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ORIGINAL ARTICLE

Optimization of *Trichoderma reesei* Medium for Increasing Xylanase Enzyme Production

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ABSTRACT
Trichoderma is one of ascomycete mesophilic fungi, which exist in all soils and are part of the majority of soil's cultivable fungi. Xylanases are part of hydrolases and possess single chains of glycoproteins whose weighs range from 6 to 8 KD. Xylanase has multiple applications in various industries. In this study, we try to optimize Trichoderma reesei medium in order to increase Xylanase enzyme Production. We provided the standard Trichoderma reesei strain from the microbe collection of Iranian Research Organization for Science and Technology (IROST). Then, we measured the activity of its Xylanase enzyme using the standard method of DNSA. Next, for the sake of optimization, different parameters of the medium like carbon, nitrogen, and pH sources, temperature and incubation time span were examined. The activity of the enzyme was measured at each stage using DNSA method. Results: The parental strain of Trichoderma reesei is featured by the xylanase enzyme activity of 2/59 U. the strain at the present of wheat bran, ammonium nitrate, yeast extract respectively as the carbon, inorganic nitrogen, organic nitrogen, and at 7/5 pH and with the average of 35 °C had the most amount of production. Xylanase enzyme is one of the most important enzymes used in diverse industries but unfortunately is not produced in our country. As it can be seen from the results of this study, we can optimize the conditions for cultivating different organisms so as to increase the industrial production of various enzymes which are not produced in Iran. We hope, thereby, to make such a production easier at the industrial scale.
Keywords; *Trichoderma reesei, Xylanase, Medium optimization*

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INTRODUCTION

Xylane is the main hemicelluloses of plants' cell wall and heteropolysaccharide is composed of xylose units that are joint via xyloside bonds and has the alternative arabinosyl, glucuronosyl, and acetyl groups [10]. Due to the intricate structure of xylane, different enzymes are required for its complete Hydrolysis [3]. Among xylanolytic enzymes, endoxylanase and β- xylosidase enzymes are the key enzymes for breaking xylane construction into smaller xylooligomers and xylose units [6]. Xylanases have many applications in improving the nutritional values of animal feed, pulp bleaching, Clarification of juice, improving the quality of bread paste, extracting vegetable oils, coffee, starch, and hydrolysis of lignocellulosic materials into fermentable sugars for oil production and other chemical materials [9].
At the industrial level, these enzymes are produced in order to be used in paper, food and animal feed industries. In paper industries, xylanases increase pulp bleaching and thus a decrease in using chlorinated compounds contaminating the medium takes place. In food industries, these enzymes are applied to accelerate cooking sweet, cakes and other foods by breaking down polysaccharides in flour. In animal feed, xylanases help poultry and pigs digest wheat by reducing its viscosity [11].
Various microorganisms like bacteria, yeasts, and Filamentous fungi are used to produce xylanase. Among these, fungi produce the highest amount of xylanase. At the industrial level, aspergillus and trichoderma are used for xylanase production [9].
Generally, xylanases are inducible enzymes, which leak in places with pure xylane or its compounds. Considering the fact that xylane cannot enter into the cell, it is assumed that xylanase production is induced by xylane chain parts produced by a slight amount of xylanase enzyme which leaks without

needing to be induced. There are some reports of induction, in the cases of some microbes, by other factors like sorbose, various kinds of xylo-oligosaccharide, xylose, and lignocelluloses end groups. On the other hand, it is glucose, which induces xylanase production to stop. Some has reported some cases of inducing the production of this enzyme by those sources containing lignocellulose such as wheat bran, rice straw, and bagasse. In some studies, easily digestible sugars like glucose and cellulose are said to be xylose synthesis inhibitors. In bacteria, the presence of amino acids has enhanced xylanase production [3] Since wheat straw contains hemicelluloses, it has been reported to induce xylanase production [12].
The cost of enzyme production is a significant factor in deciding about the cost of the process. The main purpose of studies on the industrial uses of xylanase is to reduce production costs by optimizing the medium and the process. Optimization using old methods which consider one factor at a time is time-consuming and troublesome. Optimization by statistical methods like designing factorial experiments and response surface methodology (RSM) has been effectively employed for producing enzymes. The results have revealed that statistical methods are beneficial and strong tools in optimizing xylanase production [9].The goal of this study is to optimize *Trichoderma reesei* medium in order to increase the production of xylanase enzyme.

MATERIAL AND METHODS

PARENTAL STRAIN

In this study, we have used the fungus *Trichoderma reesei* PTCC 5142, taken from the collection of Iranian Research Organization for Science and Technology (IROST), which produces xylanase enzyme.

DETERMINING THE ACTIVITY OF XYLANASE ENZYME

PREPARATION OF MATERIALS (SUPPLY)

1. Citrate phosphate buffer: using one liter of distilled water, we solve 8.7 g potassium phosphate and 2.5 g citric acid and bring the pH to 6.5.
2. Substrate solution: in order to provide 2% xylane solution, we use 2 g xylane and 100 ml of citrate phosphate buffer. In order to be used, this solution should reach a one-tenth dilution (90 ml of citrate phosphate buffer and 10 ml of 2% xylane solution).
3. Preparing primary DNSA solution: 5 g DNSA, 1 g fennel and 5 g sodium hydroxide are solved in 500 ml of distilled water.
4. 5% phosphate sodium solution: 1 g Na_2So_4 is solved in 20 ml of distilled water.
5. 5% glucose solution: 1 g glucose is solved in 20 ml of distilled water.
6. Preparing secondary dinitrosalicylic acid solution: 0.5 ml of phosphate sodium solution and 20 ml of glucose solution with 50 ml of primary DNSA solution are mixed together.
7. Preparing standard glucose: the new solution includes 1 mg in ml of glucose is obtained using distilled water.

ENZYME ASSAY PROCEDURE

1. In order to produce the enzyme by the strain, we have to provide a medium with the following features (in 1 liter of distilled water)(8)
   - ✓ 3 g $NaNO_3$, 0.5 g KC1, 1 g KH_2PO_4, 0.5 g $MnSO_4.7H_2O_4$, 0.01 g $FeSO_4.7H_2O$, and 10 g carbon source (xylane).

The pH of the medium has to be set at about 6.5 and then 100 ml is poured into a 259 ml flask and then sterile it. Then, inoculation this medium with 10 colonies with a diameter of 5 mm from our strain growth on PDA. Afterward, we incubate it for 48 hours at the temperature of 35 °C. Then, the medium is centrifuging at 6000 g× for 15 minutes and the supernatant will be contained extracellular enzyme.

2. 540 ml of substrate solution is poured into a 2 ml microtube and we add 90 ml of the surface liquid containing centrifuged enzymes.
3. 370 ml of citrate phosphate buffer is added to the previous solution so the final volume reaches 1000 ml.
4. Microtubes are place in bain-marie (water bath) at 37 ° C and for 30 minutes.
5. After 30 minutes, the microtubes are removed from the bain-marie and then 1000 miroliter of secondary DNSA is added so the final volume reaches 2000 ml.
6. To get a color, microcubes are placed in a boiling bath of 100 ° C for 15 minutes.
7. After 15 minutes, microcubes are removed from the boiling water and are quickly made cold at -4 ° C.
8. Spectrophotometer measures any sample absorption at 570 nm.

Every enzyme unit is the amount of the enzyme required for the production of one micromole xylane in one minute per milliliter.

STANDARD CURVE

1. Various amounts of glucose are poured into the microtube and then using citrate phosphate buffer we bring it to a volume of 1000 ml; and finally by adding DNSA the ultimate volume reaches 2000 ml.
2. Any absorption of the standards by spectrophotometer at 570 nm is measured and recorded.
3. After measuring glucose absorption standards according to concentration values, we obtain a linear diagram.

OPTIMIZATION OF TRICHODERMA REESEI MEDIUM FOR INCREASING THE PRODUCTION OF XYLANASE ENZYME

In order to determine most appropriate medium and obtain the best amount of xylanase production we carried out some experiments during which the suitable production temperature, pH, the most fitting carbon source, organic and inorganic nitrogen sources were examined and evaluated (5, 7).

THE IMPACT OF CARBON SOURCES ON THE PRODUCTION OF XYLANASE

In order to evaluate the results in the production of xylanase, we use different carbon sources, like sugarcane pulp, wheat bran and rice bran, each of which is individually added to the medium. After 48, 72, 96, 120, and 144 hours of shaker incubation, enzyme production is measured at 120 rpm.

The way to do it is that in the first of the above stages, we have to prepare a certain number of erlans that match our carbon sources needed to be examined; this is done for the purpose of preparing the production medium. As described in the first stage of methodology, the production medium of the enzyme contains these materials (g per liter):

3 g $NaNO_{3,\ 0.5\ g\ KC1,\ 1\ g}$ KH_2PO_4, 0.5 g $MnSO_4.7H_2O_{4,\ 0.01\ g}$ $FeSO_4.7H_2O$, and 10 g carbon source (xylane).

Having prepared this medium, we pour 50 cc of it in each flask; then, we add 1% (0.5 cc) of each carbon source to each flask (instead of the main carbon source, i.e. xylane, which is not added in this stage); next, we put the flask in the autoclave. Then, inoculation this medium with 10 colonies with a diameter of 5 mm from our strain growth on PDA. Subsequently at various times, we incubate it at 35 ° C. Then, the medium centrifuging at 6000 ×g for 15 minutes and the supernatant will be the source of extracellular enzyme. The next steps are done as before.

THE IMPACT OF ORGANIC NITROGEN SOURCES ON XYLANASE PRODUCTION

The result of production with organic nitrogen sources is studied using various sources of nitrogen such as soybean meal, beef extract, and yeast extract. The related nitrogen sources are added to the medium as the only nitrogen source; and after 48, 72, 96, 120, and 144 hours of incubation, enzyme production is measured at 35 ° C. This experiment is conducted the same as that of the effect of carbon sources with the difference that the medium we prepare for enzyme production contains various nitrogen sources.

THE IMPACT OF INORGANIC NITROGEN SOURCES ON XYLANASE PRODUCTION

Inorganic nitrogen sources include ammonium nitrate, sodium nitrate, and potassium nitrate. The related nitrogen sources are added to the medium as the only nitrogen source. After 48, 72, 96, 120, and 144 hours of incubation, enzyme production is measured at 35 ° C.

THE IMPACT OF INCUBATION TIME ON XYLANASE PRODUCTION

The effect of the time of incubation in the production of xylanase is calculated at 24, 48, 72, 96, 120, and 144 hours.

THE IMPACT OF TEMPERATURE ON XYLANASE PRODUCTION

We examine the effect of temperature on xylanase production by changing the temperature. Enzyme production in carbon and nitrogen sources is optimized and, after being incubated and shaken for an optimal amount of time, is measured at120 rpm.

THE IMPACT OF PH ON XYLANASE PRODUCTION

In order to examine the effect of pH on xylanase production, we cultivate a bacterial strain at different pH (carbon and nitrogen sources, temperature and time of incubation, in this study, are optimized at 120 rpm); then, xylanase production is calculated.

RESULTS

Glucose standard curve is drawn using Excel.

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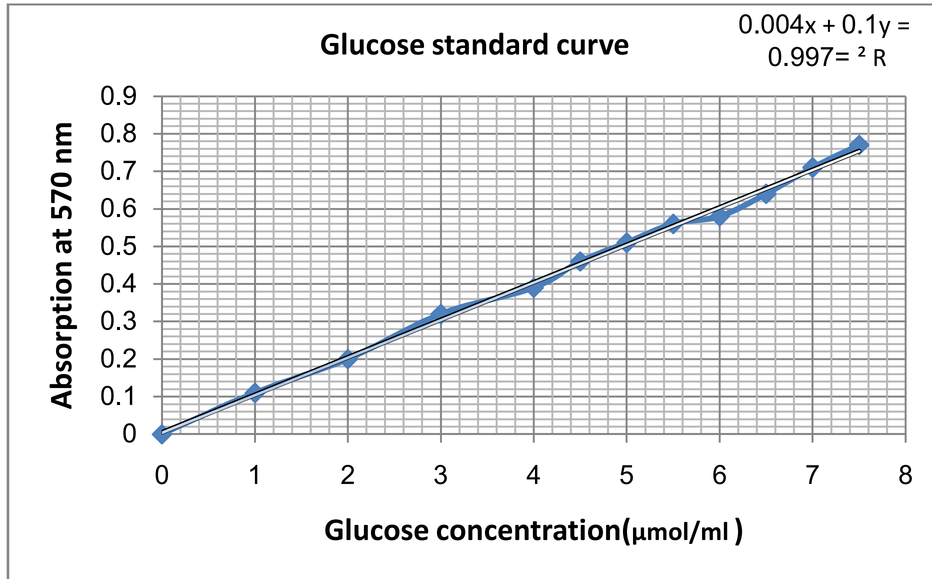

Figure 1. Glucose standard curve

The activity of produced xylanase enzyme was calculated by the parental strain using 2.59 U formula. Optimization results indicated that the highest rate of the production of xylanase enzyme, 6.4 U, was in the presence of wheat bran, as the only carbon source, at 35°C and with 96 hours of incubation. In the results of carbon source optimization it was observed that rice bran, as a carbon source, at 35°C and with 96 hours of incubation next to the existence of lactose possesses the highest rate of the production of xylanase enzyme, 5.2 U (figure 2).

Moreover, the highest rate of the production of xylanase enzyme, 5.39 U, was in the existence of yeast extract as the only source of nitrogen (organic) at 35°C and with 96 hours of incubation. The other source, which caused a considerable increase in the production of xylanase enzyme, was ammonium nitrate (inorganic nitrogen) which, at 35°C and with 96 and 120 hours of incubation, increased production up to 3.9 U.

The results showed that at 35°C and with pH being, 7.5 we had the highest rate of enzyme production in the optimized medium. That rate was respectively 6.9 and 7.1 U. In addition, there was a high amount of enzyme production at 45°C and with pH being 6 and its rate was respectively 6.8 and 7 U. The lowest rate of production was at 65°C and with pH being 3.

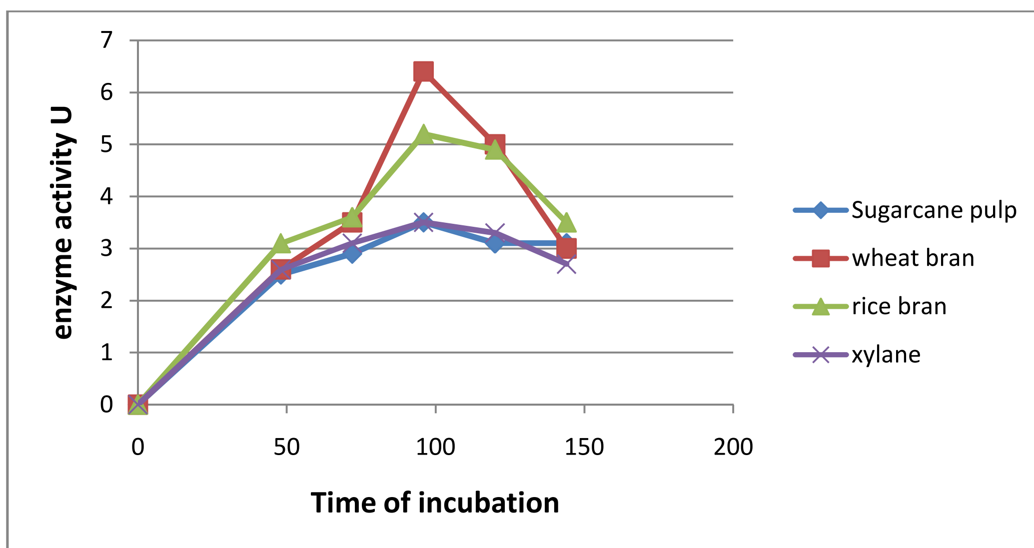

Figure2. Rates of xylanase enzyme production in the presence of various carbon sources and various incubation time at 35°C

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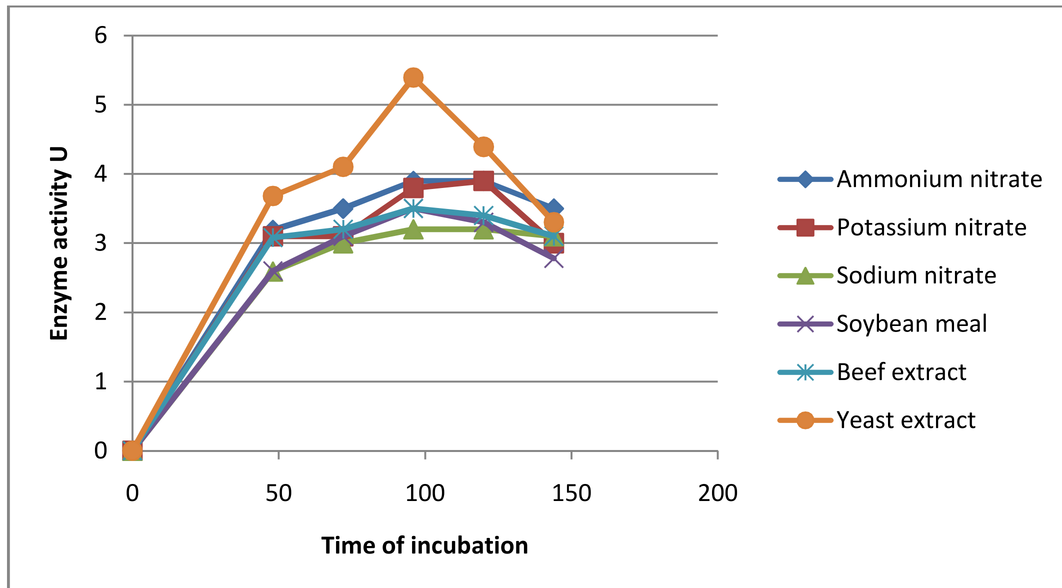

Figure 3: rates of xylanase enzyme activity in the presence of various nitrogen sources and different time of incubation at 35°C

DISCUSSION AND CONCLUSION

Medium optimization started by changing the carbon sources of the medium. Carbon source is the most essential source of energy for microorganisms and organisms have to be able to use carbon sources available in the medium. Sometime, a micro-organism uses simple compounds as a carbon source which do not need to be decomposed; but at other occasions a micro-organism is placed in a medium where simple carbon source is not at hand but there are polymeric carbon sources; in this latter case, only an organism grows competitively which can generate necessary enzymes for decomposing polymers. Xylane is a complex compound, which has to be decomposed by microorganism if it is going to be used. This task is done by xylanase enzyme.

Hence, it is logical that when carbon sources change from simple to complex, the production of decomposing extracellular enzymes increases and when we put a compound in a medium where a change happens in the amount of carbon, the rate of enzyme production changes too.

Even, when one uses sources which are related to other compounds, the rate of enzyme production and its activity differs. For instance, since it is believed that hemicellulose and lignin are closely related to each other, the higher rate of xylanase enzyme activity could have a significant advantage for decomposing lignified cell wall. The amount of lignin in the compound that is used can influence the activity of the enzyme. Lignin is a negative factor and an inhibitor for digesting and decomposing hemicellulose and thus it decreases enzyme activity.

In this study, we have used four different sources where the highest rate of production belonged to wheat bran; something that is confirmed in other studies like [1] where it was observed that the higher concentration of wheat bran causes an increase in xylanase production in aspergillusniger(1).Furthermore, in another study by Karthikeyan *et. al*. [5], on the optimization of production for three enzymes (xylanase, chitinase and asparaginase), using various carbon sources through *Trichoderma viride* they found out that *Trichoderma viride* on wheat bran has the highest rate of xylanase production [5] our results confirm those of Karthikeyan.

In the next stage, we optimized nitrogen source. The essence and concentration of nitrogen source are significant in the formation of various enzymes. Low rates of nitrogen are insufficient for enzyme production and excessive nitrogen is similarly harmful and an inhibitor to enzyme. In our results it was observed that the optimized nitrogen source was yeast abstract, an organic source of nitrogen. In 2008, Goyal *et. al*. Proposed sodium nitrate as the optimized nitrogen source for a higher rate of xylanase productio n via *Trichoderma viride* [4]; the difference was that they had not studied yeast extract and if they had done so, they would have reached to the conclusion that it yeast extract was the best nitrogen source. At any rate, there are numerous reasons, including species and strain differences, which can change the results.

We also examined the conditions of optimized medium such as pH, incubation temperature, and incubation's length of time. Among these medium conditions, pH has a crucial role in causing

morphological changes in organisms, their growth and enzyme production. A change in pH during the time when the organism grows is important in the stability of the produced enzyme. In those organisms, which grow in low or high pHs, extracellular enzymes which are secreted out of them can act in high or low pHs. Our findings indicate that 7.5 pH has the highest rate of activity and enzyme production; also, it can be seen that in those pHs with higher rates of acidity, the amount of enzyme is less than that of alkaline pHs, which is why the strain we use can grow more in alkaline pH in comparison to acidic pH. Goyal *et. al.* concluded that *Trichoderma viride* at 3.5-4 pH has the highest rate of xylanase production [4].
Temperature and the length of incubation have direct effects on the growth of microorganism and we know that if the growth increases, secondary metabolites and enzymes and secreted proteins are produced more as well. Therefore, in this study we obtained the optimal temperature and length of incubation; temperature: 35 ° C and length 95 hours. In 2008, Goyal *et. al*. Observed that *Trichoderma viride* has the highest rate of xylanase production at 25°C [4].In most studies, the optimal temperature of xylanase production for fungi is between 25-35°C.
There are many enzymes, which have several important applications in industries that are not produced in Iran. Xylanase is one of these enzymes. We concluded that by optimizing the medium of some of strains producing xylanase we may achieve to a higher production of this enzyme.

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