The Journal of Animal & Plant Sciences, 26(1): 2016, Page: 242-252
ISSN: 1018-7081

PRODUCTION OF AMYLOLYTIC ENZYME BY RUMEN FUNGI, *NEOCALLIMASTIX* SP. K7 AND *ORPINOMYCES* SP. K5

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ABSTRACT

Neocallimastix sp. K7 and *Orpinomyces* sp. K5 were studied for their amylase production capability in starch, maltose, and glucose-containing cultures. The highest level of amylase was produced in 4 and 5 mg ml^{-1} starch-containing mediums of *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7, respectively. Amylase production highly reduced in glucose-grown cultures of *Orpinomyces* sp. K5, whereas glucose stimulated the amylase production of *Neocallimastix* sp. K7 as effective as maltose. Both enzymes were optimally active at pH 5.5 and 45 °C. The pH stability range was 5.0 to 6.5 and inactivation occurred at 55 °C after 15 min for both enzymes. The highest amylase adsorption to starch was found at pH 5.5 and 6.5 in *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7, respectively. The enzyme activities were inhibited in the presence of Cu^{2+}, Zn^{2+} and Hg^{2+}, while their activities were significantly stimulated by Mg^{2+} and Mn^{2+}. Two amylase active bands were observed around 67 kDa. Glucose was determined as the only product of amylase action in *Orpinomyces* sp. K5. Glucose was the major product but maltose, maltotriose and maltotetraose were also detected in the enzymatic reaction of *Neocallimastix* sp. K7.

Keywords: *Neocallimastix* sp., *Orpinomyces* sp., starch, amylase.

INTRODUCTION

Starch is the major carbohydrate reserve in plants (Sarian *et al*., 2012) and starch containing feed grains are important components of ruminant diets. Grain based diets are used for the primary source of energy to improve meat or milk productions (Giuberti *et al*., 2014). Rumen is the major site of dietary starch digestion (Martinez *et al*., 2002), and starch is digested rapidly by microbial enzymes, to provide energy for rapid growth of rumen microorganisms and short-chain fatty acids as the main source for the host (Deckardt *et al*., 2013). The major starch utilization is performed by ruminal bacteria, therefore several amylolytic rumen bacteria and their enzymes were characterized (Kotarski *et al*., 1992). *Streptococcus bovis*, is among the most active amylase-producing ruminal bacteria, rapidly converts starch into maltooligosaccharides (Cotta and Whitehead, 1993). *Ruminococcus bromii* was found to be a dominant bacterial population in the rumen of cattle fed with a barley-based diet (Klieve *et al.*, 2007). Starch hydrolyzing bacteria possessing active cell-surface associated α-amylase constituted 19-23% of the total bacterial cells attached to particles of four different cultivars of barley grain and corn, and 70-80% of these starch hydrolyzing bacteria were members of *Ruminicoccaceae* in the phylum Firmicutes (Xia *et al.*, 2015). Rumen protozoa and fungi could also participate in ruminal grain digestion process. Holotrich protozoa have amylase activity in sufficiently large quantities for using starch as energy source (Kamra, 2005) and protozoa do not appear to produce lactic acid from starch in contrast to rumen bacteria (Van Soest, 1994).

While most anaerobic fungi can ferment both starch and maltose for their growth, some isolates of *Caeocomyces*, *Piromyces* and *Anaeromyces* do not utilize these carbohydrates (Trinci *et al*., 1994; Gordon and Phillips, 1998). Pearce and Bauchop (1985) studied an amyloglucosidase from *Neocallimastix frontalis*, which produced glucose as the only product from starch. In another study, an α-amylase was characterized from *N. frontalis* and this enzyme produced maltose and longer-chain oligosaccharides, but no glucose was produced (Mountfort and Asher, 1988). Likewise, McAllister *et al*. (1993) examined *Orpinomyces joyonii*, *N. patriciarum* and *Piromyces communis* for their ability to digest cereal starch, and found that all strains digested corn starch more readily than that from barley and wheat. *O. joyonii* exhibited the greatest ability to degrade starch in wheat and barley, whereas the degradation of these starches by *N. patriciarum* and *P. communis* was limited (McAllister *et al*., 1993).

The rumen microbial population represents an important source of hydrolytic enzymes (Yue *et al.*, 2013), therefore the rumen microbiome have been investigated for novel, underutilized and unique enzymatic activities (Gong *et al.*, 2012). Lopes *et al.* (2015) identified 28 lignocellulases, 22 amylases, and 9 other carbohydrate-active enzymes. Among the amylases, α-amylase, and α-glucosidase together represented 22.2% of the putative carbohydrate-active enzymes annotated by MG-RAST (Lopes *et al.*, 2015). Recently,

α-amylases belonging to glycoside hydrolase 13 family were found in the genome of *Orpinomyces* sp. C1A (Youssef *et al*., 2013) and *N. patriciarum* W5 (Wang *et al*., 2011). Despite their potential importance in starch fermentation, the amylolytic activities of rumen fungi are partially understood and still need further investigations. Therefore, the aim of the present study was to determine several amylolytic properties of *Neocallimastix* sp. K7 and *Orpinomyces* sp. K5 which were grown on several substrates. The enzymes from both fungi were partially characterized. The molecular weights and hydrolysis products were also determined in this study.

MATERIALS AND METHODS

Fungal isolates and culture conditions: *Neocallimastix* sp. K7 and *Orpinomyces* sp. K5 were isolated from cattle feces. Rumen fungi were maintained in anaerobic medium (Orpin, 1976) containing wheat straw as the sole energy source. Growth media for amylase production contained starch or other carbohydrates (glucose or maltose) with a concentration of 0.5%.

Enzyme preparation and assay procedures: Anaerobic fungi were cultivated for 7 days on medium containing each carbohydrate and amylase production was examined at every 24 hours by sampling from growth medium. Culture medium and fungal biomass were separated by centrifugation at 1250 g at 4 °C for 10 min. Cell-free supernatant was used as the crude enzyme extract. Fungal biomass was washed twice with 50 mM sodium phosphate buffer (pH 6.5) and then broken down by using liquid nitrogen and resuspended in the same buffer. Cellular debris was subsequently removed by centrifugation and the clarified extract was used as cell associated activity (Comlekcioglu *et al*., 2011). Cell associated and extracellular enzymes were stored at –20 °C until use. Amylase activity was measured as described by Miller (1959) using 0.5% (w/v) soluble starch (Merck) as substrates in 50 mM sodium phosphate buffer. One unit of enzyme activity was defined as 1 µmol of reducing sugar released from the substrate per minute.

Effects of pH, temperature, NaCl and substrate concentrations on amylase activity: To determine the pH optimum, the assay was carried out at various pH values from 3.5 to 12.0 by preparing the substrate in 0.05 M sodium acetate (pH 3.5-5.6), sodium phosphate (pH 6.0-8.0), Tris-HCl (pH 8.5-9.0) and sodium borate (pH 9.0-12.0). The pH stability was determined by incubating the enzyme with different buffers ranging from 3.5 to 12.0 at optimum temperature for 15 min. The effect of temperature was studied by incubating the reaction mixture at different temperatures ranging from 35 to 70 °C. For the thermal stability determination the enzyme was incubated without substrate at 35, 45 and 55 °C for different time intervals (15, 30, 45 and 60 min). Effect of NaCl was tested on amylase activity at optimum pH and temperature by using different NaCl concentrations (0.5-4.0 M) in the reaction mixture. Amylase activity was also measured with increasing substrate concentrations between 10 and 100 mg ml^{-1} with 10 increments.

Determination of amylase adsorption on substrate: The adsorption assay was conducted according to Tachaapaikoon *et al*. (2006) with minor modifications. Crude amylase was added on starch solutions (10 mg/ml) changing the pH from 3.5 to 8.0. Reaction mixture was stirred for 30 min at 4 °C. The suspension was centrifuged for 10 min at 4 °C and the clear supernatant was used to determine the remaining amylase enzyme activity.

Effects of various metal ions and chemical reagents on amylase activity: The effects of metal ions (Ba^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, $Fe^{3+}$and K^{+}) and the chemical reagents [1, 10 phenanthroline monohidrat, sodium sulfite (Na_2SO_3), sodium azide (NaN_3), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), urea, β-mercaptoethanol, Triton X-114, sodium dodecyl sulfate (SDS)] on the amylase activity were investigated by pre-incubating the enzyme in the presence of substances with final concentrations of 10 mM for 15 min at 45 °C, and then performing the assay in the presence of the same substances at the optimum temperature (Aygan and Arikan, 2009).

Zymogram analysis: For the determination of molecular weight, the enzyme preparations and protein marker (Serva) were subjected to SDS-PAGE. SDS-PAGE was performed using a 10% running gel containing 0.2% (w/v) starch (Ozcan, 2002). Zymogram analysis was carried out according to Liu *et al*. (2007).

Thin layer chromatography (TLC): For the analysis of hydrolysis products of amylase, enzyme was incubated with substrate at 45 °C for 24 hours. Prepared samples were applied on silica gel plate (Silica Gel60, Merck), and the plate was placed in a TLC chamber. Chloroform-acetic acid-distilled water (6:7:1, v/v/v) was used as mobile phase. Spots were developed by spraying the air-dried plate with aniline-diphenylamine-ortophosphoric acid (1:1:10, v/w/v) and heating it at 200 °C.

RESULTS

Location and effect of carbohydrate growth substrate: Amylase activity was found both at supernatant and cellular fractions in starch containing medium. Major activity was located in the cellular fractions for both anaerobic fungi, however supernatant fractions had also high amylolytic activity (Table 1). *Orpinomyces* sp. K5

and *Neocallimastix* sp. K7 were grown on glucose, maltose and starch as sole carbon source. Amylase production was measured everyday during 7 days (Figure 1). Starch was determined as the best inducer for amylase production in *Neocallimastix* sp. K7. Cells grown on maltose also exhibited the same productivities as obtained from starch for *Orpinomyces* sp. K5. Effect of starch concentration in growth medium on amylase production was tested and the highest amylase production was found to be 4 and 5 mg ml^{-1} for *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7, respectively (Figure 2). The production of amylase decreased above aforementioned starch concentrations. These concentrations were therefore used to produce amylase for further studies.

Enzyme characteristics: The pH profile of the amylases was determined by assaying activity using different buffers of varying pH values (Figure 3). Both enzymes were optimally active at pH 5.5 and more than 60% of the activity was observed in the range of pH 5.0-6.5. The effect of pH on stability was determined by incubating the amylases at 45 ºC for 15 min at different pH values. More than 70% of the activity remained in the pH range of 5.0-6.5. Optimum temperature was 45 ˚C for both amylases (Figure 4). Activities decreased sharply above 55 ˚C. Rapid thermal inactivation occurred after 15 min of preincubation at 45 ºC in *Orpinomyces* sp. K5 amylase. However, amylase of *Neocallimastix* sp. K7 retained more than 60% of the activity at 45 ºC after 15 min. Inactivation occurred at 55 ˚C after 15 min for both enzymes.

Different substrate concentrations were tested with equal amount of enzyme solution at 45 ˚C. Increasing the substrate concentration up to 70 and 80 mg ml^{-1} enhanced the relative amylase activity of the *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7, respectively (Figure 5). The amount of amylase adsorption on starch was investigated (Figure 6). The highest adsorption of amylase to starch was found at pH 5.5 and 6.5 in *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7, respectively. The adsorption profile was different for the tested rumen fungi. An increase in amylase adsorption rate was recorded above pH 5.5 in *Neocallimastix* sp. K7, in contrast amylase adsorption rate was decreased above pH 5.5 in *Orpinomyces* sp. K5. The enzyme activity was also determined with different NaCl concentrations (0 - 4 M) at 45 ˚C and pH 5.5 (Figure 6). Amylase activities decreased by increasing the NaCl concentrations, however, more than 70% of activity remained at 2 M NaCl for both fungal amylases. For *Neocallimastix* sp. K7 amylase, 90% activity loss was determined at 4 M NaCl, while 44% of the amylase was still active in *Orpinomyces* sp. K5 at same concentration.

Effect of metal ions on activity: The effects of various ions and reagents on the activities of amylases are given in Table 2. The amylase activities were significantly increased by Mg^{2+} and Mn^{2+} in *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7, respectively, but was strongly inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}. Among the tested reagents, SDS inhibited the activity up to 17% and 29% in *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7, respectively.

SDS-PAGE and TLC analysis: Enzyme preparations were subjected to SDS-PAGE to determine the homogenity and molecular weight of the enzyme. SDS-PAGE analysis demonstrated a single band, which shows amylolytic activity in gel. The two amylase active bands were determined to be around 67 kDa for both fungal amylases (Figure 8). The difference in molecular weight could be resulted from the amylase coding regions of the rumen fungi. The thin-layer chromatography (TLC) was used for the analysis of hydrolysis products of the amylases on soluble starch. Enzyme hydrolysis pattern of *Neocallimastix* sp. K7 was showed that the amylases hydrolyzed starch to form glucose as the main product and minor amounts of maltose, maltotriose, and maltotetraose. However, glucose is the sole product in *Orpinomyces* sp K5. Glucose was readily apparent even in the early stages of the reaction. When further hydrolysis was performed, there was an increase in glucose concentration for both fungal amylases.

Table 1. Determination of extracellular and cell associated amylase activity (U ml^{-1}) of *Orpinomyces* sp. K5 and *Neocallimastix* sp. K7 grown on media supplemented with starch

	***Orpinomyces* sp. K5**		***Neocallimastix* sp. K7**	
Time (Day)	**Supernatant amylase activity**	**Cell associated amylase activity**	**Supernatant amylase activity**	**Cell associated amylase activity**
3	8.14 ± 0.34	10.70 ± 0.24	6.72 ± 0.32	14.09 ± 0.23
5	8.08 ± 0.33	13.64 ± 0.49	8.54 ± 0.63	10.33 ± 0.38
7	10.33 ± 0.53	8.26 ± 0.18	4.95 ± 0.73	9.04 ± 0.21

Table 2. Effects of various metal ions and chemical reagents on amylase activity

Chemicals*	***Orpinomyces* sp. K5**	***Neocallimastix* sp. K7**
None	100	100
$CaCl_2$	154.8 ± 0.77	98.53 ± 0.74
$FeCl_3$	150.3 ± 1.90	114.6 ± 3.07
$BaCI_2$	150.1 ± 0.23	104.4 ± 0.75
$MnCl_2$	148.7 ± 1.19	125.2 ± 2.87
KCl	140.6 ± 0.53	100.2 ± 1.23
$CoCl_2$	128.9 ± 0.93	95.97 ± 0.90
$MgCl_2$	164.8 ± 1.63	84.47 ± 1.88
$NiCl_2$	104.5 ± 0.62	75.43 ± 2.06
$CuCl_2$	47.83 ± 1.01	15.35 ± 1.52
$ZnCl_2$	33.34 ± 0.73	15.33 ± 0.58
$HgCl_2$	27.53 ± 0.35	8.620 ± 0.38
PMSF	115.7 ± 1.61	101.7 ± 1.33
β-merkaptoetanol	132.6 ± 1.21	119.5 ± 1.05
Triton X-114	94.55 ± 1.64	95.76 ± 1.35
Urea	67.40 ± 0.47	89.44 ± 2.67
SDS	17.45 ± 0.19	29.08 ± 1.77

* All concentrations were 10 mM except β-merkaptoetanol (5 mM), Triton X-114 (1%), Urea (0.8 M) and SDS (1%).

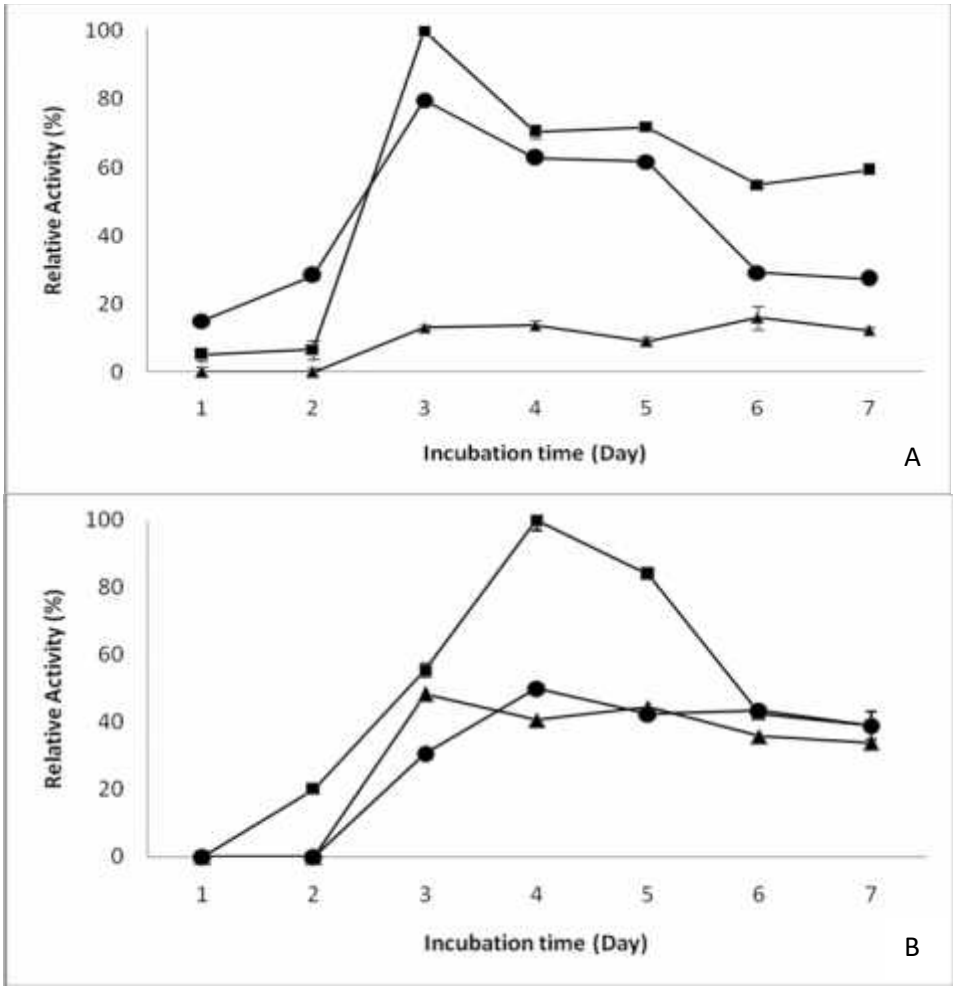

Figure 1. Time course of amylase production in *Orpinomyces* sp. K5 (A) and *Neocallimastix* sp. K7 (B) on starch (■), maltose (●) and glucose (▲) containing medium.

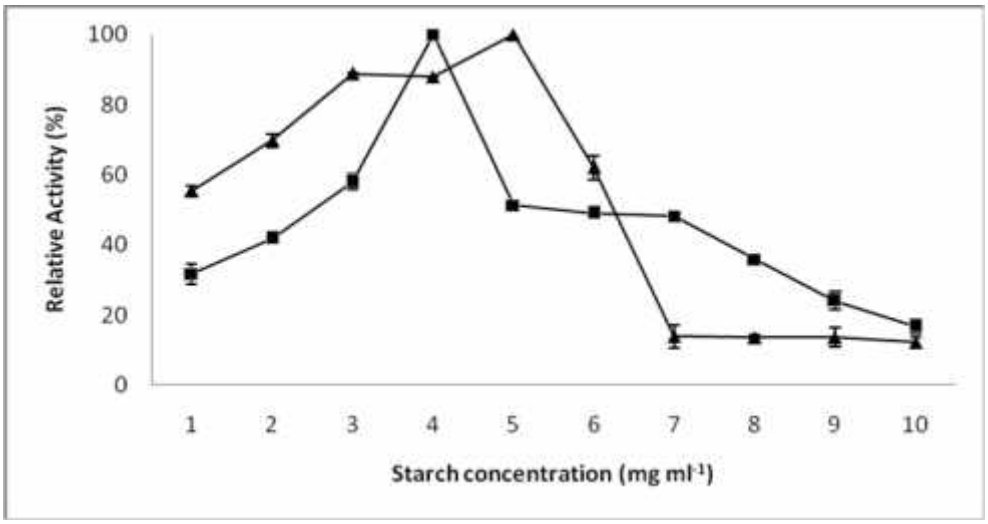

Figure 2. Effects of starch concentration in the culture on amylase production in *Orpinomyces* sp. K5 (■) and *Neocallimastix* sp. K7 (▲)

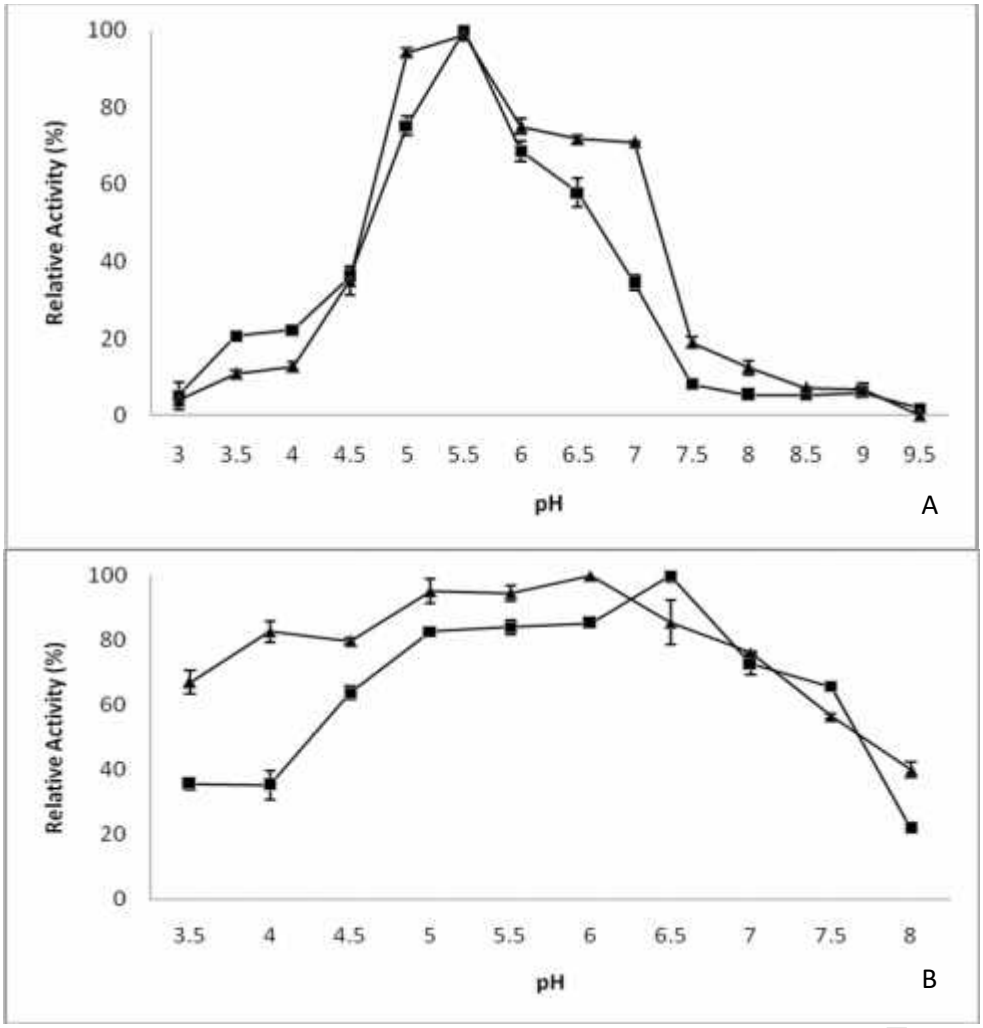

Figure 3. Effects of pH on amylase activity (A) and pH stability (B) in *Orpinomyces* sp. K5 (■) and *Neocallimastix* sp. K7 (▲).

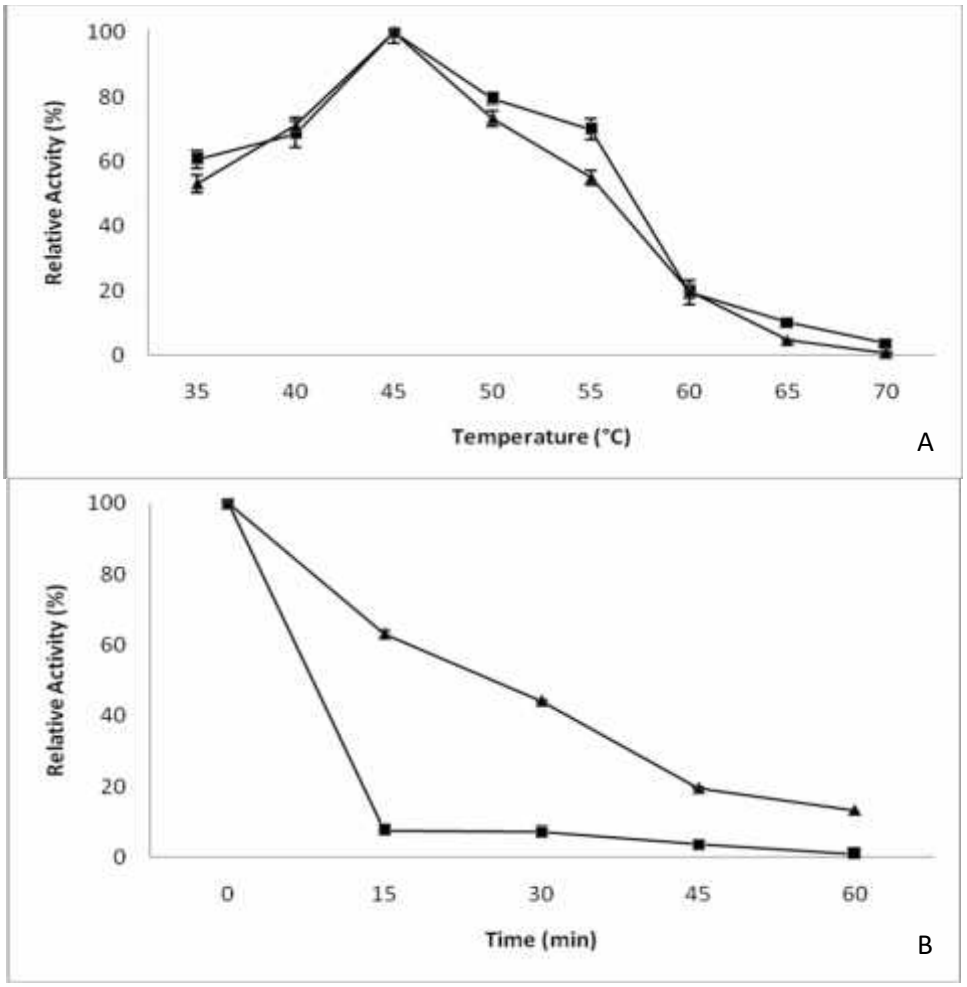

Figure 4. Effects of temperature on amylase activity (A) and thermal stability of amylases (B) in *Orpinomyces* sp. K5 (■) and *Neocallimastix* sp. K7 (▲)

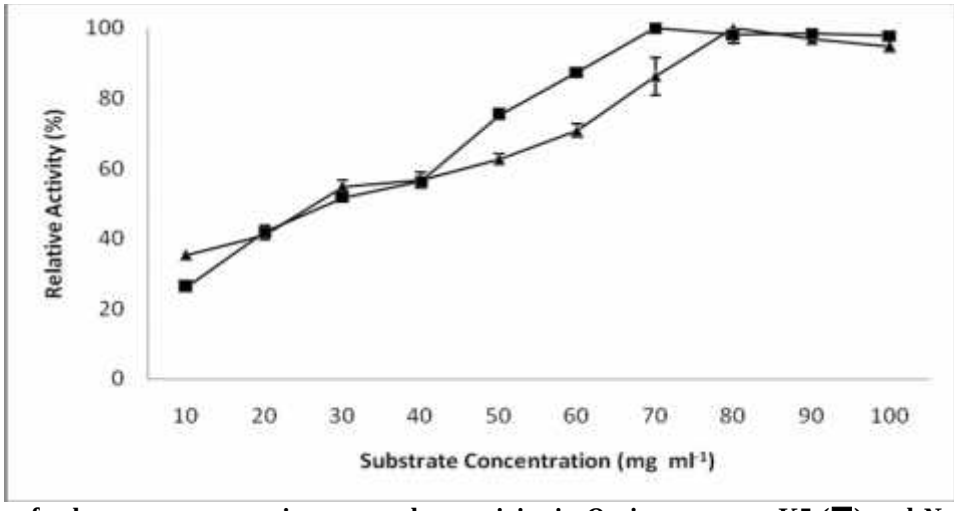

Figure 5. Effect of substrate concentration on amylase activity in *Orpinomyces* sp. K5 (■) and *Neocallimastix* sp. K7 (▲) at pH 5.5 and 45 ºC.

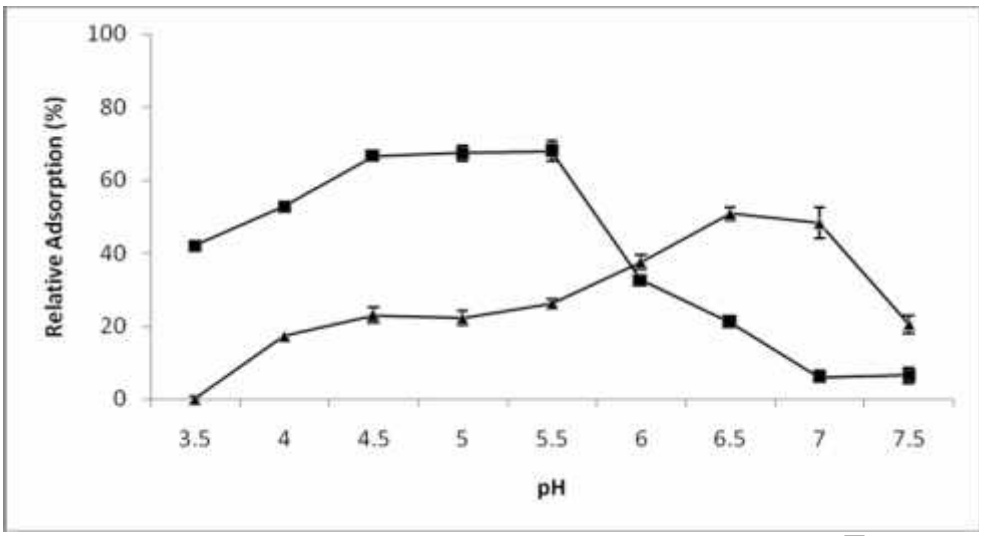

Figure 6. Effect of pH on the adsorption of amylases to starch in *Orpinomyces* sp. K5 (■) and *Neocallimastix* sp. K7 (▲) at 45 ºC.

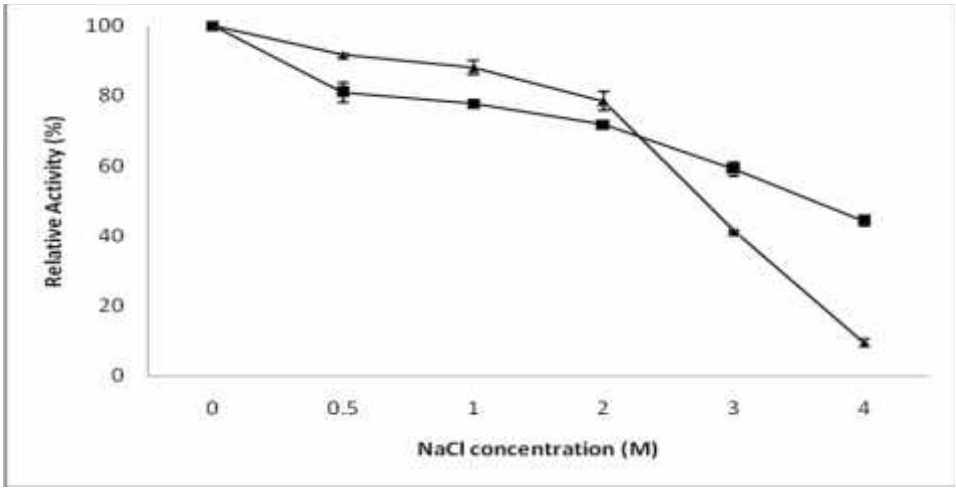

Figure 7. Effect of NaCl concentration on the activity of amylase from *Orpinomyces* sp. K5 (■) and *Neocallimastix* sp. K7 (▲).

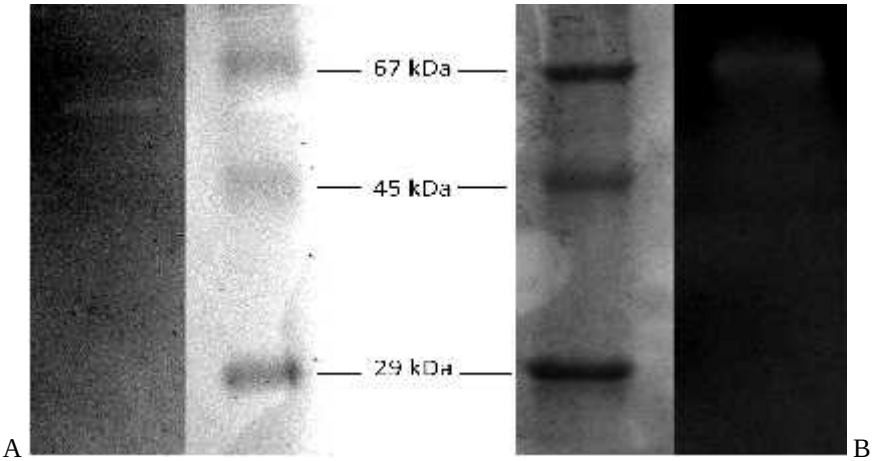

Figure 8. Zimogram analysis of amylases from *Orpinomyces* sp. K5 (A) and *Neocallimastix* sp. K7 (B).

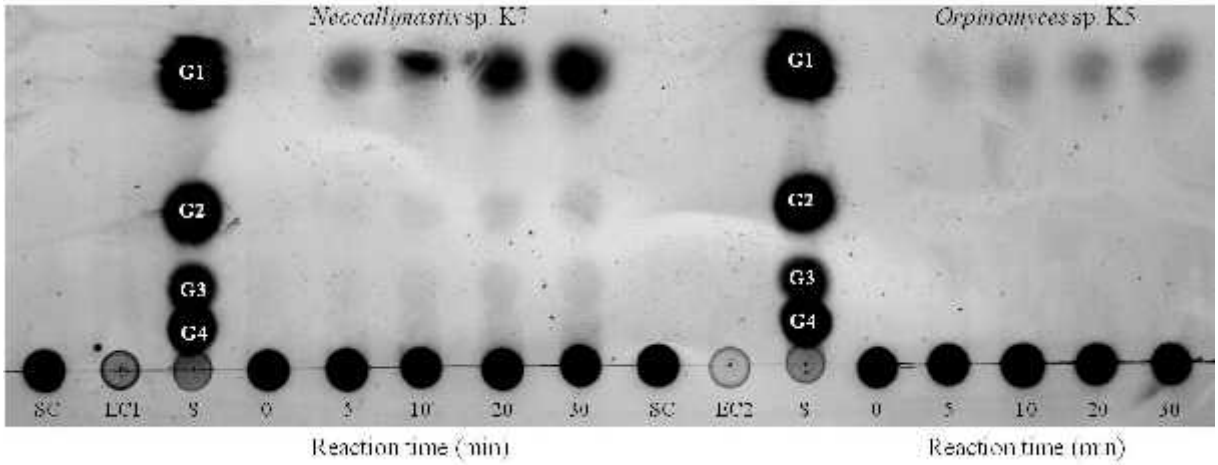

Figure 9. TLC of soluble starch hydrolysis products by amylases of *Neocallimastix* sp. K7 and *Orpinomyces* sp. K5. Standard mixture (S) contained glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4). Substrat (SC) and enzymes (EC1 for *Neocallimastix* sp. K7 and EC2 for *Orpinomyces* sp. K5) were loaded separately as controls.

DISCUSSION

Starch is the major source of energy in the diets of high-producing ruminants. Diets containing high concentration of grain have gained an interest due to its economical impact (Hernández *et al.*, 2014). However, there is a need to improve and optimize the efficiency of starch digestion, therefore researchers aimed to determine the mechanism of starch digestion in the rumen (Moharrery *et al.*, 2014). Efforts have been made to purify and characterize rumen microorganisms to gain a better understanding of starch digestion (McWethy and Hartman, 1977; Mountfort and Asher, 1988; Cotta, 1988; Freer, 1993; McAllister *et al*., 1993; Yanke *et al*., 1993; Klieve *et al.*, 2007). Rumen fungi are indigenous to the rumen and produces highly active cellulases and hemicellulases (Gordon and Phillips, 1998). Ruminal fungus *N. frontalis* has been shown to produce an α-amylase, which was released mainly into the culture fluid (Mountfort and Asher, 1988). A rumen fungus isolate F, *N. patriciarum* and *P. communis* were reported to produce highest amylolytic activities in cells grown on starch. The extracellular activities were less for these anaerobic fungi (Williams and Orpin, 1987). In the present study, ruminal fungi *Neocallimastix* sp. K7 and *Orpinomyces* sp. K5 produced both extracellular and cell associated amylase. Glucose repressed the amylase production of *Orpinomyces* sp. K5. Amylase productions of *S. bovis* JB1, *Bacteroides ruminicola* 23 and B_14, and *Butyrivibrio fibrisolvens* 49 and A38 were greatly reduced in glucose-grown cultures (Cotta, 1988). A regulatory role of glucose on α-amylase production was shown for *N. frontalis* (Mountfort and Asher, 1988), however glucose stimulated the amylase production as much as maltose for *Neocallimastix* sp. K7. The tolerance to catabolite repression in *Neocallimastix* sp. K7 reveals a different mechanism of amylase synthesis from *Orpinomyces* sp. K5. Starch was found to be the most effective substrate for amylase production and the maximum production was obtained at 4-5 mg ml^{-1} starch concentration. *N. frontalis* produced the highest amylase on 2.5 mg ml^{-1} starch containing medium, and a sharp decline was reported beyond this level, and the amylase production virtually ceased at 5 mg ml^{-1} and above starch concentrations (Mountfort and Asher, 1988). Amylases are generally induced in the presence of starch or maltose (Gangadharan *et al.*, 2006), however several amylases are produced even in the glucose containing medium (Sreekanth *et al.*, 2012, Sankaralingam *et al.*, 2012).

The studied fungal strains digested the starch optimally at pH 5.5 and 45 ˚C. Optimum pH of α-amylase from *N. frontalis* was also 5.5, however optimum temperature was 55 ˚C (Mountfort and Asher, 1988), which was higher than our findings. Thermal stability profiles were found to be different in *Neocallimastix* sp. K7 and *Orpinomyces* sp. K5. Amylase from K7 was determined more stable than amylase from K5. α-amylases from *N. frontalis* (Mountfort and Asher, 1988) and *S. bovis* (Freer, 1993) were found to be relatively stable at 50 ˚C for 1 h. Strong inhibiton of amylase by Cu^{2+}, Zn^{2+} and Hg^{2+} was reported (Lee *et al.*, 2015; Zhou *et al.*, 2015), and studied amylases were also inhibited by these ions. Anaerobic fungal enzymes seemed to be sensitive to SDS inhibition, such as β-D-xylosidase from *N. frontalis* (Garcia-Campayo and Wood, 1993), β-glucosidase from *Orpinomyces* sp. PC-2 (Chen *et al*., 1994), CMCase from *Neocallimastix* sp. GMLF7 (Comlekcioglu *et al*., 2008), and Cel1A and Xyn1B from *Neocallimastix* sp. GMLF1 (Comlekcioglu *et al*., 2010). PMSF did not produce an inhibitory effect

on amylase activity in K5 and K7 and similar findings was also reported by Menon *et al*. (2014).

Zymogram studies revealed an amylase active band for both fungi with an approximate molecular weight of 67 kDa. A wide range of molecular weights from 10 to 210 kDa has been reported for amylases (El-Sayed *et al.*, 2013). The molecular weights of purified amylases from *Bacteroides amylophilus* (Mcwethy and Hartman, 1977) and *S. bovis* JB1 (Freer, 1993) were 92 and 77 kDa, respectively. SDS-PAGE zymograms of ruminal extracts showed the presence of a certain number of amylase-active bands with different molecular weights ranging from 36 to more than 100 kDa (Martinez *et al*., 2002). Products of starch digestion were found to be different for K5 and K7. Glucose was the only product of K5 amylase, however maltose, maltotriose and maltotetraose were also detected in the hydrolysis products of K7 amylase. Pearce and Bauchop (1985) was found that the glucose is the only product of starch digestion, and they concluded that an amyloglucosidase was responsible for starch hydrolysis. On the other hand, Mountfort and Asher (1988) reported that the hydrolysis products of α-amylase from *N. frontalis* were mainly maltotriose, maltotetraose and longer-chain oligosaccharides, however no glucose was produced.

In conclusion, starch is an important diet component for highly productive ruminants, hence it is essential to study the starch degrading microorganisms in the rumen. Anaerobic rumen fungi play a vital role in biodegradation of the plant biomass and the present research represents an effort to describe some biochemical characteristics of rumen fungal amylases. Amylases belonging to glycosyl hydrolase 13 family were identified in the *Orpinomyces* sp. C1A genome (Youssef *et al.*, 2013) and cloning the amylase coding regions from rumen fungi will expand our understanding on the role of these microorganisms in ruminal starch degradation.

Acknowledgements: We thank Kahramanmaras Sutcu Imam University for the funding of this study (Grant no: 2012/4-14M). Valuable helps of Sedat KOSTEKCI are also gratefully acknowledged.

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