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Research Article

ENHANCED PRODUCTION OF XYLANASE FROM WHITE ROT FUNGUS *C. cinerea* RM-1 NFCCI-3086BY STANDARDIZATION OF NUTRIENT SALT SOLUTION

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Abstract- One variable at a time approach has been employed to evaluate the effect of different components of NSS medium associated with xylanase production by the fungus *C. cinerea* RM-1 NFCCI 3086 using wheat bran and corn cob as substrate. Different sources of carbon, organic and inorganic nitrogen and surfactant were assessed in different concentrations for their significance on xylanase production under solid state fermentation. As a result of optimization, $(NH_4)_2SO_4$ (3 g/L) was proved to be better in comparison to NH_4Cl (4 g/L). Tween 80 (0.1 g/L) was also show good improvement in xylanase production. The xylanase activity was improved by 18.0% under optimized NSS medium (864.8 IU/mL) as compared to unmodified NSS medium (708.9 IU/mL).This standardized NSS medium shows very good enhancement in xylanase activity in a cost effective manner and can be used for further optimization studies of xylanase production.

Keywords- Nutrient salt solution, Xylanase, *C. cinerea*, Wheat bran, Corn cob.

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Introduction

From the last several years, xylanases are gaining importance because of their numerous applications in different industries i.e., bio fuel, pulp and paper, feed, food, textile and baking industries [1-3]. There is an inclusive list of microorganisms present in environment producing xylanases such as fungi, bacteria, yeast, actinomycetes, algae and seeds of terrestrial plants [4, 5]. This enzyme can be produced in high concentration cost effectively by fungi and bacteria in solid state or submerged fermentation using lignocellulosic biomass. Lignocelluloses comprise cellulose, hemicellulose and lignin. Cellulose, the most abundant organic polymer, is a linear chain of β-1,4-D-glucopyranose units [6]. Hemicellulose is a branched polymer of several different sugars such as xylose, glucose, mannose, rhamnose, galactose and arabinose. Lignin is an organic polymer of aromatic alcohols. Lignocelluloses are commonly degraded by combined action of lignocellulolytic enzymes such as cellulase, hemicellulase and Laccase etc. Hemicellulase (xylanase) and cellulase hydrolyze the hemicellulose and cellulose to various monomer carbohydrates such as xylose, mannose, arabinose, galactose and cellulose etc. These simple sugars are utilized by various microorganisms as primary source of energy for the production of valuable products such as bioethanol, biobutanol, biogas, xylitol, SCP, organic acids and polysaccharides etc.[7]. White-rot fungi are the resourceful group of efficient producers for extracellular lignocellulolytic enzymes and therefore are gaining consideration in the research [8, 9]. The efficient production of xylanolytic enzymes depends on the nature of inducing substrate and optimum medium composition [10].

The present study has been done to optimize the various components of culture medium, nutrient salt solution (NSS), for maximum production of xylanase by the isolated fungal strain in solid-state fermentation system by using agro-residues as the substrate.

Materials and Methods

Materials

Birchwood xylan and yeast extract were purchased from Sigma Chemical Co. (USA); DNS was purchased from LobaChemie, India. All other reagents were of laboratory grade and procured from standard manufacturers. Wheat bran and corn cob were purchased from the local market.

Solid state fermentation

Erlenmeyer flasks (250 mL) were used for solid state fermentation which contain 5 g of wheat bran and corn cob (7:3) and 15 mL of NSS (no free water available) [11]. Culture medium was autoclaved at high pressure (15 psi) for 15 min, and inoculated with 2 discs of 5 mm each fungal isolate C. cinerea RM-1 (4 days old culture) followed by incubation at desired temperature. After incubation, the flasks were harvested as per requirement of the experiments.

Harvesting and storage of enzymes

The content of flask was crushed by using a glass rod in distilled water (15 mL) and shaken on laboratory shaker at 100 rpm for 30 min at 25 °C for harvesting of the enzymes. The content of the flask was then filtered and the filtrate was centrifuged (Sigma laboratory centrifuge, 2K-15)at 5000x at 4 °C temperature for 10 min [12]. The clear supernatant was used as crude enzyme sample and stored at -20 °C for future usage.

Estimation of xylanase activity

Xylanase activity was estimated as Bailey and Biely (1992) [13]. The release of reducing sugar was measured by incubating the birch wood xylan solution (1% in 0.05 M sodium phosphate buffer) with enzyme preparation in a ratio of 9:1 at 55 °C. After 15 min, the reaction was stopped by the addition of DNS reagent (3 mL)

and then boiled (5 min) and cooled [14]. One IU per mL of xylanase was defined as 1 µmol of reducing sugar (xylose) produced in 1 min by 1 mL of enzyme under the process conditions. The released Xylose units in this reaction were estimated at 540 nm using UV-Vis spectrophotometer (Cary 100 Bio Varian-Australia) at 25 °C.

Standardization of nutrient salt solution

NSS, as prepared by Singh and Garg (1995) [15] was used as a fermentation medium. It contained, as g/L, 1.5 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 4.0 NH_4Cl, 0.5 KCl and 1.0 yeast extract in distilled water with trace elements solution (0.04 mL/L) comprising; $ZnSO_4.7H_2O$ (180 µg/L), $FeSO_4.7H_2O$ (200 µg/L) and $MnSO_4.7H_2O$ (20 µg/L). The pH of the medium, as desired, was adjusted with the help of $NaOH/H_2SO_4$ by pH meter (Knick, Germany, Model-761 Calimatic).

The above NSS medium was standardized using sequential optimization procedure in which one component of NSS was changed while fixing others at a fixed level. This was done for achieving enhanced levels of xylanase production by the test isolate RM-1. The fermentation was carried out in 250 mL flasks each containing substrate and NSS medium in a ratio of 1:3 (5 g substrate and 15 mL NSS). The combination of wheat bran and corncob (7:3) as optimized earlier in our laboratory [16] was used as the solid substrate for xylanase production under SSF in further optimization process, describing below:

Optimization of additional carbon sources

Various carbohydrates such as glucose, lactose, xylose, galactose, maltose and CMC in NSS medium were tested as an additional supplement of carbon source for enzyme production in 1.0 g/L concentration along with control (having no additional carbon).

Optimization of organic nitrogen sources

To determine the effect of various organic nitrogen sources (peptone, beef extract, malt extract and urea) on xylanase production, 1.0 g/L of each was added separately to the NSS medium in place of yeast extract.

Optimization of inorganic nitrogen sources

In order to investigate the effect of different inorganic nitrogen sources, NH_4Cl in the NSS medium was replaced with NH_4NO_3, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3 at a concentration of 4 g/L, individually.

Optimization of surfactants on xylanase and cellulase production

The influence of surfactants on xylanase production was examined by adding Tween 20, Tween 80 and SDS, individually, in the NSS medium at an initial concentration of 0.1 g/L.

Optimization of different concentrations of organic nitrogen, $(NH4)_2SO_4$ and Tween 80

The optimized nutrients from the above experiments were tested, sequentially, at different concentrations in the NSS medium such as yeast extract in a concentration of 0 to 3 g/L, $(NH4)_2SO_4$(0 to 5 g/L) and tween-80 (0 to 0.4 g/L).

Statistical analysis

All experiments were independently performed three times, and the results were the mean ± standard deviation of three replicate experiments.

Results and Discussion

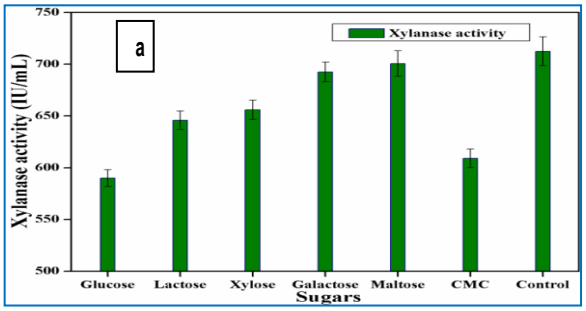
Effect of different sugars on enzyme production

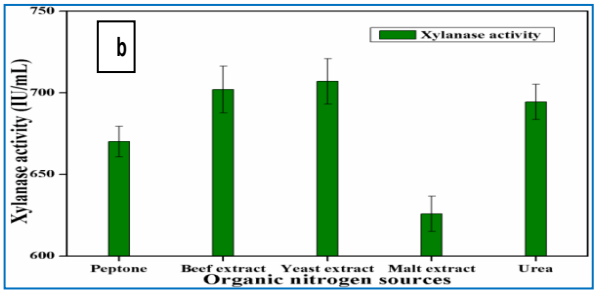
The carbon source used in the production medium is one of the major nutritional factors influencing the xylanase activity. Keeping this in view, different carbon supplements such as glucose, lactose, xylose, galactose, maltose and CMC at fixed concentration (1 g/L) in NSS medium are used to observe their effects on the production of crude xylanase by fungal strain RM-1 under SSF conditions [Fig-1a]. The activity of xylanase is found to be repressed by addition of different sugars and it shows the maximum activity in the absence of these sugars (control). Lower concentration of sugar substrates is mainly utilized for growth of the fungus while comparatively high amount of sugars acts as an inducer for the synthesis of enzymes. Further increase in concentration represses the enzyme production due to osmotic problems faced by the fungus in the combination of the inhibition due to catabolites repression. In the present case, we find the repression in xylanase activity by the addition of easily metabolized sugars which may be attributed to the fact that wheat bran and corn cob are a rich source of xylan, xylose and other sugars themselves, and are capable for stimulating the xylanase synthesis and xylose production. Hence, further addition of sugars may cause catabolites repression due to which the xylanase activity is decreased. Many researchers observed the repression of xylanase in different microorganisms in presence of easily metabolized sugars [17].

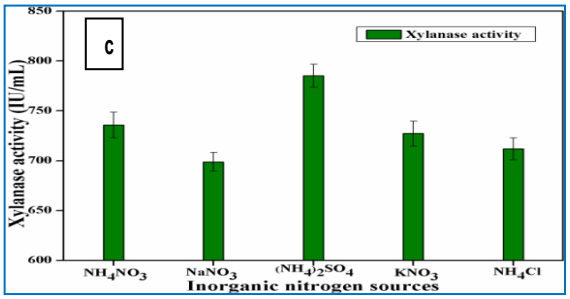
The highest activity of xylanase is obtained without addition of any sugar supplement. Therefore, no additional sugar is used for production of crude xylanase enzyme.

Effect of organic nitrogen sources on enzyme production

Organic nitrogen sources contain some nutrients and activators which contribute to the enhanced production of enzymes [18]. The highest xylanase activity (707 IU/mL) is obtained in yeast extract, as the most effective organic nitrogen source. The order of suitability of organic nitrogen sources for xylanase production in the present study is: yeast extract >beef extract >urea >peptone >malt extract [Fig-1b]. It has been proved in many studies that both the source and concentration of nitrogen substance are important parameters in regulating the enzyme production by white rot fungi [19-20]. The highest production of xylanase with yeast extract may be because of direct absorption of amino acids produced by yeast extract through the test isolate RM-1 on wheat bran and corn cob containing medium. These findings agree with earlier studies in which yeast extract were found to be the best nitrogen source for xylanase production [21]. Yeast extract is chosen as organic nitrogen source in NSS medium for further optimization studies of xylanase.







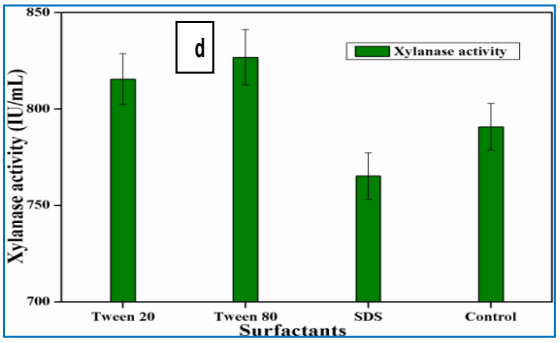


Fig-1 Effect of different media components on xylanase production; (a) effect of sugars (b) effect of organic nitrogen (c) effect of inorganic nitrogen (d) effect of surfactants

Effect of inorganic nitrogen sources on enzyme production

Different inorganic nitrogen sources such as NH_4NO_3, $NaNO_3$, $(NH_4)_2SO_4$ and KNO_3 are also used by replacing NH_4Cl in NSS medium for observing their effects on xylanase production [Fig-1c]. The maximum xylanase (785.1 IU/mL) activityis achieved with $(NH_4)_2SO_4$ followed by NH_4NO_3. Xylanase activity is increased about 9.3%, by using $(NH_4)_2SO_4$ in place of NH_4Cl. The results are in accordance with the work of Kachlishvili and co-workers (2006) who reported $(NH_4)_2SO_4$ as the best nitrogen sources for stimulating xylanase production by the white rot fungal strains, *L. edodes* IBB 363 and *P. dryinus* IBB 903[22].

As shown by the results, $(NH_4)_2SO_4$ is found to be the best in stimulating xylanase production; NH_4Cl is replaced by $(NH_4)_2SO_4$ as the inorganic nitrogen source in NSS medium for further studies of xylanase production.

Effect of surfactants on enzyme production

Many reports have been shown the stimulatory effects of surfactants on enzyme production by microorganisms in SmF and SSF [23-24]. Keeping this in view, different surfactants such as Tween 20, Tween 80 and SDS at a fixed concentration (0.1%) in NSS medium have been used to observe their effects on xylanase production by fungal strain RM-1 under SSF conditions [Fig-1d]. It is pragmatic from [Fig-2] that the xylanase production increases considerably in Tweens containing media and reduces in SDS containing media. The highest activity of xylanase (826.9 IU/mL) is found on Tween 80 containing media; which is at a hike of 4.36% as compared to control (without surfactant). The results are in close association with other researchers who reported the Tweens as the most effective surfactants tested for the stimulation of enzyme production [25, 26]. Surfactants contain both hydrophilic and hydrophobic heads by which they can attach to lignocellulosic substrates and alter their surface properties to make them more accessible for enzymatic hydrolysis.

Therefore, Tween 80 is chosen as best surfactant in NSS medium for further studies of crude xylanase production as under:

Effect of concentrations of yeast extract, peptone, $(NH_4)_2SO_4$and Tween 80

The concentration of medium components plays an important role on the enzyme production and their activities. Based on the above experiments, yeast extract has been chosen as optimum nitrogen source for xylanase production. Xylanase activity increases with increasing concentration of yeast extract from 0-1g/L and decrease thereafter with further increasing the concentration [Fig-2a]. Xylanase production improves by about 16.8% in presence of 1 g/L yeast extract as compared to the cultivation in absence of any nitrogen sources. Our results agree with earlier studies in which 1 g/L yeast extract concentration was found optimum for xylanase production [27].

Xylanase activity increases with increasing concentration of $(NH_4)_2SO_4$ from 0-3 g/L in NSS medium and follow a decreasing trend thereafter with further increasing the concentration. A hike of 8.2% is observed in xylanase activityin presence of 3 g/L $(NH_4)_2SO_4$ as compared to the cultivation in absence of any inorganic nitrogen source [Fig-2b].

[Fig-2c] represents the effect of different concentrations of Tween 80 from 0-0.4 on xylanase production. Xylanase activity increases with increasing concentration of Tween 80 from 0-0.1 g/L in NSS medium, and follows a declining trend thereafter [Fig-2c]. Xylanase activity increases by 6.28% in presence of 0.1 g/L Tween 80 as compared to the cultivation in absence of surfactant. Ding and co-workers (2004) observed an about two fold increment in xylanase activity produced by *S. olivaceoviridis* E-86 with increasing the concentration of Tween 80 from 0-1.5% [28].

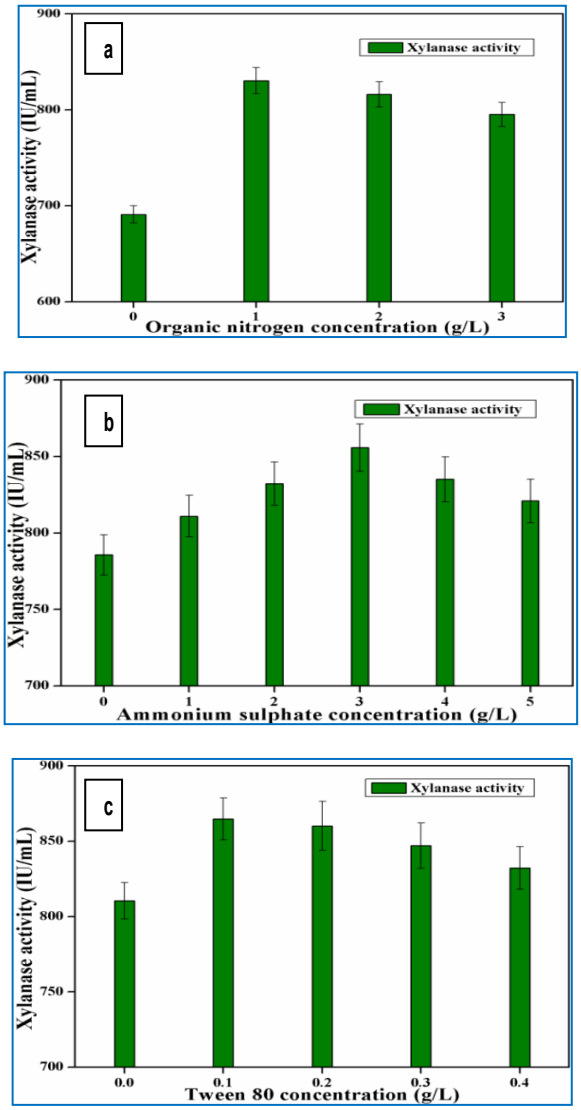


Fig-2 Effect of different concentrations of media components on xylanase production

Conclusions

Based on the above results, the NSS medium may be modified for maximum xylanase production as under:

KH_2PO_4, 1.5 g/L; $MgSO_4$. $7H_2O$, 0.5 g/L; $(NH_4)_2SO_4$, 3.0 g/L; KCl, 0.5 g/L; Tween-80, 0.1 g/L; and yeast extract, 1.0 g/L, in distilled water with trace elements solution (0.04 mL/L) comprising $ZnSO_4$. $7H_2O$ (180 µg/L), $FeSO_4$. $7H_2O$ (200 µg/L), and $MnSO_4$. $7H_2O$ (20 µg/L).The fungal strain RM-1 produces quite high xylanase activity (864.8 IU/mL), representing 18.0% improvement as compared to that obtained under unmodified NSS medium (708.9 IU/mL). Xylanase enzyme has different biotechnological applications which increased the demand of these enzymes manifold.*C. cinerea* RM-1 produced quite high activity of these enzymes and therefore, may be of great interest for different industries.

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Conflict of Interest: None declared

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