrup, ethanol and inulo-oligosaccharide. The present study was carried out for the production of exo- inulinase from an indigenous *Aspergillus oryzae* Bot-HS.L5 in Vogel's medium supplemented by wheat bran as a basal carbon source using submerged fermentation (SmF) technique. Different citrus fruits such as sour orange, sweet orange, lemon, grapefruit and citron were evaluated for the isolation of *A. oryzae* having superior enzyme activity. Among them, the strain isolated from lemon exhibited the highest productivity. It was coded as Bot-HS.L5. The enzyme activity of 10.34 ± 0.93 , 176.5 ± 8.82 protein content and 17.2 ± 0.86 DCM were observed, respectively with intermediate pellet (1.2-2 mm diameter) of fermented broth. Different parameters for nutritional requirements were investigated. For nutritional requirements, maximum enzyme activity (61.34 U/ml) was obtained when 2.5% maltose as an additional carbon source, 0.4% oxalic acid, 1.25% of KH_2PO_4 and 0.04% MgNO_3 were used. An overall 65% improved enzyme activity was accomplished at the optimal batch conditions which is highly significant (LSD 15.11, Df 12, $p \leq 0.05$). The enzyme preparation could be exploited for the production of high- fructose syrup.

Introduction

Inulinases catalyze the hydrolysis of inulin, producing inulo-oligosaccharides, fructose and glucose as main products. These constitute an important class of hydrolyzing enzymes. The general reaction mainly involves action of two enzymes: exoinulinase (E.C. 3.8.1.80) which splits the terminal fructose units from inulin and endoinulinase (E.C. 3.2.1.7) that breaks down the inulin into inulo-oligosaccharides

(IOS). The former can be used for the production of high fructose syrup from natural inulins, and the later can be used for producing inulo-oligosaccharides of varying lengths (Sumat et al., 2012). Inulin is reserved carbohydrate consisting mainly of β -(2-1)-D-fructosyl-fructose links terminated by a source residue (De Leenher et al., 1996). Inulinases has been receiving much attention since the discovery of their

benefits in human nutrition. The functional properties of inulin in food and non food products depend partly on the chain length of the polymer (Ongen-Baysal et al., 1994). Fructose is a sweetener largely used in food and beverage industries with a sweetening power 70% higher than sucrose. It is well tolerated by diabetics, improves iron absorption by children and aids the removal of ethanol from the blood of alcoholics (Turner et al., 2007). Fructo-oligosaccharides (FOS) are regarded as functional foods, positively influencing the composition of the gut micro flora, increasing the population of bifidobacteria and improving mineral absorption. The enzyme can also produce inulo-oligosaccharides (IOS) by the hydrolysis of inulin as these are functionally similar to other oligosaccharides and can be used as a soluble dietary fiber, a functional sweetener or a prebiotic (Hidaka et al., 1987; Roberfroid, 1993). These can be used as abundant substrates for oligosaccharides production due to their high inulin contents (Cho et al., 2001; Aslam et al., 2013).

A number of microorganisms including molds, yeasts, actinomycetes, and other bacteria are known to produce inulin hydrolyzing enzymes (Vandamme and Derycke, 1983; Pandey et al., 1999; Singh and Gill, 2006; Djegui et al., 2014). The selection of such microorganisms depends on their physiological characteristics and the fulfillment of Generally Recognized as Safe (GRAS) and Food and Drug Administration (FDA) criteria for food products. A number of molds have been reported to produce inulin hydrolyzing enzymes. *Aspergillus* species are one of the most explored fungus for production of inulinases. Several strains of this fungus have been reported to produce high levels of exo- inulinase (Sumat et al., 2012). Attempts are being made to develop different techniques for enzyme production.

Although the commercial production and almost all works presented in the literature related to microbial inulinases employs submerged fermentation (SmF), solid-state fermentation (SSF) have also been successfully used to produce this enzyme by employing several agro-industrial residues as substrate (Mazutti et al., 2010). A typical pattern of growth follows a response to the nutrients in the environment, modified by other environmental factors. Typically, a single unit of fungus will grow rapidly in the beginning. The exponential phase will be followed by a plateau, commonly called the stationary phase, which as the organic nutrients become depleted is followed by a decline phase. These stages are typical of any organism growing in fixed quantities of nutrients, particularly under laboratory conditions. Response of fungi to nutrients is highly variable. Some fungi are unable to sporulate under conditions of high nutrients. These fungi continue to grow vegetatively in abundant nutrient conditions (Chen et al., 2009). Fungal nutritional conditions are dependent upon many supplements such as additional carbon sources, weak organic acids, phosphate sources and various magnesium sources. All these supplements play a major role in fungal growth, sporulation and production of enzymes and other metabolites so an appropriate nutrient along with its suitable concentration should be employed to maximize enzyme production.

Among nutritional requirements carbon is of prime importance for mycelium growth. Almost half of the dry weight of fungal cell consists of carbon (Chi et al., 2011). The study of carbon nutrition, therefore, is fundamental to understand the physiology of fungi. It is understandable that fungi in general, are the most versatile organisms, with respect to their ability to utilize wide variety of carbon sources such as

monosaccharide, disaccharide, polysaccharide, organic acids and lipids. This shows that they possess the ability to synthesize having broad substrate specificities (Cruz et al., 1998). Weak acid adaptation probably evolved to facilitate growth at low pH. Phosphorus is known to play an important physiological and biochemical role in microbes. Therefore, it regulates the microbial growth and yield to the greater extent (Nuggehalli and Montanhalli, 1936). The present study deals with an insight on the optimization of nutritional requirements for exo- inulinase production from *Aspergillus oryzae* Bot-HS.L5 in submerged culture.

Materials and Methods

Equipments such as an autoclave (KT-40L, ALP Co. Ltd., Tokyo, Japan), cooled incubator (11-679-25C, Gallenkamp, England), rotary shaking incubator (Iremeco GmbH, Germany), and spectrophotometer (SP-300, Optima, Tokyo, Japan), were used. The chemicals including citric acid, ammonium nitrate, potassium dihydrogen phosphate, ammonium sulphate, and bovine serum albumin (BSA) were of analytical scale. These were procured from Acros (Belgium) and E-Merck (German). All other chemicals were also of the highest possible purity.

Isolation, maintenance and identification of *A. oryzae*: Twelve different strains of *A. oryzae* were isolated from different citrus fruits such as sour orange (*Citrus aurantium*), sweet orange (*Citrus sinensis*), lemon (*Citrus limonia*), grapefruit (*Citrus paradisii*) and citron (*Citrus medica*) which were collected from District Lahore. The samples were cut into horizontal pieces and transferred into individual sterilized Petri plates. Later, the pieces were moistened by a light spray of sterile distilled water. The

plates were wrapped with a cling tape and placed in a growth chamber at 30°C for 4-7 days. The initial colonies of *A. oryzae* were picked up and transferred to malt extract agar (MEA), pH 4.8 slants and incubated at 30°C (3-5 days) for maximum sporulation. The slant cultures were stored in a refrigerator at 4°C. The culture was examined microscopically, and compared with the master culture (*A. oryzae* NRRL-1560). Most distinguishing features including septate mycelium, branched hyphae and generally white color of its conidia which turns green later on, and presence of one to a few nuclei per cell were observed to purify the culture from rest of the lot after Sharma (2005).

Inoculum preparation and spore count: Ten milliliter of slightly warm sterile distilled water was transferred to a slant culture of *A. oryzae* having adequate growth under aseptic conditions. A sterilized inoculum needle was used to disrupt the clumps of conidiospores. The tube was shaken gently to obtain a homogeneous spore suspension. A haemocytometer was used to count the conidiospores which was found to be 1.25×10^7 CFU/ml.

Solid substrate, fermentation technique and culture conditions: Wheat bran collected from a local market of Lahore district was used as a solid substrate. It was dried at 60°C for 1 h in an oven, and stored in sterile polythene bags at room temperature (25°C) under dark. The production of inulinase was carried out using submerged fermentation (SmF) in 250 ml Pyrex flasks. Twenty five milliliter of Vogel's medium containing 2.5 g/l citric acid, 2 g/l NH_4NO_3 , 5 g/l KH_2PO_4 , 4 g/l $(\text{NH}_4)_2\text{SO}_4$, 0.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g/l peptone, 1 g/l yeast extract was used as basal fermentation medium. Wheat bran (10%) was dispensed in Vogel's medium as an agricultural by

product. The flasks were cotton plugged and sterilized in an autoclave at 15 lbs/in² pressure (121°C) for 15 min. Later the medium was allowed to cool at room temperature (25°C) and seeded with 1 ml of spore suspension, aseptically. The flasks were placed in a rotary shaking incubator at 30°C (200 rpm) for 48 h. All the batch culture experiments were performed in a set of three parallel replicates.

Analytical techniques

Enzyme extraction: At the termination of fermentation process, the flasks were removed from the shaking incubator and the contents were filtered through a pre-weighed Whatmann filter paper No. 44. The samples were centrifuged at 3200×g for 15 min and the clear supernatant was used for further analysis.

Enzyme assay: Inulinase activity was assayed by measuring the amount of reducing sugars released from inulin (as a substrate) using Nelson's method (1944). For the determination of enzyme activity, the reaction mixture containing 0.5 ml of crude enzyme and 0.5 ml 1% inulin (prepared in 0.1M acetate buffer, pH 5.5) was incubated at 50°C. After 20 min of incubation, 1.5 ml of 3,5-dinitrosalicylic acid (DNS) reagent was added and the sample was analyzed according to DNS method (Miller 1959). It was placed in boiling water for 15 min then it was allowed to cool at room temperature (25°C) with the addition of 0.5 ml Rochelle salt along with 7 ml of slightly warm distilled water so that the final volume was raised up to 10 ml. Control samples were run parallel using the same procedure but replacing crude enzyme with distilled water. The A_{595nm} of reaction mixture was measured using a spectrophotometer. One unit of inulinase activity is defined as the amount of enzyme which produces 1 µg/ml of reducing sugars

per minute under the controlled assay conditions. Following relationship was used to determine enzyme activity,

Enzyme activity (U/ml) =

$$\frac{\text{Sugar released} \times \text{DF} \times \text{NF}}{1000} \times 100$$

Where, DF represents 'dilution factor' while NF denotes 'normalization factor'.

Protein estimation: Protein contents were estimated after Bradford (1976). Five milliliter of Bradford reagent was taken in a test tube followed by the addition of 0.1 ml of enzyme extract. The tubes were swirled and incubated at room temperature (25°C) for 5 min. A blank was also run parallel by replacing enzyme extract with 0.1 ml of distilled water. Absorbance was measured at 595 nm. The slope of curve was used for protein estimation, following the relationship.

Protein content (µg/ml) = Slope × 5 × Dilution factor

Determination of dry cell mass (DCM): The fermented mash was filtered in a pre-weighed Whatman filter paper No. 44 and washed twice with distilled water. Later, it was placed in an oven at 105°C for 30 min. After drying, DCM was calculated following the relationship.

DCM (g/l) = Total dry weight – (Weight of filter paper + Wheat bran)

Determination of mycelial morphology: Mycelial morphology was determined for each of the twelve isolates of *A. oryzae* following the procedure of Moreira et al. (1996). The mycelia were categorized on the basis of their pellet diameter which included fine (0.2-0.5 mm), small (0.5-1 mm), large

(2.5-3.5 mm), large broken (3.8 mm) and mixed (0.5-3.2 mm). In some cases, a disrupted morphology viz. viscous, gelatinous and dumpy mass was also observed.

Optimization of nutritional requirements

Additional carbon sources: The effects of various carbon sources like glucose, maltose, fructose, sucrose, xylose and glycerol was studied on the enzyme activity. Maltose proved to be more effective carbon source, so various concentrations (0.5, 1, 1.5, 2, 2.5 and 3 %) were employed to study their effects on enzyme production.

Weak organic acids: Various weak organic acids such as citric acid, oxalic acid, casamino acid, acetic acid, molybdic acid and ascorbic acid were used to further enhance enzyme production. The effects of various levels of oxalic and citric acids (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 %) were also studied.

Phosphate sources: Effect of different phosphate sources (KH_2PO_4 , K_2HPO_4 , NaH_2PO_4 and NaHPO_4) was studied to obtain maximal enzyme production. Pre-existing source (KH_2PO_4) appeared to be more suitable so its concentrations (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5%, w/v) were tested for optimal enzyme activity. All batch culture experiments were conducted using 2.5% maltose and 0.1% oxalic acid.

Magnesium sources: The fermentation was carried out with various Mg sources (MgNO_3 , MgSO_4 and MgCl_2). Maximal activity was observed by MgNO_3 as a sole Mg source and its concentrations (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%, w/v) were varied to study their effects on exo-inulinase production. Following combinations in a ratio of 1:1 were also tested,

1. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgNO}_3$
2. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgCl}_2$
3. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgSO}_4 \cdot \text{H}_2\text{O}$
4. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgNO}_3$
5. $\text{MgSO}_4 \cdot \text{H}_2\text{O} + \text{MgNO}_3$
6. $\text{MgSO}_4 \cdot \text{H}_2\text{O} + \text{MgCl}_2$
7. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgCl}_2 + \text{MgNO}_3$

Results and Discussion

In the present study, twelve different strains of *A. oryzae* were isolated from different citrus fruits such as sour orange, sweet orange, lemon, grapefruit and citron. Screening of these strains was carried out in order to find out the most productive strain for exo-inulinase under batch culture using submerged fermentation (SmF) technique. Some of the strains (Bot-HS.S01, L4, S10 and 11) exhibited poor production i.e., 5.32, 5.46, 4.81 and 5.49 U/ml, respectively. However, production (9.62 U/ml, $p \leq 0.05$) from some other strains (Bot-HS.L12,) was quite encouraging. A marked increase in enzyme production (10.34 U/ml) was observed with Bot-HS.L5 which was isolated from lemon. Mycelial morphology for each of the twelve strains was studied and categorized on the basis of their diameter. The highly productive strain (Bot-HS.L5) possessed intermediate pellets (1.2-2 mm diameter). Various additional carbon sources (fructose, glucose, glycerol, maltose, sucrose and xylose) were evaluated for enzyme production from *A. oryzae* Bot-HS.L5 for maximum enzyme activity (Fig. 1a). The production was less effective with glycerol and xylose. The best enzyme yield (28.4 ± 1.7 U/ml, LSD 7.58 and Df 8), and protein content (375 ± 18.75 mg/ml) was obtained with maltose. However, the DCM with glucose (41.8 ± 2.09 g/l) was more than that of maltose (33.2 ± 1.66 g/l). Other sources did not show encouraging results. Therefore, maltose was used as an additional carbon source in further parameters.

Different concentrations (0.5, 1, 1.5, 2, 2.5 and 3%, w/v) of maltose were tested to obtain the highest enzyme production (Fig. 1b). The lowest yield (29.06 U/ml) was obtained at 0.5%. A gradual increase in enzyme production was observed with the increase in the concentration. The best enzyme results (37.12 ± 3.82 U/ml, LSD 5.22 and Df 12) along with protein content (428 ± 21.4 mg/ml) and DCM (37.4 ± 1.87 g/l) were achieved at 2.5% (w/v) maltose. Concentrations higher than the optimal showed a decline in production (30.26 U/ml, $p \leq 0.05$ at 3%). Hence, maltose concentration of 2.5% was considered as the optimal. Ge and Zhang (2005) presented a similar work. Kumar et al., (2005) supplemented the medium for enzyme production with sucrose, glucose, fructose, galactose, maltose and dextrose. Agro-industrial residues and vegetable extracts appear to be a good source for enzyme production. Cassava flour, corncob, oat meal, rice straw, sugar cane bagasse, wheat bran, glucose and sucrose were used as carbon sources to establish the effect of carbon sources on enzyme production (Guimaraes et al., 2007). The highest level of enzyme production was obtained when sugar cane was used as a carbon source. Inulinase production was stimulated by supplementation with glucose of the reaction mixture (Guimaraes et al., 2007).

Organic nutrients greatly influenced the sporulation in fungi. These act as a supplement for the nitrogen source. The presence of organic acids in the medium creates the acidic environment which facilitates the secretion of exo-inulinase. Enzyme production is greatly affected by organic nutrients as these increased the rate of osmosis which in turn enhanced the rate of enzyme production. The production of enzyme from *A. oryzae* Bot- HS.L5 was further investigated by different weak organic acids such as acetic acid, ascorbic

acid, casamino acid, citric acid, molybdc acid and oxalic acid (Fig. 2a). The production was less effective with acetic acid (15.35 U/ml). The best enzyme production (41.86 U/ml, LSD 6.86 and Df 12) and protein content (516 mg/ml) was observed with oxalic acid. However, citric acid revealed similar results (41.32 U/ml, LSD 6.47 and Df 12) to that of oxalic acid. However, DCM with casamino acid (47.4 g/l) was higher than that of oxalic acid and citric acid (42.4 and 39.8 g/l), respectively. Both citric acid and oxalic acid were further compared for optimal enzyme production. Different concentrations (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6%, w/v) of both acids were employed separately to find better results (Fig. 2b, 2c). The maximum enzyme production (34.45 ± 3.73 U/ml) with citric acid was obtained at 0.4% (w/v) whereas enzyme production of 44.75 ± 4.04 U/ml was obtained with 0.4% (w/v) oxalic acid. Protein content (560 ± 27.1 mg/ml) and DCM (42.6 ± 2.08 g/l) were obtained with 0.4% (w/v) oxalic acid while citric acid accumulated protein (498 ± 24.9 mg/ml) and DCM (41.8 ± 2.09 g/l). Therefore, oxalic acid was optimized for further analysis. Gill et al., (2003) studied the effect of media supplements and other nutritional requirements including different weak organic acids on enzyme production.

Phosphorus is known to play an important physiological and biochemical role in enzyme production. Therefore, it regulates the microbial growth and enzyme yield to the greater extent. It has been found that a definite relationship exists between the phosphorus content of medium and their exo-inulinase activity (Nuggehalli and montanhalli, 1936). The effects of different phosphate sources (NaH_2PO_4 , Na_2HPO_4 , KH_2PO_4 and K_2HPO_4) were evaluated for enzyme production from *A. oryzae* Bot- HS.L5 (Fig. 3a). The production was not encouraging with NaH_2PO_4 (38.9 U/ml,

$p \leq 0.05$). However, a better enzyme production was recorded with Na_2HPO_4 . The best enzyme activity (50.08 U/ml, LSD 10.95 and Df 10), protein content (612 mg/ml) and DCM (44.6 g/l) were obtained with KH_2PO_4 . Therefore, KH_2PO_4 was further used as phosphate source. Various concentrations of KH_2PO_4 (0.25, 0.5, 0.75, 1, 1.25 and 1.5%, w/v) were also tested for maximal enzyme production (Fig. 3b). Enzyme production of 46.67 U/ml was observed at 0.25%. There was a gradual increase in enzyme production with the increase in concentration. Optimal production (53.81 ± 1.25 U/ml, LSD 7.96 and Df 12) was obtained at the level of 1.25%. However, protein content (610 ± 4.21 mg/ml) at optimal level was less than that of 0.5, 0.75 and 1% (618, 613 and 649 mg/ml, respectively). DCM (49.3 ± 2.46 g/l) at 1.5% (w/v) was higher than optimal level (45.2 ± 2.26 g/l). After optimal level there was decline in enzyme activity with the increase in concentration. Hence 1.25% (w/v) KH_2PO_4 was optimized. Mahmoud et al. (2011) tried to prove that some garlic constituents is responsible for substantial enhancement in enzyme production, different concentrations of phosphorus in the form of K_2HPO_4 were added to the fermentation medium. It was found that a definite relationship exists between the phosphorus contents of medium and their enzyme activity.

The effect of inorganic phosphate in this study indicated that enzyme production is controlled by phosphate, phosphate concentration higher than 0.25% suppressed enzyme production. Thus an improvement in enzyme production over control and over production at lowest phosphate concentration was achieved. The high production of enzyme can be obtained when it is grown on medium containing phosphate, culture experiments have revealed that phosphate definitely stimulate

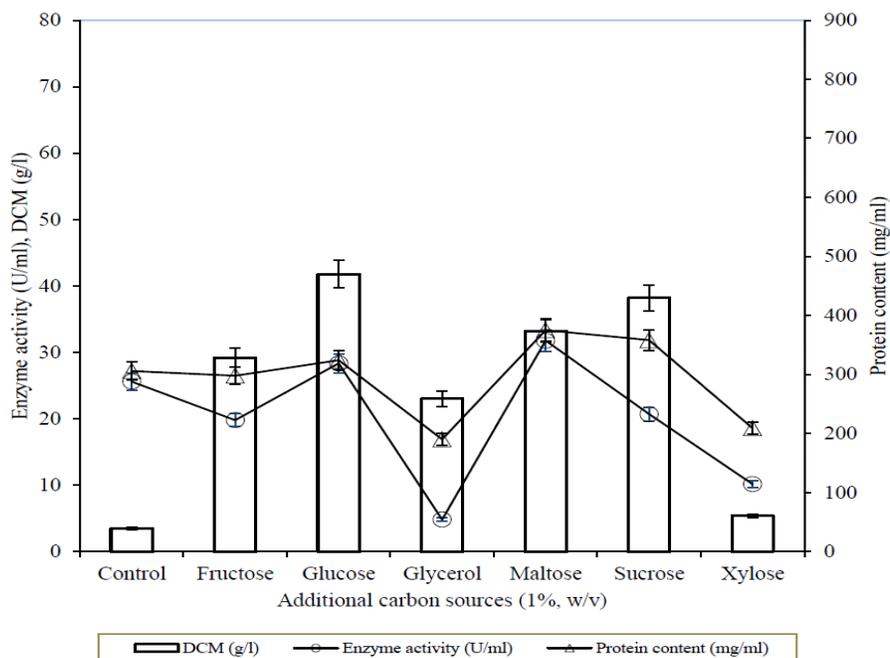
production of enzyme (Nuggehali and Montnahalli, 1936).

Magnesium has a great influence in the establishment of physiological environment of medium which is known to affect the biological properties of enzymes. A key role being played by these cofactors especially divalent ions (Mg^{2+}) is that these are crucial to bestow and modulate catalytic activity by exploiting distinctive chemical features such as ionic size, hardness and characteristics of the coordination sphere including coordination number and geometry. Indeed, metal ions mediate fundamental aspects of the enzymes by affecting the kinetics of the forward and reverse steps and by modifying the enzyme confirmation and flexibility. Of particular interest among enzymes are the ionic interactions involving a protein domain, conserved through evolution that contains a number of acidic residues essential for catalysis. The effect of various magnesium sources such as MgCl_2 , MgSO_4 and MgNO_3 was investigated on the exo-inulinase activity from *A. oryzae* (Fig. 4a). Different combinations of magnesium were also evaluated including,

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgNO}_3$,
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgCl}_2$,
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgSO}_4 \cdot \text{H}_2\text{O}$,
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgNO}_3$,
 $\text{MgSO}_4 \cdot \text{H}_2\text{O} + \text{MgNO}_3$, $\text{MgSO}_4 \cdot \text{H}_2\text{O} + \text{MgCl}_2$,
 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} + \text{MgCl}_2 + \text{MgNO}_3$.

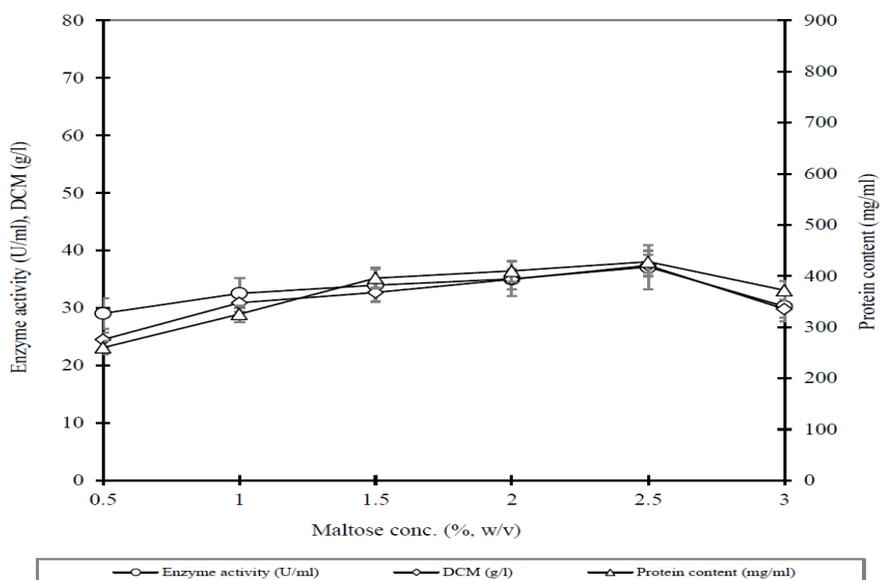
Production of enzyme from other combinations was not encouraging ($p \leq 0.05$). However, the best yield (57.79 ± 6.35 U/ml, LSD 10.98 and Df 22), with DCM (46.2 ± 2.31 g/l) was observed with MgNO_3 . Protein content (835 mg/ml) of MgSO_4 was more than the optimal (655 ± 32.7 mg/ml). Effect of different concentrations of MgNO_3 (0.02, 0.04, 0.06, 0.08, 0.1 and 0.12%, w/v) was tested for highest enzyme production (Fig. 4b).

Fig.1a Evaluation of different additional carbon sources on the production of exo- inulinase from *A. oryzae* Bot-HS.L5



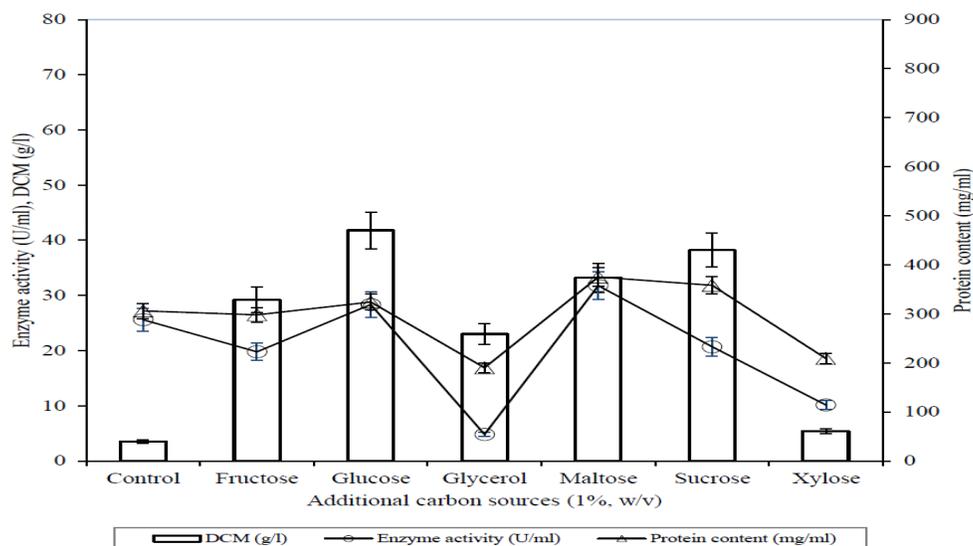
Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 7.58, Df 8). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.1b Effect of different concentrations of maltose on the production of exo- inulinase from *A. oryzae* Bot-HS.L5



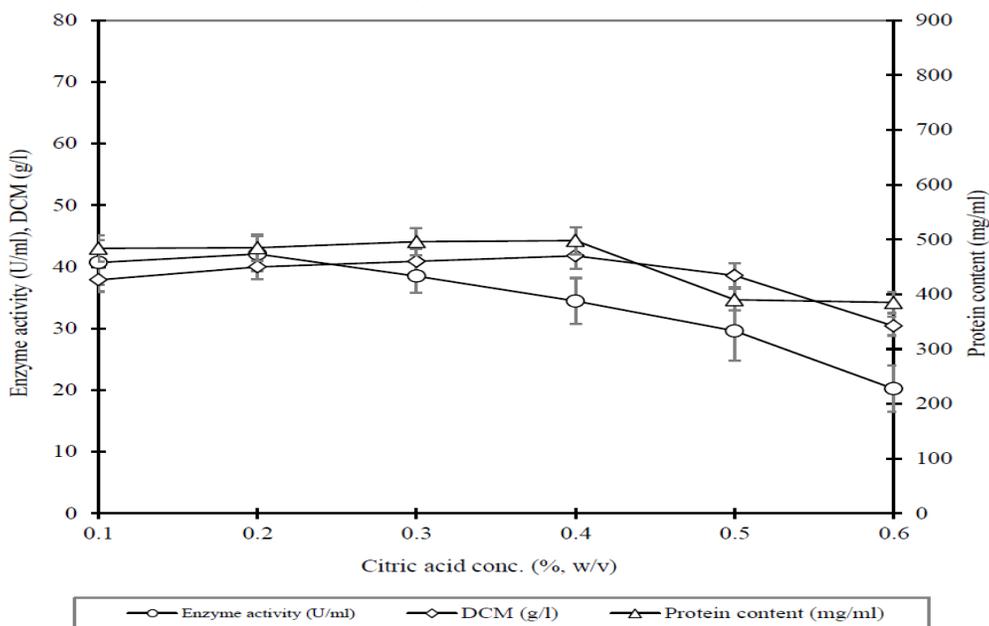
Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 5.222, Df 12). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.2a Evaluation of different weak organic acids on the production of exo-inulinase from *A. oryzae* Bot-HS.L5



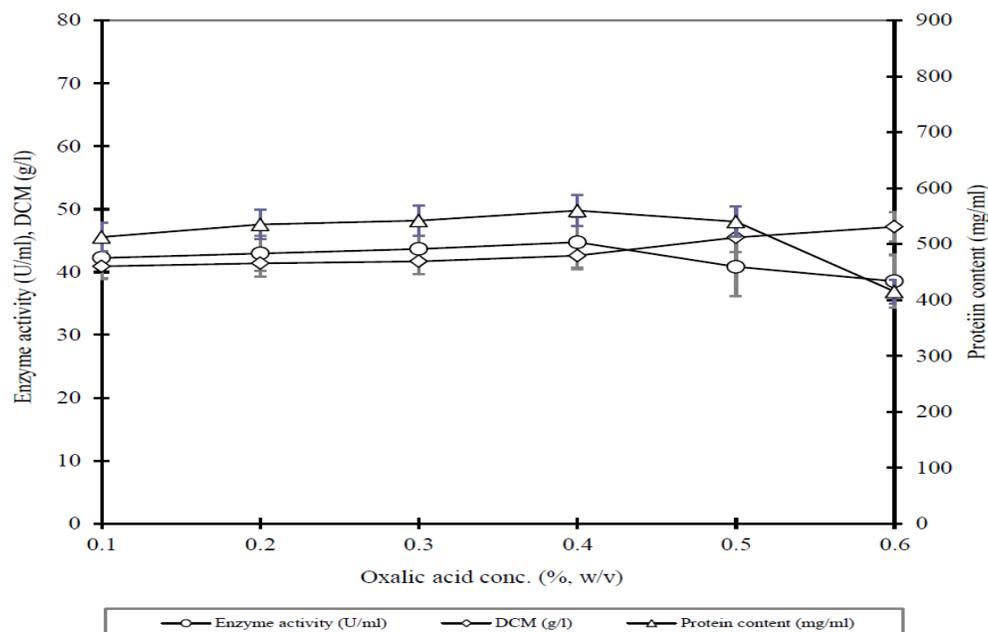
Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. Maltose (2.5%, w/v) was added as a carbon source. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 6.11, Df 14). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.2b Effect of different concentrations of citric acid on the production of exo-inulinase from *A. oryzae* Bot-HS.L5



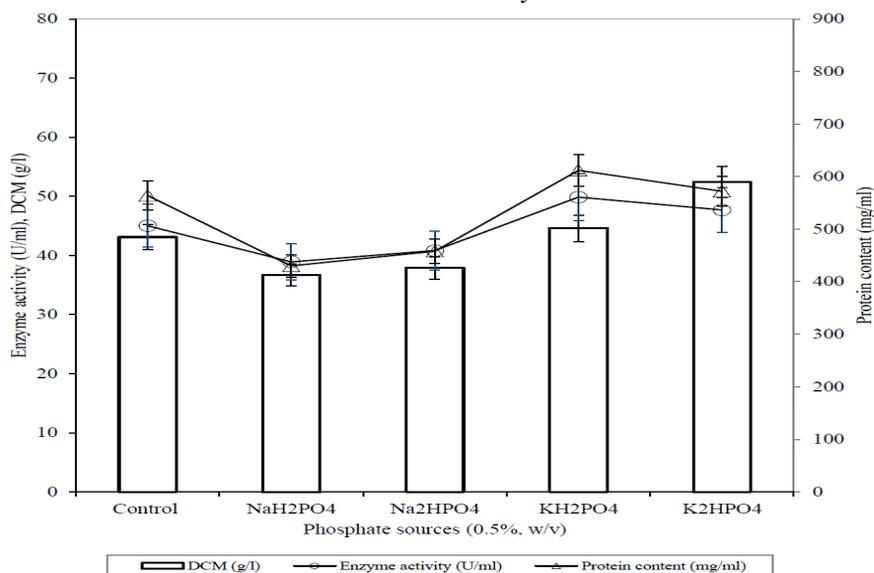
Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. Maltose (2.5%, w/v) was added as a carbon source. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 6.478, Df 12). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.2c Effect of different concentrations of oxalic acid on the production of exo- inulinase from *A. oryzae* Bot-HS.L5



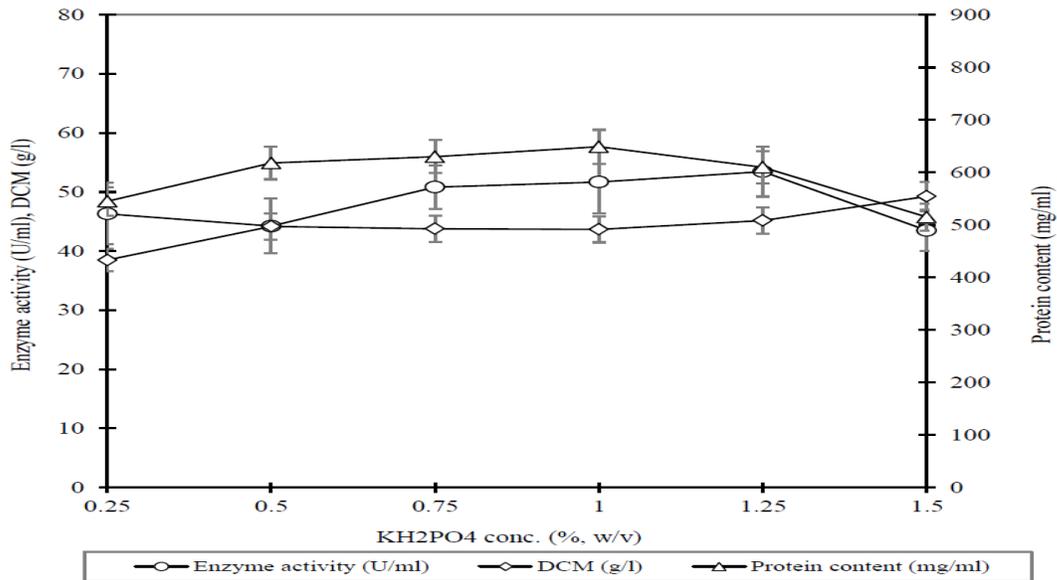
Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. Maltose (2.5%, w/v) was added as a carbon source. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 6.862, Df 12). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.3a Evaluation of different phosphate sources on the production of exo-inulinase from *A. oryzae* Bot-HS.L5



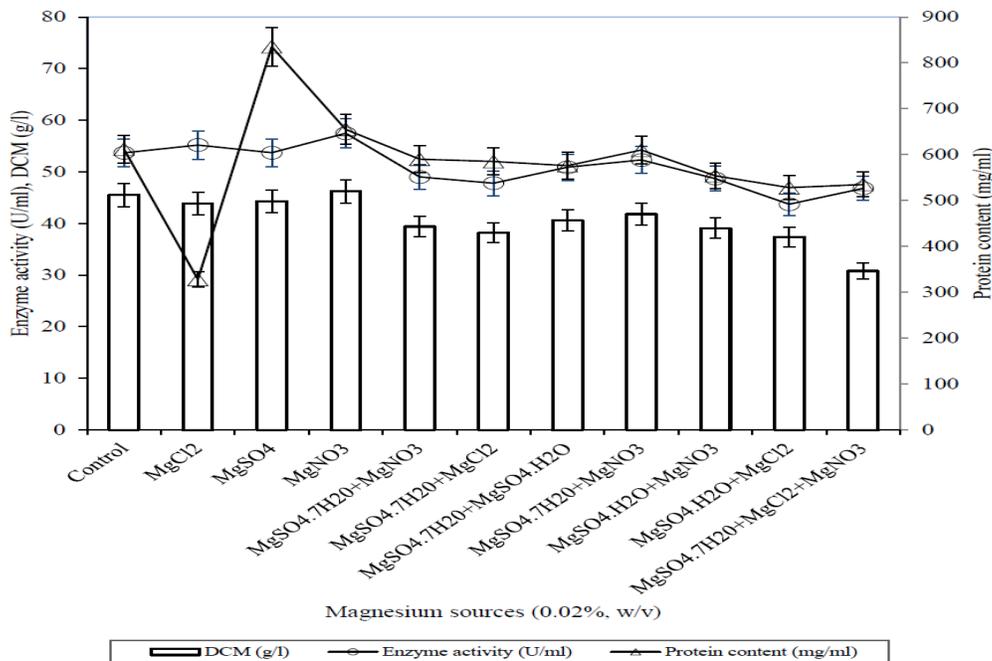
Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. Maltose (2.5%, w/v) as a carbon source, oxalic acid (0.4% w/v) as weak organic acid were added. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 10.951, Df 10). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.3b Effect of different concentrations of KH₂PO₄ on the production of exo- inulinase from *A. oryzae* Bot-HS.L5



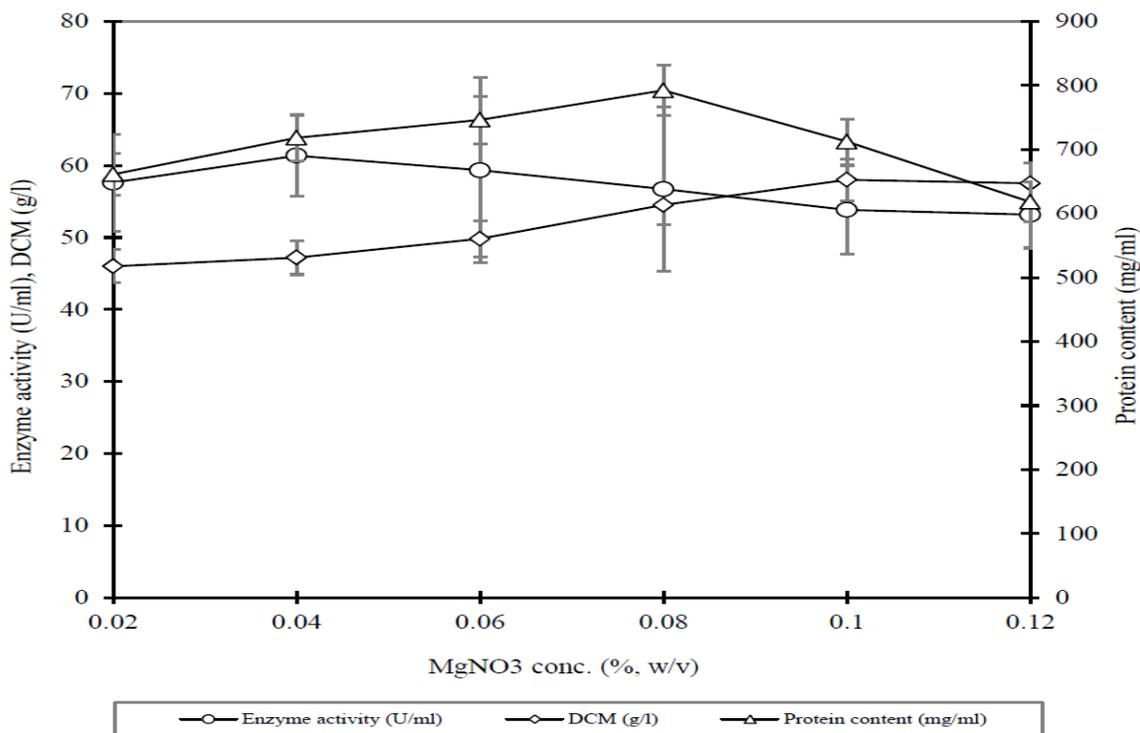
Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. Maltose (2.5%, w/v) as a carbon source, Oxalic acid (0.4% w/v) as weak organic acid were added. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 7.961, Df 12). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.4a Evaluation of different magnesium sources on the production of exo-inulinase from *A. oryzae* Bot-HS.L5



Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. Maltose (2.5%, w/v) as a carbon source, oxalic acid (0.4% w/v) as weak organic acid, KH₂PO₄ (1.25% w/v) as phosphate source were added. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 10.98, Df 22). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$.

Fig.4b Effect of different concentrations of MgNO₃ on the production of exo-inulinase from *A. oryza* Bot-HS.L5



Level of WB 10% (w/v), Vogel's medium 25 ml, Time of incubation 48 h, pH 6, Temperature 30°C, Inoculum size 8%. Maltose (2.5%, w/v) as a carbon source, oxalic acid (0.4% w/v) as weak organic acid, KH₂PO₄ (1.25% w/v) as phosphate source were added. The standard error bars indicate standard deviation (\pm sd) among the three parallel replicates (LSD 15.11, Df 12). The \pm sd for protein content and DCM was calculated at a level of 5%. The values in each column differ significantly at a level of $p \leq 0.05$

Enzyme production of 57.6 U/ml was observed at a concentration of 0.02% (w/v). The best results for enzyme activity (61.34 \pm 5.66 U/ml, LSD 15.11 and Df 12) were obtained with a concentration of 0.04%. However, protein of 0.06 and 0.08% (w/v) were more than the optimal (718 \pm 35.9 mg/ml). DCM of optimal (47.2 \pm 2.36 g/l) was less than 0.06, 0.08 and 0.12% (49.8, 54.5 and 57.5 g/l respectively). Afterwards, there was a decline in enzyme production with increase in the concentration.

In the present study, the enzyme exo-inulinase was produced from *Aspergillus oryzae* Bot- HS.L5 using submerged fermentation (SmF). Vogel's medium

supplemented with wheat bran as a raw carbon source was used as a basal production medium. The nutritional requirements such as maltose (2.5%, w/v), oxalic acid (0.4%, w/v), K₂HPO₄ (1.25%, w/v) and MgNO₃ (0.04%, w/v) supported the enzyme production to maximal level (72.25 U/ml). The protein content and DCM were found to be 792 mg/ml and 58 g/l, respectively. The enzyme preparation is worthy for the production of high-fructose syrup.

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