Determination of In-vitro Optimum Conditions of Xylanase Enzyme Activity Produced by *Orpinomyces* sp. and *Neocallimastix* sp.

Abstract

This study was aimed to determine the in-vitro optimum conditions of xylanase enzyme activity produced by *Orpinomyces* sp. and *Neocallimastix* sp. In the study, xylanase enzyme activity was investigated in terms of extracellular and intracellular total activity (TA) and specific activity (SA) levels at different time (day), pH and temperature levels.

Orpinomyces sp. and Neocallimastix sp. when different days (time) were considered in terms of xylanase enzyme activity of fungi species, it was determined that there was a statistically significant positive correlation between TA=0.732 and SA=0.546 ($p<0.01$). It was determined that there was a statistically positive and significant relationship between TA=0.622 and SA= 0.520 at different pH levels. This situation differs in terms of temperature levels. It was determined that there was a statistically negative significant relationship between genders and SA=-0.354 ($p<0.05$). In this study, it is thought to contribute by determining the optimum conditions in in-vitro and industrial uses.

Keywords: *Orpinomyces* sp., *Neocallimastix* sp. pH, In-vitro optimum conditions and fungal growth

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Introduction

Lignocellulotic enzymes, which are members of the carbohydrate active enzyme (CAZy) family, are organic compounds synthesized by some microorganisms (Leggieri ~~et al~~*et al*., 2021). Although CAZy enzymes are the most widely used enzyme group in different biotechnological fields, especially in biofuel production, it has been observed that the cost of optimization processes can be high in some cases (Ranganathan ~~et al~~*et al*., 2017; Hanafy ~~et al~~*et al*., 2018; Dehhaghi ~~et al~~*et al*., 2020).

In order to produce these enzymes by other microorganisms at a cheaper cost, it has led to the research of different living groups and the examination of the enzyme activities of these organisms, and the presence of AGFs among these organisms has increased the research on these microorganisms (Haitijema ~~et al~~*et al*., 2014; Hooker ~~et al~~*et al*., 2019; Singh ~~et al~~*et al*., 2019; Flad ~~et al~~*et al*., 2020).

Anaerobic Gut Fungi (AGF) are eukaryotic microorganisms that spread symbiotically in the rumen and have lignocellulotic enzymes that provide hydrolysis of the plant cell Wall (Henske et al*et al*., 2018; Swift et al*et al*., 2019; Flad et al*et al*., 2020; Wilken et al*et al*., 2021). These microorganisms and other fungi have maximal CAZy activity (Barret et al*et al*., 2020).

Xylanase, a member of the CAZy family, is an important enzyme synthesized by these microorganisms, and it is a new enzyme that is isolated from some AGFs and purified for use in biotechnological fields as a result of cloning, and potentially used in different fields such as bread making. (Malik et al*et al*., 2018; Wen et al*et al*., 2021). At the same time, the use of xylanases, which are thermostable, in paper, feed, food and various bio-converting systems increases the demand for purification from different microorganisms due to the increasing interest in these enzymes (Bajaj et al*et al*., 2019; Chadha et al*et al*., 2019).

As a result of the comparison of xylanase enzyme activity, which is a member of the CAZy system of AGFs, *Orpinomyces* sp. and *Neocallimastix* sp. It has been observed that this enzyme activity is high in species (Dagar et al*et al*., 2018). Therefore, the potential use of xylanase enzyme in new technological areas and the discovery of this enzyme from AGFs *Orpinomyces* sp. and *Neocallimastix* sp., it was aimed to determine the optimum conditions for xylanase activity in vitro.

Materials and Methods

Anaerobic Gut Fungi (AGF) (*Orpinomyces* sp. and *Neocallimastix* sp.) used in the present study were obtained from Kahramanmaraş Sütçü İmam University Biotechnology Laboratory stock culture collection. Orpin (1976) anaerobic nutrient medium was used for the development of AGFs in vitro.

Determination of xylanase enzyme activity

Extracellular: For the enzyme study, the samples were centrifuged at 1200 g for 15 minutes and the upper liquid was separated for extracellular (supernatant) enzyme activity and transferred to sterile eppendorf tubes.

Intracellular: In order to determine the intracellular enzyme activity, the sample was centrifuged at 1200 g for 15 minutes and the pellet remaining at the bottom was taken into a separate epondorphan for intracellular enzyme activity, washed twice with distilled water, and then used with liquid nitrogen to break the cell wall. Afterwards, the obtained samples were

taken into 25mM potassium phosphate buffer solution and centrifuged at 10000 g for 3 minutes and the supernatant was taken. (Flint ~~et al~~*et al*.,1991). Enzyme activity of the obtained samples was done according to Miller (1959) method. The slope standard curve from xylose was used to calculate xylanase activity. One unit of xylanase activity (IU) was defined as the amount of enzyme capable of producing 1 µmole of xylose in 1 minute under reaction conditions at 40 ℃.

Determination of in-vitro optimum parameters

Orpinomyces sp. and *Neocallimastix* sp. the effects of different time intervals, different temperatures and pH on the raw enzyme were examined. 1-7 days as different time intervals, temperature values between 30-80℃ with 5℃ increments; For pH, it was adjusted in 0.5 unit increments in the range of 3-9. The activity of xylanase at different time, temperature and pH changes was determined.

Statistical Calculation

The data obtained in the study were analyzed statistically with the help of SPSS-23 program. Descriptive statistical analysis of the data and Pearson correlation statistical test were applied. $p<0.05$ and $p<0.01$ were considered statistically significant.

Results and Discussion

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In this study, *Orpinomyces* sp. and *Neocallimastix* sp. descriptive statistical data according to their extracellular and intracellular status at different time, pH and temperature ranges, and the descriptive statistics data were associated with the person correlation coefficients and the enzyme levels were determined at optimum time, pH and temperature.

Table 1. *Orpinomyces* sp. and *Neocallimastix* sp. descriptive statistics according to the environmental conditions in different variables.

Variable	Medium	N	Minimum	Maximum	Mean	Std. Deviation	Variance
Day	**TA**	28	1,20	58,70	25,47	16,37	267,97
	SA	28	22,10	505,40	203,90	153,62	23597,84
pH	**TA**	52	0,00	49,10	16,50	13,50	182,29
	SA	52	0,00	398,70	77,82	86,91	7552,92
Temperature	**TA**	44	2,50	57,60	28,13	15,25	232,54
	SA	44	44,10	501,20	203,10	130,16	16941,09

TA: Total Activity (µmol/min/ml), **SA:** Specific Activity (µmol/min/mg

Orpinomyces sp. and *Neocallimastix* sp., depending on time, the lowest value TA=1.20 µmol/min/ml, the highest value TA=58.70 µmol/min/ml and the lowest value SA=22.10 µmol/min/ mg, the highest value was found to be SA=505.40 µmol/min/mg. When examined

in terms of pH levels, the lowest value TA=0 µmol/min/ml, the highest value TA=49.10 µmol/min/ml and the lowest value SA=0 µmol/min/mg, the highest value SA=398.70 µmol It was found as /min/mg. In the temperature ranges, the lowest value TA=2.5 µmol/min/ml, the highest value TA=57.60 µmol/min/ml and the lowest value SA=44.10 µmol/min/mg, the highest value SA=501, It was found to be 2 µmol/min/mg. According to the analysis of variance findings, TA=267.97 and SA=23597.84 at different times, pH levels TA= 182.29 and SA= 7552.92, and at temperature levels TA=232.54 and SA= 16941.09 (Table 1).

Time-dependent findings of xylanase enzyme activity

Orpinomyces sp. and *Neocallimastix* sp. on different days, the changes in xylanase enzyme activity extracellular and intracellular in terms of TA and SA values were investigated. In the study, time dependent xylanase enzyme activity of Orpinomyces sp. In the study, extracellular TA=47.5 µmol/min/ml intracellular TA=36.9 µmol/min/ml on the 5th day, while extracellular SA=201.4 SA= It was found to be 204.7 µmol/min/mg. Neocallimastix sp. For the 5th day, extracellular TA=58.7 µmol/min/ml intracellular TA=42.7 µmol/min/ml, while extracellular SA=505.4 SA= 502.4 µmol/min/mg (Table 2).

Table 2. *Orpinomyces* sp. and *Neocallimastix* sp. time dependent xylanase enzyme activity

Day	***Orpinomyces* sp.**				***Neocallimastix* sp.**			
	Extracellular		**Intracellular**		**Extracellular**		**Intracellular**	
	TA	**SA**	**TA**	**SA**	**TA**	**SA**	**TA**	**SA**
1	4,12	38,7	1,2	22,1	7,65	50,1	2,1	25,1
2	9,5	76,4	6,8	44,6	15,8	101,5	8,6	129,4
3	21,5	106,5	8,4	88,6	25,4	208,5	14,7	279,5
4	33,2	184,6	19,4	125,6	42,8	327,6	29,6	426,8
5	47,5	201,4	36,9	204,7	58,7	505,4	42,7	502,4
6	44,5	104,2	30,1	197,2	50,2	402,7	35,1	479,6
7	22,6	99,4	22,6	100,1	45,1	328,1	26,5	348,3

Highest Lowest **TA:** Total Activity (µmol/min/ml), **SA:** Specific Activity (µmol/min/mg)

Enzyme production activity of *Neocallimastix* sp was investigated in different media. In the study, it has been reported that there are differences in enzyme activity according to the medium, and it changes depending on the time (Karaman ~~et al~~*et al*., 2022). The effect on xylanase production by *Aspergillus niger* at different temperatures was investigated. In the study, it was stated that 92 hours (approximately 4 days) were required to achieve the optimum temperature of 28°C and maximum xylanase activity (Yuan ~~et al~~*et al*. 2005). In our study, the optimum xylanase enzyme activity was reached on the 5th day. This explains the genus-specific differences for xylanase activity. The optimum enzyme activity level was determined on the 5th day in our study. The xylanase enzyme activity was determined as 290

U/ml under optimum conditions. (Yuan ~~et al~~*et al*. 2005). In our study, the highest value in terms of enzyme activity was *Neocallimastix* sp. for 502.4 µmol/min/mg in the cell. *Orpinomyces* sp. the highest value for intracellular SA=204.7 µmol/min/mg was determined.

Findings of xylanase enzyme activity at different pH levels

Extracellular and intracellular optimum pH level of *Orpinomyces* sp. was determined to be 7. Extracellular TA= 37.6 µmol/min/ml and SA= 94.3 µmol/min/mg at pH=7, intracellular TA=41.2 µmol/min/ml and SA= 124.3 µmol/min/ determined in mg. *Neocallimastix* sp. intracellular xylanase was determined at the pH level of TA=41.4 µmol/min/ml with the highest value at 6.5 pH, and SA=398.7 µmol/min/mg at a pH level of 7 (Table 3).

In this study, *Orpinomyces* sp. and *Neocallimastix* sp. were found at pH values between 6.5 and 7.5. It was determined that the enzyme activity was at the lowest values below the average pH value of 5. The pH range was determined as 6.50 in the study in which the biogas efficiency was examined using *Saccharomyces cerevisiae*. (Vallejo-Hernández ~~et al~~*et al*. 2018). It is thought that the use of xylanase enzyme, which will be obtained according to the environment, will be beneficial and guide in researches where the enzyme activity changes according to the cell environment.

Table 3. *Orpinomyces* sp. and *Neocallimastix* sp. xylanase enzyme activity at different pH levels

pH	*Orpinomyces* sp.				*Neocallimastix* sp.			
	Extracellular		Intracellular		Extracellular		Intracellular	
	TA	SA	TA	SA	TA	SA	TA	SA
3	0	0	0	0	0,12	0,1	0,2	0
3,5	0,19	0,09	0,1	0,3	0,19	0,1	0,2	0,4
4	0,2	0,11	1,6	7,5	3,1	0,9	2,4	14,1
4,5	2,4	6,8	4,5	19,4	6,5	20,4	6,5	37,5
5	9,6	11,8	7,9	26,4	7,3	34,8	9,7	69,4
5,5	14,6	22,9	18,4	45,6	15,2	50,4	22,4	97,6
6	17,9	33,7	22,7	67,7	19,9	97,1	26,7	142,1
6,5	25,4	62,1	34,1	86,3	37,8	127,8	41,4	297,5
7	37,6	94,3	41,2	124,3	49,1	325,9	34,2	398,7
7,5	22,4	88,6	30,5	104,5	38,7	223,4	31,4	224,6
8	19,4	72,4	21,1	93,4	28,6	120,4	26,5	201,4
8,5	15,2	69,4	12,2	68,3	23,7	102,5	19,1	96,5
9	12,1	57,7	9,1	42,1	14,4	87,4	12,3	70,1

Highest | Lowest | **TA:** Total Activity (µmol/min/ml), **SA:** Specific Activity (µmol/min/mg)

Findings of xylanase enzyme activity at different temperatures

Orpinomyces sp. and *Neocallimastix* sp. at different temperatures, it was determined that the xylanase enzyme activity had different intracellular and extracellular temperature values. The highest extracellular and intracellular total (TA) activity value of *Orpinomyces* sp. was 55 µmol/min/ml at 50 ^{o}C, and the specific (SA) activity value was 309.1 µmol/min/mg at 50 ^{o}C. However, the highest values of TA=44.2 µmol/min/ml and SA=304.3 µmol/min/mg were obtained for intracellular xylanase enzyme at 55 ^{o}C. It was observed that the xylanase enzyme activity degraded with the increase in temperature and therefore decreased significantly (Table 4).

Neocallimastix sp. the highest extracellular total (TA) activity value was 57.6 µmol/min/ml at 50 ^{o}C and specific (SA) activity value was 501.2 µmol/min/mg at 50 ^{o}C. In intracellular, the highest total (TA) activity value was 43.1 µmol/min/ml at 50 ^{o}C and specific (SA) activity value was 501.2 µmol/min/mg at 50 ^{o}C (Table 4). The interactive effects of temperature (20-60 ^{o}C) and pH (2-8) were investigated for xylanase production from *Aspergillus niger* DFR-5. In the study, it was reported that the maximum activity of the enzyme was 4354 IU/ml at 40 ^{o}C. (Pal and Khanum, 2011). Xylanase enzyme optimization was achieved at different pH and temperatures with the mutated strain *Neocallimastix patriciarum*. In the study, it was reported that it provides the highest activity at pH=6 and temperature at 62 ^{o}C. (Huang ~~et al~~*et al*., 2021).

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Table 4. *Orpinomyces* sp. and *Neocallimastix* sp. xylanase enzyme activity at different temperatures

Temperature (^{o}C)	*Orpinomyces* sp.				*Neocallimastix* sp.			
	Extracellular		Intracellular		Extracellular		Intracellular	
	TA	SA	TA	SA	TA	SA	TA	SA
30	22,1	104,2	26,2	77,5	30,1	102,1	34,2	97,6
35	29,4	168,2	28,9	109,2	35,7	201,4	38,4	124,1
40	38,4	197,5	37,5	154,2	47,9	397,1	39,8	226,7
45	49,8	296,2	41,2	197,6	55,7	498,2	40,2	396,4
50	55	309,1	42,1	247,6	57,6	501,2	43,1	501,2
55	30,1	204,2	44,2	304,3	37,1	402,4	36,4	406,5
60	19,5	178,2	30,1	205,3	21,2	301,1	34,1	305,4
65	10,2	100,4	19,9	185,3	11,5	207,5	28,7	227,1
70	8,2	95,2	17,6	93,2	10,2	108,8	20,4	108,7
75	4,2	79,9	10,3	70,5	6,2	97,6	15,1	93,1
80	2,5	45,2	7,6	44,1	5,5	94,5	13,4	70,5

Highest Lowest **TA:** Total Activity (µmol/min/ml), **SA:** Specific Activity (µmol/min/mg)

Orpinomyces sp. and *Neocallimastix* sp., it was determined that there was no significant difference between the breeds and the environment ($p<0.01$). However, it was statistically determined that there was a negative relationship between the breeds in terms of SA values. When different days (time) were considered, it was determined that there was a statistically significant positive correlation between TA=0.732 and SA=0.546 ($p<0.01$). It was determined that there was a statistically positive and significant relationship between TA=0.622 and SA= 0.520 at different pH levels. This situation differs in terms of temperature levels. It was determined that there was a statistically negative significant relationship between genders and SA=-0.354 ($p<0.05$). In addition, a statistically positive and significant relationship was found within the SA values. TA values were not found to be statistically significant (Table 5).

Orpinomyces sp. in study was conducted on thermostability improvement of xylanase obtained from Xylanase from *Orpinomyces* shows optimum activity at neutral or acidic pH. In the study, xylanase activities using different mutant species were maximum at 60 ^{o}C and pH range of 5-7. It was stated that the temperature ranges of xylanase activities of different mutants changed. (Trevizano ~~et al~~*et al*., 2012). The synergistic effect of pH and temperature on xylanase enzyme was investigated by using *Aspergillus niger* species. In the study, the optimum temperature values for the enzyme were found between 35 ^{o}C and 60 ^{o}C, and the optimum pH range was between 4 and 5.5 (Farinas ~~et al~~*et al*. 2010).

Table 5. *Orpinomyces* sp. and *Neocallimastix* sp. according to the environment in different variables, Pearson correlation analysis data

Correlations

	Species	Ortam	Day	pH	Temperature	TA	SA
Species	1						
Medium	0,000	1					
Day	0,000	0,000	1				
pH	0,000	0,000		1			
Temperature	0,000	0,000			1		
TA-Day	-,215	,320	***,732*** **			1	
SA-Day	***-,597*** **	-,057	***,546*** **			***,751*** **	1
TA-pH	-,140	-,021		***,622*** **		1	
SA-pH	***-,365*** **	-,140		***,520*** **		***,825*** **	1
TA-Temperature	-,132	-,092			***-,692*** **	1	
SA-Temperature	***-,354*** *	,078			-,296	***,767*** **	1

***.** Correlation is significant at the 0.05 level (2-tailed).
****.** Correlation is significant at the 0.01 level (2-tailed).
TA: Total Activity (µmol/min/ml), **SA:** Specific Activity (µmol/min/mg)

Conclusion

Orpinomyces sp. and *Neocallimastix* sp., with different applications, optimum endoxylanase and exoxylanase TA and SA values were determined. Since the concepts of pH, temperature and time are important for enzyme activity in industrial and in-vitro laboratory applications, it

has been interpreted statistically by making it with both sexes. Although both sexes showed similar characteristics in the study, a statistically negative significant relationship was found ($p<0.01$). In industrial applications, it is important to apply the data obtained from these parameters in order to obtain the optimum product.

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