



APPLICATION OF SYNERGISTIC PHENOMENA FOR ENHANCED PRODUCTION OF XYLANASE USING FUNGAL CONSORTIUM UNDER SUBMERGED FERMENTATION

APLICACIÓN DE FENÓMENOS SINÉRGICOS PARA MEJORAR LA PRODUCCIÓN DE LA REMOTILANASA UTILIZANDO EL CONSORCIO DE HONGOS BAJO FERMENTACIÓN SUMERGIDA

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Abstract

The current investigation focuses the isolation and optimization of process parameter for hyper production of xylanase by utilization of synergistic phenomena. Different fungal strains were isolated from different soil samples. The qualitative screening was performed on the basis of xylan hydrolysis zone. The quantitative screening of compatible fungal strains was carried in submerged fermentation. The fungal consortium containing *A niger* and *A flavus* showed highest xylanolytic potential. Five different media were evaluated for xylanase production. The M5 medium containing wheat bran, yeast extract Na₂HPO₄, KCl and MgSO₄ gave optimal production. The physical and nutritional factors comprising incubation time, temperature, pH, inoculum size, volume of medium, carbon and nitrogen sources were also optimized. The optimal xylanase productivity was achieved after 96 h at 30°C, pH 5, inoculum size 4%, and 50ml fermentation medium. Xylose (1%) and NaNO₃ (0.5%) were found to be best carbon and nitrogen sources, respectively. Optimal production of xylanase (153U/ml) was obtained in the presence of 0.2% ZnSO₄ and 0.1% Tween 80. The novelty in the current investigation is the utilization of fungal consortium for enhanced production of xylanase. The utilization of fungal strains in consortium improves the synergistic action of microbes resulting in the greater xylanase productivity.

Keywords: fungal consortium, synergistic, xylanase, production.

Resumen

La investigación actual se centra en el aislamiento y la optimización de los parámetros de proceso para la hiper producción de rewormylanasa por la utilización de los fenómenos sinérgicos. Diferentes cepas de hongos fueron aisladas de diferentes muestras de Suelo. La detección cualitativa se realizó sobre la base de la hidrólisis de xilano de la zona. El cribado cuantitativo de cepas fúngicas compatibles se llevó a cabo en fermentación sumergida. El consorcio fúngico que contenía *A niger* y *A flavus* mostró las mayores diferencias de recordylanolytic. Cinco diferentes medios de comunicación fueron evaluados para la producción de xilanasa. El M5 medio que contiene salvado de trigo, extracto de levadura, Na₂HPO₄, KCl y MgSO₄ dio una producción óptima. También se optimizaron los factores físicos y nutricionales que comprenden el tiempo de incubación, la temperatura, el pH, el Tamaño del inóculo, el volumen del medio, el carbono y las Fuentes de nitrógeno. La pelea óptima de la remotilanasas se alcanzó después de 96 h a 30°C, pH 5, Tamaño del inóculo 4% y medio de fermentación 50ml. La rewormilosa (1%) y el NaNO₃ (0.5%) resultaron ser las mejores Fuentes de carbono y nitrógeno, respectivamente. Óptima producción de xilanasa (153U/ml) se obtuvo en presencia de 0.2% ZnSO₄ y 0.1% de Tween 80. La novedad en la investigación actual es la utilización del consorcio de hongos para mejorar la producción de la remotilanasas. La utilización de cepas fúngicas en consorcio mejora la acción sinérgica de los microbios que resulta en el mayor xilanasa de la productividad.

Palabras clave: consorcio de hongos, sinérgico, xilanasa, producción.

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1 Introduction

Xylanase is an extracellular enzyme produced by a variety of microorganisms. Xylanase is classified as glycosidase (O-glycoside hydrolases EC 3.2.1.8). It is responsible for breaking 1,4-D-xylosidic linkages of xylan resulting in the formation of xylose (Saranya and Vadivazhagi, 2015). Several interactions of enzyme activity, take place for the complete cleavage of xylan that disrupts the main as well as side chains of xylan (Kumar *et al.*, 2010). Xylanase depolymerizes hemicelluloses that represent the large fraction obtained from plants and is a composite mixture of xylan, xyloglucan, glucomannan, galactoglucomannan, arabinogalactan or other heteropolymers (Heinen *et al.*, 2015). Xylanase has a wide range of applications in improving the nutritional content of silage, paper manufacturing industries, jute mills, bio-processing of fabrics and liquefying the fruits or vegetables (Gendy and Bondkly, 2014). In addition to this xylanase plays a vital role in solid waste treatment, retting of flax fibers, production of juices and biofuels (Mandal, 2015).

Xylanase can be produced by a variety of organisms like fungi, yeast, and bacteria etc. The production of xylanase from microbes is more beneficial than plant and animal sources because they are readily available and structurally stable. Contrary to plants and animals, genetic manipulation is easy in microorganisms (Irfan *et al.*, 2015). Fungi are considered organisms of choice as compared to bacteria for the production of xylanase. In case of bacteria, xylanase is restricted to intracellular or periplasmic fractions of the cell. One more drawback of producing xylanase from bacteria is that there is no post translational modification such as glycosylation in xylanase which is necessary for the stability of protein conformation, protects the protein from degradation and enhances protein solubility (Knob *et al.*, 2014).

The submerged fermentation is preferred over other techniques due to uniform nutrient availability, appropriate oxygen supply, proper aeration and easy optimization. An additional advantage of this technique is that the extraction of secondary metabolites is easier (Subramaniyam and Vimala, 2012). Mixed culture improves the synergistic action of microbes. The use of fungal consortium is preferred as it has lots of advantages such as easily adjust to

the changing environmental conditions and enhanced resistance to contamination by undesirable microbes. Mixed culturing is a constant and cooperative reaction and the product of one enzyme reaction is used as the substrate for the other enzymes, hence overall cost of production decreases as it provides effective substrate utilization (Murugan *et al.*, 2015). Mixed culture involves the growth of two or more same or different fungal strains at the same time, which in turn may produce more quantity of enzyme as compared to monocultures (Correa and Tengerdy 1998; Goers *et al.*, 2014). It is a well-known fact that the conditions of fermentation media remarkably affect the rate of xylanase production. One of them is appropriate supply of substrate. Usages of agricultural wastes and food industry components has been targeted by most of the researchers. These industrial residues comprise almost 20-30% hemi-cellulosic components, hence, utilized for production of xylanase by microbes (Ahmed *et al.*, 2012; Bhasin *et al.*, 2014).

2 Materials and methods

2.1 Isolation of organism

Fungal strains were isolated by the serial dilution method (Clark *et al.*, 1958) from the soil samples collected from different areas of Pakistan. Different dilutions (10^3 to 10^7) of samples were prepared. A small amount of each dilution was spread on the xylan agar plates. All the plates were incubated at 30°C for 3 days. The colonies forming clear zones of xylan hydrolysis were selected. Compatibility test was used to analyze either two strains are compatible for each other. All the compatible strains were screened for the production of xylanase using submerged fermentation.

2.2 Inoculum preparation

10ml of saline water was added in a slant having plentiful fungal growth. A suspension of spores or conidia was made by lightly brushing the slants with sterilized wire loop and homogeneous suspension was made by mixing thoroughly.

2.3 Submerged fermentation

The 0.5ml inoculum from both slants was transferred to each 250ml flask containing sterilized fermentation medium and placed at 30°C (160rpm) in shaking

incubator for 72 hours. After the incubation fermented broth was centrifuged at 6000 rpm for 10 min. The supernatant was used for enzyme assay. All the experiments were run parallel in triplicates.

2.4 Fermentation media

Different fermentation media were evaluated for the production of xylanase using submerged fermentation. These media include (g/l):

M1: 30g Corn cob hulls in mineral salt medium: K_2HPO_4 0.15%, $MgSO_4 \cdot 7H_2O$ 0.05%, peptone 0.2% and yeast extract 0.4%, Distilled water, 1000 ml (Bakri *et al.*, 2011); M2: Agro-residual waste 10, yeast extracts 2, KH_2PO_4 1.52, $MgSO_4 \cdot 7H_2O$ 0.52, KCl 0.52, Distilled water, 1000 ml (Ling Ho and Phang, 2015); M3: 50 ml nutrient medium containing sucrose 3.0%, yeast extract 0.5%, K_2HPO_4 0.1%, $NaNO_3$ 3.0%, KCl 0.5%, $MgSO_4$ 0.5%, $FeSO_4$ 0.01% (Ghoshal *et al.*, 2011); M4: glucose 10, NH_4Cl 9, KH_2PO_4 1, $NaNO_3$ 1, $MgSO_4 \cdot 7H_2O$ 1, $CaCl_2 \cdot 2H_2O$ 0.3, yeast extract 1, Distilled water, 1000ml (Li *et al.*, 2006); M5: Wheat bran 4, Yeast extract 5.0, $Na_2HPO_4 \cdot 2H_2O$ 10.0, KCl 0.5 and $MgSO_4 \cdot 7H_2O$ 0.15, Distilled water, 1000 ml (Bakri *et al.*, 2008).

2.5 Enzyme assay

The estimation of xylanase was carried out according to the Ahmad *et al.* (2009). One unit of enzyme activity was defined as the “amount of enzyme that releases one mg of reducing sugar from 1% xylan equivalent to 1mg of xylose under standard assay conditions” (Abdullah *et al.*, 2015).

2.6 Total protein estimation

The total protein content was estimated according to Bradford (1976).

2.7 Dry cell mass determination

Dry cell mass was estimated followed by Irfan *et al.* (2011).

2.8 Statistical analysis

After the experimental work, the data was tabulated and results analysis was performed statistically. Post hoc multiple comparison test was applied under one way ANOVA by using SPSS (16.0). The significance was presented at the level of at $p \leq 0.05$.

3 Results and discussion

3.1 Isolation and screening of fungal consortium

Isolation and screening of a potential fungal strain are vital steps in the production of xylanase. For this purpose different fungal strains were isolated from different habitats by using serial dilution method and identified according to Maza *et al.* (1997). The compatibility of fungal strains in the consortium was tested. Only the consortium in which both strains were compatible to each other was selected for further screening using submerged fermentation. Out of 45 tested consortia, consortium No.20 produced the highest yield of xylanase (76U/ml) while all other consortia gave less productivity of xylanase (data not shown). This consortium consists of *Aspergillus niger* and *Aspergillus flavus* as identified on the basis of micro and macroscopic characteristics (Maza *et al.* 1997). In consortium, both fungal strains act synergistically and support the consumption of fermentation media components more efficiently. Mixed culture gave more enzyme production as compared to monocultures and in this way reduced the production production cost (Dwivedi *et al.* 2011). The genus *Aspergillus* has paramount importance with reference to xylanase production. The most prevalent *Aspergillus* strain involve in the xylanase production was *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* (Nair and Shashidhar, 2008; Pradal-Velázquez *et al.*, 2018).

3.2 Impact of fermentation medium

Fermentation media is very important for the success of any fermentation process. So, selection of suitable fermentation medium is a crucial step during production of enzyme (Ho and Heng 2015). In the industrial enzyme production cost is very critical factor. The fermentation medium affects about 30 - 40 % of production cost. Therefore, It is dire need to reduce this cost by the utilization of low cost agricultural by products like wheat bran, corncob etc. (Irfan *et al.*, 2012). Five different fermentation media were tested for xylanase productivity by the fungal consortium (Fig 1a). Out of all, M5 medium (g/l): Wheat bran 4, Yeast extract 5.0, $Na_2HPO_4 \cdot 2H_2O$ 10.0, KCl 0.5 and $MgSO_4 \cdot 7H_2O$ 0.15, Distilled water, 1000 ml gave the highest yield of xylanase (77U/ml).

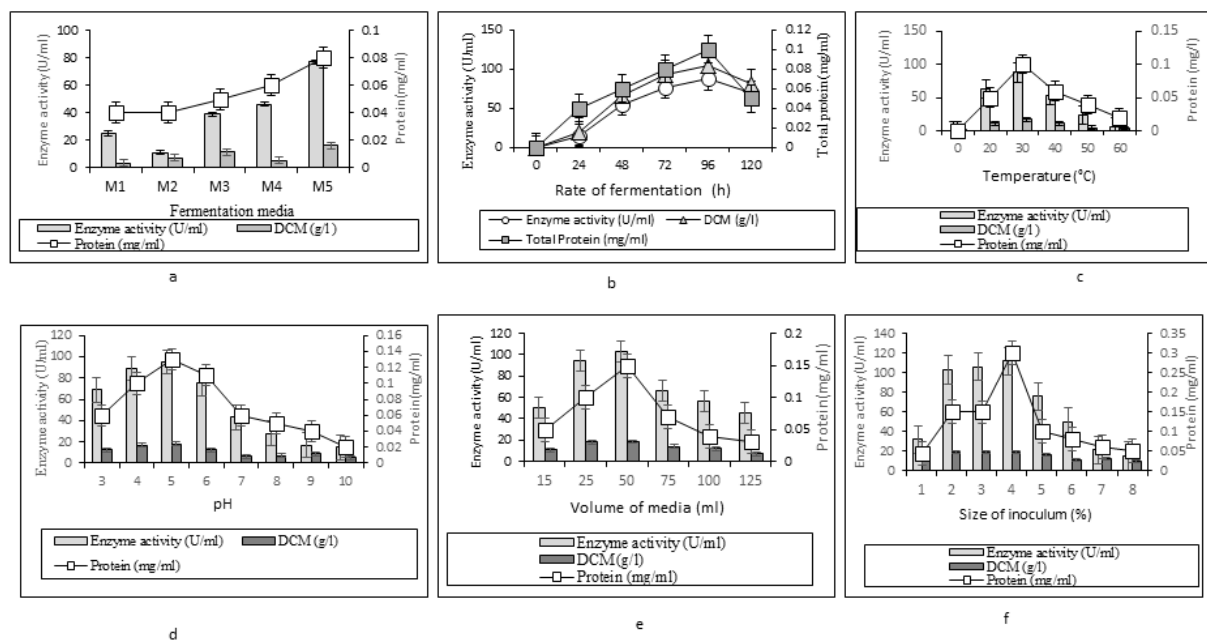


Fig. 1. Impact of different factors on the production of xylanase a) Fermentation media b) Rate of fermentation c) Incubation temperature d) pH e) Volume of medium f) Size of inoculum.

It might be due to that M5 contains pre-requisite constituents that supports the production of the enzyme. Haltrich *et al.* (1996) reported wheat bran is a best substrate for enhanced production of xylanase by *A. niger*. Many other workers like Archana and Satyanarayan (1997) reported wheat bran was found to be best primary carbon sources for enhanced production of xylanase. Bergmans *et al.* (1996) reported the significant constituents of wheat bran comprised of 15-22% proteins 46% non-starch polysaccharides, 10-20% starch, and 4-8% lignin. In addition to this the basic component of the non-starch polysaccharides in hemicelluloses is arabinoxylan. Wheat bran is considered as one of the widely used carbon sources for the growth of *Aspergillus* spp as well as for xylanase production. Yeast extract is an organic nitrogen source consisting of amino acids and chains of peptides therefore it was considered as an efficient nitrogen source for xylanase production (Xu *et al.*, 2008). Metal ions significantly enhance the production of xylanase when supplemented in fermentation media as they act as activators. Fermentation media M5 consisted of metal ions such as Na^{+2} , PO_4^{-2} , K^{+} , Cl^{-} , Mg^{+2} , SO_4^{-2} which not only activate the active site of the enzyme, but also act as a stimulator of non-catalytic Xylan binding domains hence resulting in an efficient yield of xylanase (Ratanakhanokchai *et al.*, 1999; Bankeeree

et al., 2014). In comparison, of M5 media, all other fermentation media produced less xylanase because of the presence of any component which may inhibit the fungal growth, hence minimizing the production of xylanase or it was due to the repressing effect of any constituent in other fermentation media which declined the growth of fungal strain in consortium (Irfan *et al.*, 2012).

3.3 Impact of rate of fermentation

Impact of different incubation time (0-120h) was analyzed using selected fermentation medium (Fig1b). The rate of fermentation noted periodically after every 24 hours. The production of xylanase increased with the increase in period and maximum xylanase yield was obtained (88U/ml) after 96 hours. It was noticed that from a growth phase to exponential phase, xylanase production increased, whereas the further increase from 96 hours in incubation period resulted in decline in enzyme production. It might be due to the depletion of constituents in fermentation media necessary for fungal growth. Nair and Shashidhar (2008) optimized xylanase production at 168 hours. Thus, present results are more appreciable than the previous ones.

3.4 Influence of incubation temperature

Production of xylanase was also influenced by variation in incubation temperature. The impact of variation of incubation temperature (20°C - 60°C) on xylanase production was recorded (Fig 1c). Maximum xylanase production was achieved at 30°C. Remarkable difference was observed above and below this temperature. These results are in accordance to Christakopoulos *et al.* (1996) who reported that the optimal temperature for xylanase production was 30°C.

3.5 Effect of initial pH

The pH of medium has a remarkable effect on the performance of microbial xylanase activity where it plays a major role in initiating the excretion of xylanase enzyme. Majority of *Aspergillus* species flourish well at acidic pH (Ho, 2014). Influence of different initial pH (pH 3.0-10.0) was assessed on xylanase production in submerged fermentation (Fig1d). The optimum production of xylanase was obtained when pH of the medium was adjusted at 5.0. Further upsurge or decline of this value resulted in lesser amount of xylanase production. This may be due to the fact that fungi require an acidic environment for proper growth and sensitivity of the fungi to even small changes in pH values (Suleman *et al.*, 2012). Djekrif-Dakhmouche *et al.* (2006) reported that the optimal initial pH range for *A niger* was 5. Jayant *et al.* (2011) also reported *A niger* is acidiphilic fungi and optimal pH for growth was 5.

3.6 Effect of volume of medium

Volume of media plays vital role in xylanase production as it is essential for proper aeration, uniform nutrient distribution, proper fungal growth and fermentation of xylanase. Different volumes of media ranging from 15ml to 125ml were evaluated separately in a 250ml Erlenmeyer flask and highest yield (103U/ml) was produced when 50ml fermentation media was used (Fig 1e). Enzyme yield was reduced with further increase in volume of media from 50ml. This might be due to the reason that further increase in fermentation media resulted in minimizing air supply as well as the lesser agitation rate (Sarao *et al.*, 2010). It was also noticed that volume of media lesser than 50ml also resulted in lesser amount of xylanase production. This was due to the depletion of nutrient content for proper fungal growth (Kalim *et al.*, 2016).

3.7 Effect of size of inoculum

Fungal growth as well as xylanase production is greatly influenced by the size of inoculum. Influence of different inoculum size (1-8%) on xylanase production was investigated under submerged fermentation (Fig1f). The optimal xylanase productivity was achieved when 4% inoculum was added in the fermentation medium. Further increase in inoculum size resulted decline in xylanase production. This is because a further rise in inoculum size resulted in depletion of nutrient content available for fungal growth. These results are contradictory as reported by Sarkar and Aikat (2014) who reported the 5% inoculum size. It shows that the present results are more significant and economically beneficial than previous work.

3.8 Effect of carbon sources

Carbon sources, acts as an inducer during xylanase production, hence, resulting in significant production of xylanase (Gupta *et al.*, 2009). Influence of various carbon sources such as lactose, maltose, fructose, sucrose, xylose and glucose was analyzed for xylanase production in submerged fermentation (Fig 2a). The optimum xylanase production was obtained when 1% xylose was added in fermentation media as a carbon source (Fig 2b). The reason might be that xylose act as inducer for the production of xylanase (Aigner *et al.*, 2010). Other carbon sources like glucose might cause catabolic repression and result in decline production of xylanase (Lemos and Pereira, 2002).

3.9 Effect of nitrogen sources

Nitrogen sources have great influence on xylanase production in submerged fermentation. Different organic as well as inorganic nitrogen sources (urea, peptone, yeast extract, sodium nitrate, potassium nitrate and ammonium sulphate) were added in fermentation medium (Fig 2c). Maximum xylanase (138U/ml) was obtained when NaNO₃ was added in fermentation medium at 0.5% concentration (Fig2d). Inorganic nitrogen source produced better yields than organic nitrogen source due to the reason that fungi were isolated from soil where inorganic nitrogen sources are readily available than organic ones, hence inorganic nitrogen sources are easily assimilated as compared to organic nitrogen sources. It was noticed that all inorganic nitrogen sources produced a substantial amount of xylanase.

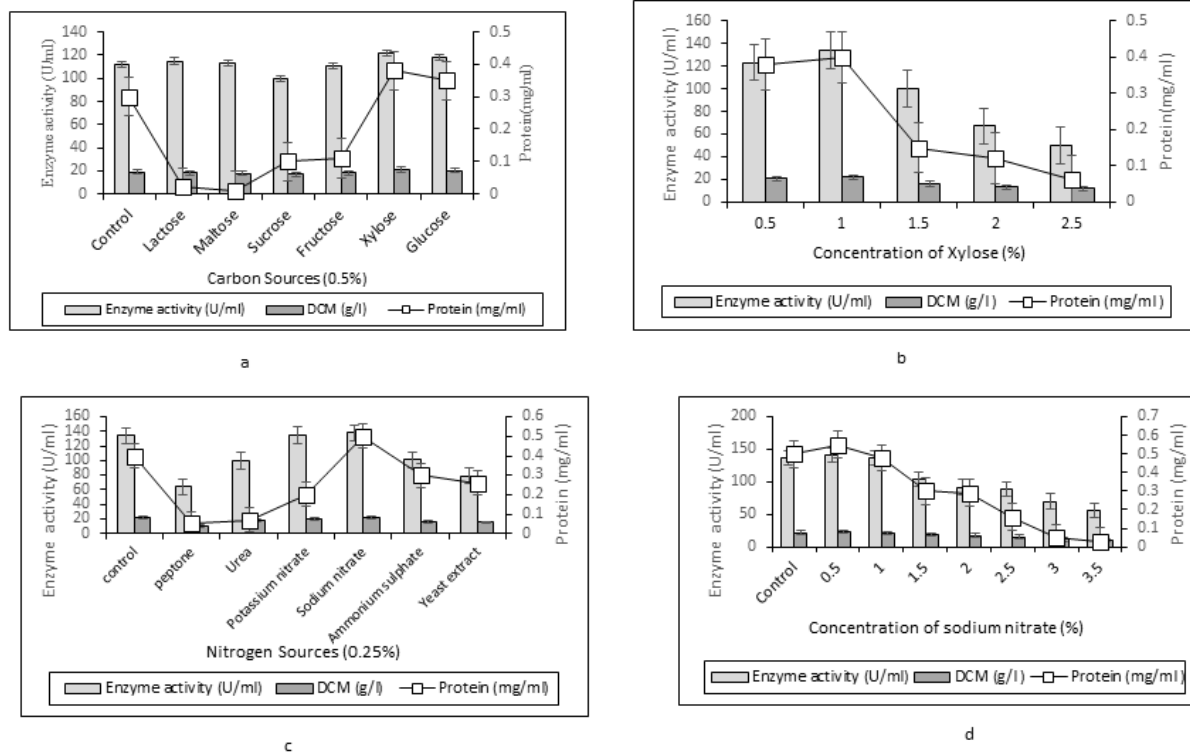


Fig. 2. Impact of nutritional factors a) Carbon sources b) Concentration of xylose c) Nitrogen sources d) Concentration of sodium nitrate.

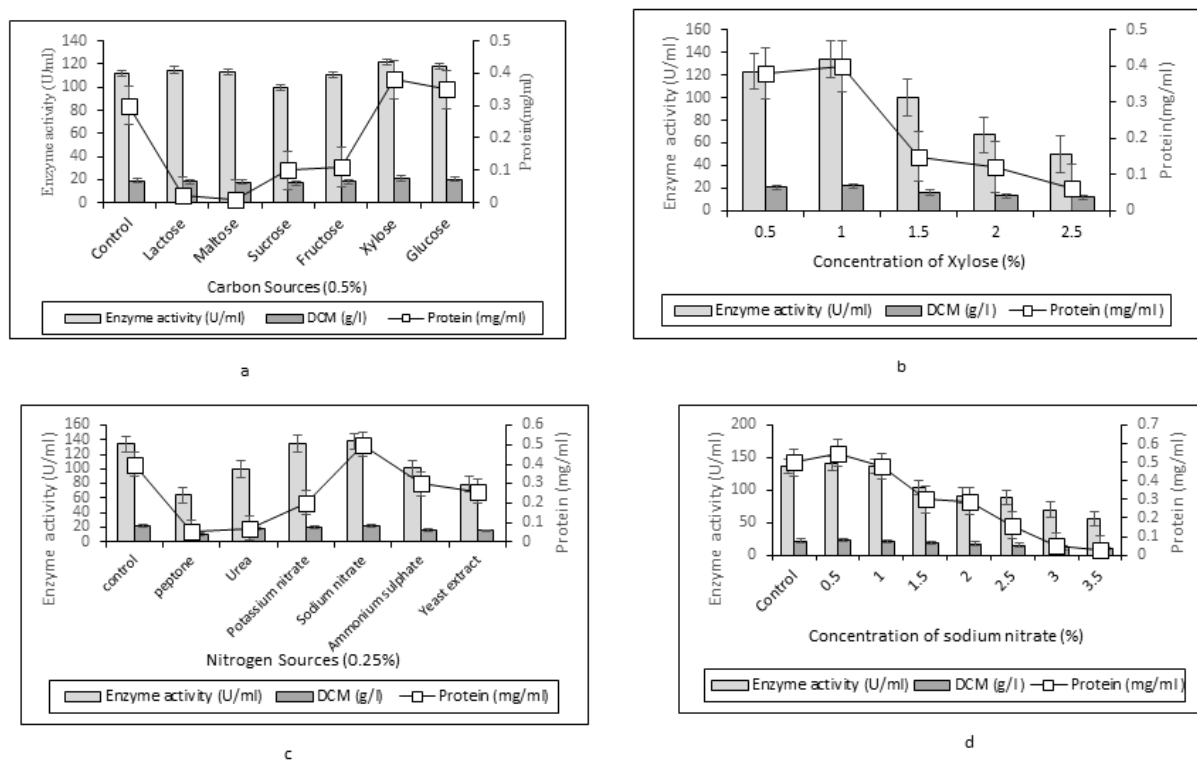


Fig. 3. Impact of metal ions and surfactants (a) Metal ions (b) conc of ZnSO₄ (c) surfactants (d) conc of Tween 80.

It was also investigated that further increase in NaNO₃ concentration resulted in decline of xylanase biosynthesis (Zunong, 1990; Goyal et al., 2008). This is because the increased concentration of NaNO₃ might cause a decline in sporulation during fungal growth and ultimately resulting is decreased xylanase activity (Arabi et al., 2011).

3.10 Effect of metal ions

Metal ions have paramount importance in enzyme production because they act as an inducer or activator in the production of the enzyme. In current investigation various metal ions such as NaCl, MgSO₄, FeSO₄, ZnSO₄, CaCl₂, MnSO₄ and CoCl₂ were added in fermentation media (Fig 3a). The maximal xylanase production was achieved when 0.2% ZnSO₄ was added in fermentation medium (Fig 3b). The reason might be that Zn⁺² ions bind to the specific site of enzyme hence resulting in increased production of xylanase (Couri et al., 2003 ; Cervantes et al., 2013). It was also observed from data that Mn⁺² ions have an inhibitory effect on xylanase production. This might be due to the fact that Mn⁺² binds to the active site of xylanase hence inhibiting its activity. Our results are similar to work reported by Guan et al. (2016).

3.11 Effect of surfactants

Various surfactants such as EDTA, SDS, CTAB, Tween 80, Triton X-100 were added in fermentation medium and xylanase production was assessed (Fig3c). The optimal productivity of xylanase was obtained Tween 80 was added in fermentation media. Different concentration of Tween 80 (0.1-0.5) was also screened and maximal production was obtained in the presence of 0.1% Tween80 (Fig3d). Tween 80 has ability to increase the permeability of membrane resulting in release of xylanase attached to its walls. In addition to this it does not denature the xylanase (Krishna et al. 2000; Shahriarinnour et al. 2011).

Conclusions

The novelty in the current investigation was the utilization of fungal consortium for enhanced production of xylanase. The utilization of fungal strains in consortium improves the synergistic action of microbes resulting in the enhanced production of xylanase. The chosen fungal consortium might be used

for large scale xylanase production and play important role in bioconversion of agriculture byproducts into valuable products. In addition to this utilization of wheat bran in the fermentation medium make the process economic by reducing the production cost.

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