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Comparing Cellulotic Enzyme Activities of *Neocallimastix* sp. in Orpin's and Menke's Media

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Abstract: *Neocallimastix* sp. is a member of the Anaerobic Gut Fungi (AGF) family with unique lignocellulotic enzymes, and it is usually grown in two basic media *in vitro*. Orpin's mediais preferred for the detection of enzymatic activites while Menke's is mostly chosen for evaluating the gases produced during the fermantation. Although these two media were shown to be effective for their targeted purposes, no attempt has been made to compare the activities of various cellulotic enzymes in them. In this study, we measured the growth rate of *Neocallimastix* sp. in these two media up to 7 days and A similar progress was observed in both ($p>0.05$). We also discovered that Menke's media was better for the 5th ($p<0.001$) and 7th ($p<0.0.1$) days of Avicellase and for up to 5 days [0 ($p<0.001$), 3 ($p<0.01$), 5 ($p<0.0001$)] of Xylanase activity. Orpin's media, on the other hand, displayed superior CMCase activity in all time points[0 ($p<0.001$), 3 ($p<0.0001$), 5 ($p<0.05$, 7 ($p<0.001$)]. As for Cellulase, the activities were measured virtually the same for the both media on the 0th and the 3rd days whereas they were higher in Menke's ($p<0.0001$) on the 5th, and in Orpin's on the 7th day ($p<0.01$). As a result, in Xylanase enzyme studies, it has been determined that menke media gives better results.

Keywords: Anaerobic fungi, Enzyme activity, Orpin, Menke

Orpin ve Menke Besi Ortamlarında *Neocallimastix* sp'nin Selülotik Enzim Aktivitelerinin Karşılaştırılması

Öz: *Neocallimastix* sp., benzersiz lignoselülotik enzimlere sahip Anaerobik Gut Fungusları (AGF) ailesinin bir üyesidir ve genellikle in vitro olarak iki temel besi ortamında da yetiştirilir. Enzimatik aktivitelerin tespiti için Orpin besi ortamı tercih edilirken, fermantasyon sırasında oluşan gazların değerlendirilmesi için daha çok Menke besi ortamı tercih edilmektedir. Bu iki ortamın hedeflenen amaçlar için etkili olduğu gösterilmiş olmasına rağmen, bu besi ortamlarındaki çeşitli selülotik enzimlerin aktivitelerini karşılaştırmak için hiçbir girişimde bulunulmamıştır. Bu çalışmada *Neocallimastix* sp.'nin bu iki besiyerinde de 7 güne kadar büyüme hızı ölçülmüş ve her ikisinde de benzer bir gelişim gözlenmiştir ($p>0.05$). Ayrıca Menke besi ortamının Aviselaz'ın 5. ($p<0.001$) ve 7. ($p<0.0.1$) günleri ve 5. güne kadar [0 ($p<0.001$), 3 ($p<0.01$), 5 ($p<0.0001$)] Ksilanaz aktivitesi için daha iyi olduğu belirlenmiştir. Orpin besi ortamı ise tüm zaman noktalarında [0 ($p<0.001$), 3 ($p<0.0001$), 5 ($p<0.05$, 7 ($p<0.001$)] üstün KMSaz aktivitesi sergilemiştir. Selülaz için,

aktiviteler her iki ortamda 0. ve 3. günlerde hemen hemen aynı ölçülürken, Menke'de ($p<0.0001$) 5. günde ve Orpin'de 7. günde ($p<0.01$) daha yüksek olduğu görülmüştür. Sonuç olarak Ksilanaz enzim çalışmalarında menke besi ortamınız daha iyi sonuç verdiği tespit edilmiştir.

Anahtar kelimeler: Anaerobik fungus, Enzim aktivitesi, Orpin, Menke

Introduction

Ruminants are mainly fed on herbal materials (Canbolat, 2012), yet they cannot produce cellulolytic or hemi-cellulolytic enzymes to digest them. This task is accomplished by the microorganisms in their gut with which they are in a symbiotic relationship (Vinzelj et al., 2020). Polymeric components in plant cell walls such as hemicellulose, cellulose and lignin make up lignocellulosic biomass (Bobleter, 1994). Anaerobic gut fungi (AGF) are one of the most important microorganisms that form lignocellulosic by-products in the rumen ecosystem (Kamra, 2005; Yazdic et al., 2021). By rapidly colonizing plant cell walls, rumen fungi can break down cell wall carbohydrates (Grenet et al., 1988) thanks to a group of highly active enzymes such as cellulases, xylanases, glycosidases and xylosidases (Comlekcıoglu et al., 2010). As one of the most widely studied AGF's, *Neocallimastix* sp., has a wide range of extremely promising and largely undiscovered enzymes for the complete and efficient break down of lignocellulosic bio mass that can be used for industrial applications (Dagar et al.,2018; Banerjee et al., 2010). Appropriate media is needed to study the growth of microorganisms in vitro. Orpin's medium is generally used in in vitro studies for the determination of the activity of lignocellulosic enzymes (Orpın, 1976). However, Menke's medium, which is thought to represent the rumen environment as Orpin's does (Menke et al.; 1979), has not been utilized for such a purpose. Menke medium is a preffered for the in vitro analysis of gases such as CO_2 and CH_4 formed as a result of fermentation in the rumen (Sanni et al.,2020). Although the two media have been widely used for different purposes, there have been no study to investigate the growth rate of *Neocallimastix* sp. in these media and how the lingocellulosic enzyme producing potential of the organism is affected by them. Here we compared not only the number of viable individuals in Orpin's and Menke's media, but also the activities of Xylanase, Avicelase, Cellulase and Carboxy Methyl Cellulase (CMCase) enzymes per individual on the 0th, 3rd, 5th and 7th days of inoculation. It has been studied to minimize the starter cost of commercially used enzymes such as xylanase.

Material and Metod

Revitalization of anaerobic gut fungi

The Anaerobic Gut Fungi (AGF), namely GMLF 35 (*Neocallimastix* sp.), was obtained from the culture collection of Kahramanmaraş Sütcü Imam University, Faculty of Agriculture, Department of Animal Science, Biotechnology and Gene Engineering Laboratory (BIGEM).It was taken from liquid nitrogen (-196°C), thawed at room temperature and placed into hay-fattening environment under 2 atm carbondioxide (CO_2) pressure (Balch and Wolfe, 1976). Antibiotic mixture (chloromphenicol, amphicillin, streptomycin, erythromycin) was added into the media to prevent bacterial growth. It was then left in incubation (38°C) and its development was observed on the 0th, 24th, 48th and 72nd hours.

Classical fattening medium: This medium was prepared based on Orpin (1976). Glucose was added to the medium as a source of energy, with the final concentration of 0.5 (w/v) %. Then the medium was boiled and saturated with CO_2 until its color is pale yellow. Later, it is transferred into hungate tubes (8 mL) under 2 atm CO_2 pressure and autoclaved at 110°C for 10 minutes. The basal medium content required for the development of AGFs in vitro is given in the table 1. Therewithal, while preparing the anaerobic medium, L-Cystein is added for oxygen induction and Resazurin is added for the oxygen indicator in the medium.

Modified fattening environment: This environment was inspired by the fattening environment prepared by Menke (1979), yet some modifications on the content of the media were performed for the study. After artificial saliva was prepared as Menke described, and rumen fluid and glycose with a final concentration of 0.5 (w/v) % were added to it. The environment was then saturated with CO_2 and distributed in hungate tubes. The tubes were autoclaved at 110 °C for 10 minutes and let it cool before use (Table 2).

Table.1. Medium Content (Orpin,1975)

Content	Liquid Media
Mineral Solution-1 [K_2HPO_4-3g/L]	150ml/L
Mineral Solution-2 [KH_2PO_4-3g/L, $(NH_4)SO_4$-6g/L, $MgSO_4.7H_2O$-0,6g/L, $CaCl_2$-0,4g/L, NaCl-6g/L]	150ml/L
Rumen fluid	150ml/L
$NaHCO_3$	6 g/L
Yeast Extract	2,5g/L
dH_2O	550ml/L

Preparation of substrates

All substrates used in the study [Carboxy methyl cellulose (CMC), Cellulose, Avicel and Xylane] were purchased from Sigma (UK). 500 mg of each was weighed and dissolved in 100 mL (50 mM) of sodium phosphate buffer (pH:6.0).

Preparation of enzyme extracts

0.5 mL of AGFs were added to two different medium prepared and incubated for 0, 24, 48 and 72 hours. At the end of each time point, tubes were centrifuged at 1200 g for 10 minutes and the supernatants were transferred to a new tube to store at -20 °C for later use.

Determination of enzyme activities

Enzyme activities of supernatants in two different fattening environents were determined by the method described by Miller (1959). Briefly, each substrate [Carboxy methyl cellulose (CMC), Cellulose, Avisel and Xylane] was treated with all supernatants. Samples were incubated at 50°C for 45 minutes. By adding DNS to them, enzyme activity was stopped and the mixtures were boiled for 5 minutes. Absorbances were read at 545 nm. The standard graph for calculating enzyme activities was obtained using glucose. One unit of enzyme activity was defined as the mmol amount of reducing sugar released within 1 min. Protein concentrations were determined by Bradford Assay.

Table 2. Medium Content (Menke,1979)

Content	Liquid Media
Solution A	13,2g/L
$CaCl_2.H_2O$	10g/L
$MnCl_2.4H_2O$	1g/L
$CaCl_2.6H_2O$	0,8g/L
$FeCl_3.6H_2O$	Liquid Media
Solution B (Buffer)	35g/L
$NaHCO_3$	Liquid Media
Solution C	5,7g/L
Na_2HPO_4	6,2g/L
KH_2PO_4	0,6g/L
$MgSO_4.7H_2O$	13,2g/L

Determination of population quantity

The most probable number (MPN) is one of the best methods for calculating the population density of Anaerobic Gut Fungi (AGF) in vitro and in this method, which is used to calculate the number of viable individuals in the population of fibrolytic organisms, the thallus forming unit (tfu) is preferred as the unit. (Theodorou et al., 1990). In this study, MPN table (De Man,1975) and 'most probable number calculator.epa.gov' database were used to calculate tfu amount. To determine the number of individuals grown in the fungal population, 1 mL of fungi was taken and inoculated into 9 mL of

fattening medium. This process was repeated several times and dilution was performed (10^{-1},10^{-2},10^{-3},10^{-4},10^{-5}). The tubes were kept in incubation at 38°C for 15 days and the tubes were checked every day. The tubes in which the fungi were develop were marked with '+' while the ones without any growth were marked with '-' .Each condition was prepared in triplicates.

Enzymatic activity per cell

The enzyme activity value per individual was calculated by the division of the total enzyme activity values found through Miller's method (1959) by the number of individuals in the population determined through MPN table (De Man, 1975) and database calculate programs.

Statistical analysis

Enzyme acitivites were calculated and graphs were drawn by using GraphPad Prism (CA, USA). Activities of the same enzyme in two different media were compared via Two-Way ANOVA, and the difference was considered statistically significant if the P value for interaction was equal to or lower than 0.05. For any specific time point, enzyme acitivties were compared by student t-test. Again, P values which were equal to or lower than 0.05 indicated a statistical significance.

Results

According to the counts made with the help of MPN technique and database calculator, it has been revealed that *Neocallimastix* sp. was able to grow in the nutrient medium of Orpin and Menke. However, it was also realized that the growth rate of *Neocallimastix* sp. in the nutrient medium belonging to Orpin appeared to beslightly higher than it was in the culture medium of Menke even though no statistical analysis could be performed because of the difficulty of having biological replicates. Yet, it was still quite observable that number of individuals in both environments increased until the 5th day and decreased on the 7th day.

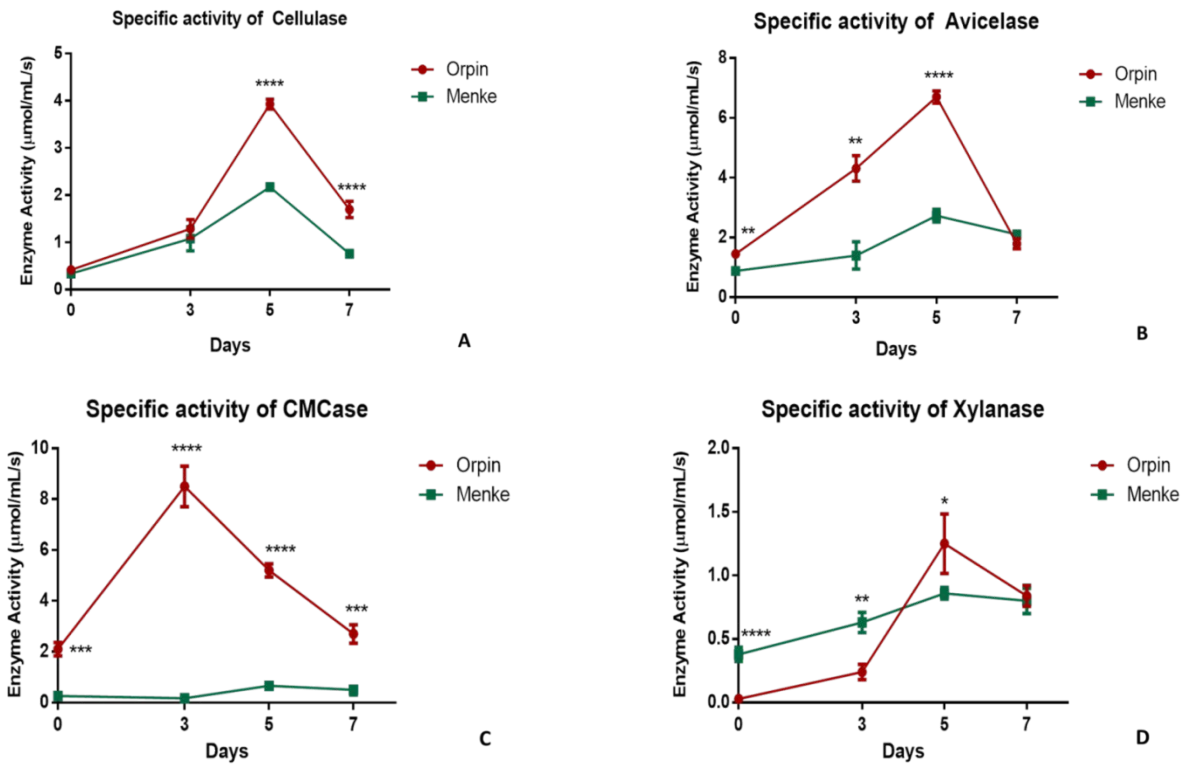

Figure.1. Specific enzyme activity of A) Cellulase B) Aviselase C) CMC D) Xylanase. Specific activites of all enzymes at four different time points. Red line respresents Orpin's media while the green one is for Menke's. Asterisks were placed (*: P <0.05, **: P <0.01, ***: P <0.001, ****: P <0.0001)

To calculate the extra cellular enzyme activity of *Neocallimastix* sp. the microorganism was left to incubate for 0, 3, 5 and 7 days. At the end of each incubation, the supernatant of the culture was separated from the fungal biomass and the Xylanease, Avicelase, CMCase and Cellulase activities were determined. According to the results obtained, the CMCase enzyme activity of *Neocallimastix* sp. grown in Orpin's medium was determined to have the highest activity on the 3rd day where all other enzyme in both media were at their maximum capacity on the 5th day. The results obtained were presented in Figure 1.

As it can be seen in Figure 1, When 2-way ANOVA analysis were performed on specific activities of all

enzymes studied, Orpin and Menke's media were observed to cause a significant change in all. In general, Orpin's media seemed to be superior to Menke's for each condition examined.

Later, specific enzyme activities were normalized to the actual numbers of individual *Neocallimastix* sp.in each case, and distinct patterns were emerged for all enzymes compared to their specific activities. Considering all times for CMCase enzyme activity, Orpin's medium gives information that it will be a suitable medium for this enzyme study (Figure 2A). For cellulase enzyme activity, the activity of the first 3 in Orpin's nutrient medium and Menke's nutrient medium was found to be parallel to each other. However, it was observed that the enzyme activity on the 5th day increased in Menke's broth (Figure 2 B). It was observed that Menke's medium was more suitable for avicelase and xylanase enzyme activity of *Neocallimastix* sp, which was grown on two different media Figure 2C-D). It was observed that xylanase enzyme activity was more effective in Menke's medium than in Orpine's medium in all preferred times throughout the study (Figure 2D).

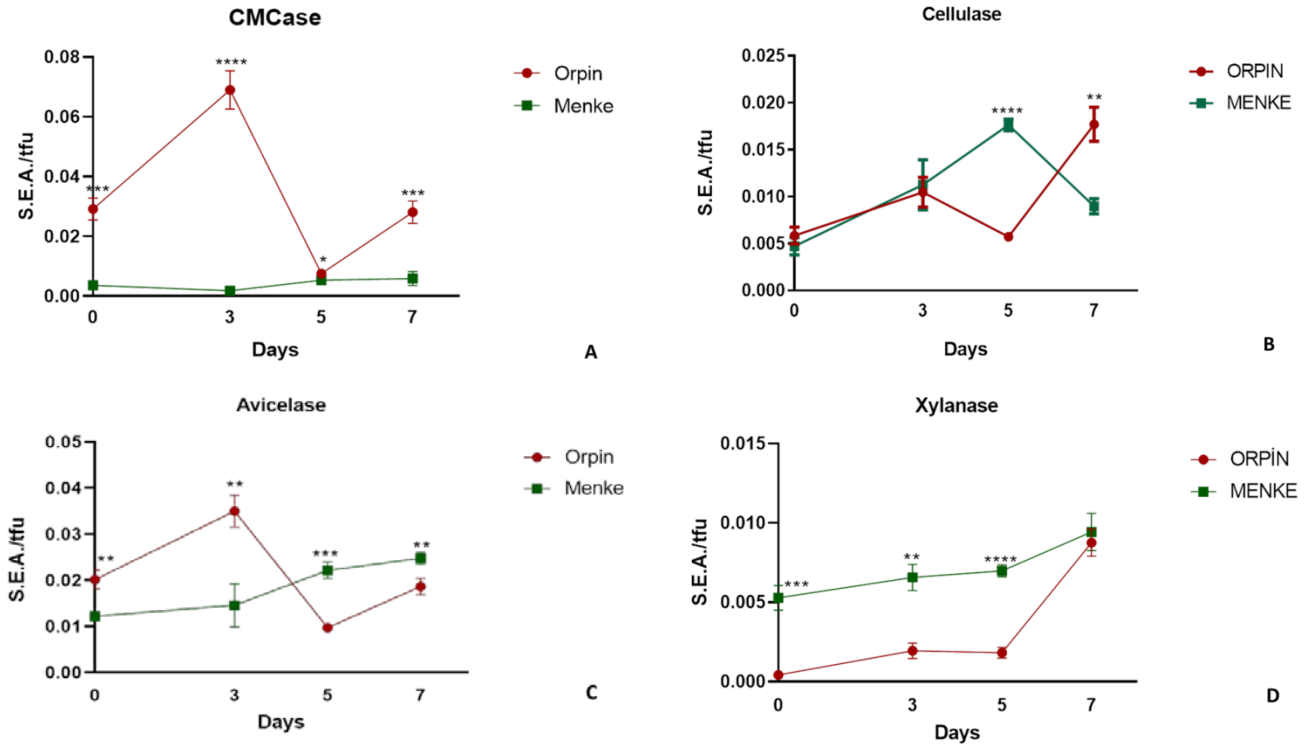

Figure.2. Enzyme activities tfu (thallus forming unit) of AGFs that have completed their incubation in the nutrient medium of Menke and Orpin. A) Avicelase B) CMCase C) Xylanase D) Cellulase. Specific enzymatic activities were divided by the number of the individuals in each condition. Results for each enzyme at each time point were compared through unpaired t test. Asterisks were placed when the differences were statistically significant (*:P <0.05, **: P <0.01, ***: P <0.001, ****: P <0.0001).

Discussions

It has been known that the cellulotic enzyme activity of AGFs is more effective than other bacteria and anaerobic fungi that synthesize these enzymes (Steenbakkers et al., 2003). In this study, we culture done of the most widely studied AGF, *Neocallimastix* sp, in the media suggested by Orpin (1976) and Menke (1979) to measure their growth rates as well as specific and individualistic activites of certain fibrolitic enzymes.

Firstly, we observed that *Neocallimastix* sp. has grown in Menke's almost as sufficiently as it has in Orpin's. As seen in Table 1., the maximum growth of the species was detected on the 5th day in Orpin's medium, which was expected because there a great number of studies reporting a similars observation (Dagar et al., 2018). Menke's, in spite of presenting a similar trend with Orpin's (i.e., showing the highest number on the 5th day), appeared to slightly lower the growth rate of the species. Unfortunately; however, the data has not allowed us to perform a statistical analysis, therefore, our conclusion remained only suggestive.

Although there have been many studies in which Orpin was declared as the choice of medium for *Neocallimastix* sp.culturing (Comlekcıoglu et al., 2017), the number of the studies investigating the growth rate of the species in Menke's has been rather limited. Menke's

medium, on the other hand, has been prefered in calculating the fermantation rate of various anaerobic microorganisms (Totakul et al., 2020; Sarnataro et al., 2020) including *Neocallimastix* sp. (Cao and Yang, 2011).

Secondly, when we measured the specific activities of four fibrolitic enzymes (Avicelase, CMCase, Cellulase, Xylanase) in both medium, we found that the maximum enzymatic activites were determined on the 5th day in all conditions except for CMCase measured in Orpin's, whose highest efficiency was obtained on the 3rd day (Figure 1). Here, the results presented for Orpin were quite in agreement with the previous results in the literature (Comlekcıoglu et al., 2012) even though, for Menke's, no such study has been found to compare our results to.

Later on, however, by considering the findings in Figure 1 along with the fact that the species had its highest growth also on the 5th day, we suggested that the specific activities of these enzymes could be highly depending on the cell number. To investigate this assumption, we divided the values of the specific enzyme activity by the number of the viable cells in each condition and labeled them as individualistic enzyme activity (Figure 2). Again in most cases, the values were higher for the cells cultured in Orpin's. For the individualistic activities of Xylanase and Avicelase; however, the results were pointing another direction. Individualistic Xylanase activities were detected to be higher in Menke's than Orpin's at virtually all time points ($p<0.05$) whereas Avicelase presented a significantly higher individualistic enzyme activity in Menke's ($p<0.05$) at the longest time point (7 days). Convientiently, our results for Xylanase were in accordance with the report of (Cao and Yang 2011), who demonstrated a significant activity of Xylanase in Menke's medium.

In a closer examination of the graphs in Figure 2, we realized that all individualistic enzyme activites were at their highest on the 0th day. Then we reflected that this fact might be caused by a possible back-up mechanism, where the amount of secreted enzymes could be kept at a certain range regardless of the viable cells present. However, since we have not performed any experiment nor have we encountered such an observation in the literature, this possiblity must be considered as a bare speculation. As far as our knowledge is concerned, the present study is the first one on the comparison of the activites of any cellulotic enzymes in Menke's and Orpin medium. Therefore, not only does it provide a novel set of data and a fresher look to the field but it aslo provokes many questions for further evaluations.

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