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Effects of Essential Oils Supplementation on Survival Rate and Lignocellulolytic Enzyme Activities of Rumen Fungi Isolated From Cattle

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ABSTRACT: Due to the ban on antibiotics and ionophores, which were used as feed supplements, scientists have become more interested in evaluating other alternatives to control the rumen fermentation. Plant essential oils might be alternative to that kind of supplements for farm animals. However, our knowledge about the possible effects of plant essential oils on survival rate and enzyme activity of anaerobic fungi (besides other rumen microbes) is still limited. Current study, therefore, aimed to determine the effects of plant essential oils on this unique group of rumen microorganisms. Effects of various dosages of myrtle (*Myrtus communis*), juniper (*Juniperus communis*), melissa (*Melissa officinalis*) and thyme (*Thymus vulgaris*) plant essential oil, which have antimicrobial properties, were investigated on survival rate and enzyme activity of *Neocallimastix* sp. GMLF2 and *Orpinomyces* sp. GMLF18. Accordingly, plant essential oils were dissolved in ethyl alcohol and added into medium. Fungal growth (*Neocallimastix* sp. GMLF2 and *Orpinomyces* sp. GMLF18) was completely inhibited in the 1 mg/ml essential oil concentration of culture medium. Furthermore carboxymethylcellulase and xylanase activities were decreased remarkably when 0.5 mg/ml concentration of essential oil was added into culture media regardless of type of the oil.
Keywords: Rumen, anaerobic fungi, plant essential oil, carboxymethylcellulase, xylanase, enzyme activity

Esansiyel Yağların Eklenmesinin, Sığırlardan İzole Edilen Rumen Funguslarının Yaşama Oranları ve Lignoselülolitik Enzim Aktivitelerine Etkileri

ÖZET: Antibiyotik iyonoforların yasaklanmasından sonra bilim insanları rumen fermantasyonunun kontrolü amacıyla diğer alternatiflerin değerlendirilmesi üzerine yoğunlaşmışlardır. Bitkisel kaynaklı esansiyel yağlar çiftlik hayvanları için bu tip katkı maddelerine alternatif oluşturabilir. Fakat bitkisel esansiyel yağların anaerobik fungusların yaşama oranları ve enzim aktiviteleri üzerine olan bilgilerimiz oldukça kısıtlıdır. Bu çalışma bitkisel esansiyel yağların rumen mikroorganizmaları içerisinde özel bir konuma sahip bu grup üzerine olası etkilerini araştırmayı amaçlamıştır. Antimikrobiyal özelliklere sahip olan mersin, ardıç, melisa ve kekik esansiyel yağlarının farklı dozajlarının *Neocallimastix* sp. GMLF2 ve *Orpinomyces* sp. GMLF18 izolatlarının yaşama oranları ve enzim aktiviteleri üzerine etkileri araştırılmıştır. Bu amaçla esansiyel yağlar etil alkol içerisinde çözündürülerek besi yerlerine eklenmiştir. Besi yerlerine 1 mg/ml esansiyel yağ eklendiğinde fungal (*Neocallimastix* sp. GMLF2 ve *Orpinomyces* sp. GMLF18) yaşamın tamamen durduğu gözlemlenmiştir. Besi yerlerine 0.5 mg/ml^{-1} veya daha fazla esansiyel yağ eklenmesi durumunda KMSaz (Karboksi metil selülaz) ve ksilanaz enzim aktivitelerinde önemli bir azalma söz konusu olmuştur.
Anahtar kelime: Rumen, anaerobik fungi, bitkisel esansiyel yağ, Karboksi metil selülaz, ksilanaz, enzim aktivitesi

INTRODUCTION

Anaerobic gut fungi (AGF) are indigenous to the rumen and they actively degrade plant cell wall proteins, pectins and carbohydrates to their monomers. Overall, 30-35% of total biodegradation of the plant biomass ingested by ruminant herbivores could be attributed to this group of rumen microorganisms. AGF produce highly active cellulases and hemicellulases and especially β-glucanase, xylanases and xylan debranching enzymes (Borneman et al., 1993, Comlekcioglu et al, 2008; Ozkose et al., 2009). They possess the unique capacity to penetrate the surface cuticle of plants, and the lignified tissues of cell walls (Akin et al., 1990; Akin, 1989). Furthermore, they digest recalcitrant substrates such as wheat and barley straws more extensively (37-50 %) than do ruminal bacteria (Joblin et al., 1989).

There are persuasive number of reports that cellulose degradation by microbial enzymes is adversely affected in the presence of soluble sugars such as cellobiose, glucose and maltose in culture medium (Stewart et al. 1990; Ekinci et al., 2001); by the existence of some end products such as acetate, formate, lactate, malate, H_2 and ethanol (Joblin and Naylor, 1993) or by the presence of polyphenolic compounds in the culture medium (Ozkose et al., 2011). That kind of reductions in enzyme activity might be caused by cumulative mutation in the regulatory mechanism (Stewart et al., 1990; Ekinci et al., 2006). Since discovery of the AGF by Orpin (1975) from the rumen digesta of sheep, more than 20 species under 8 genera have been reported and degradation of fibrolytic plant material is a common property of all of them including recently erected species including *Caecomyces sympodialis* (Chen et al., 2007), *Oontomyces anksri* (Dagar et al., 2015a) and *Buwchfawromyces eastonii* (Callaghan et al., 2015).

The population size and biodiversity of AGF is tend to be affected by diet and feed additives ingested by the host animals. The enzyme activities of this special group of gut microbes might be inhibited or stimulated by the supplementation of various feed additives such as *Albizia lebbeck, Lawsonia inermis, Terminalia arjuna* (Dagar et al., 2015b). Addition of antibiotics as feed supplement into the animal rations as growth stimulators is strictly limited due to its potential adverse effects on human health. Therapeutic plants might be an alternative to antibiotics as feed additives for farm animals. Usage of the essential oils, whose antimicrobial effects have been demonstrated previously (Patra, 2011), as feed additives could be a feasible alternative to antimicrobial products.

Supplementation of essential oil mixture remarkably reduced the size of fungal population and colonization which were determined according to fungal sporangia counts and, similar effects were observed when monensin was supplemented to beef cattle ration (Tomkins et al., 2015). The literature does contain convincing evidence that addition of various essential oils can have remarkable positive effects on the reduction of methane production, particularly for ruminant herbivores (Yadeghari et al., 2015). However, our knowledge about potential effects of essential oil supplementations on the biodegradation of lignocellulolytic plant biomass by rumen fungi is quite limited.

Main objective of current study was to determine the possible effects of essential oils on the survival rate and enzyme activities of two fungal cultures namely *Neocallimastix* sp. GMLF2 and *Orpinomyces* sp. GMLF18 isolated from cattle.

MATERIALS and METHODS

Microorganisms and Culture Maintenance

The anaerobic rumen fungi, *Neocallimastix* sp GMLF2 and *Orpinomyces sp.* GMLF18 were isolated from freshly collected cow feces and frozen fecal sample of cow respectively as described earlier (Comlekcioglu, 2009) and identified with the aid of molecular techniques (Ozkose et al., 2015). Both isolates are deposited in culture collection of Biotechnology and Gene Engineering Laboratory of Kahramanmaraş Sutcu Imam University, Turkey. Cultures were maintained at 39±1 ^{0}C without shaking in complex liquid medium containing 30 % (v/v) clarified rumen fluid (Orpin, 1976), and 0.5 % (w/v) carboxymethylcellulose (CMC) or 0.5% (w/v) xylan as sole energy sources (pH 6.7). To obtain the anaerobic conditions, the medium was reduced using CO_2 and possible trace amount of O_2 was removed by heating the medium gently. Cysteine-HCl was also used as reducing agent and resazurin was used as O_2 indicator. The medium was prepared by dispensing 10 ml of medium into Hungate tubes (16 X 125 mm, Bellco Glass Inc., Vineland, NJ, USA) under strict anaerobic conditions then sterilized by means of autoclaving at 121°C for 15 min for both carboxymethylcellulose and xylan, included media. To inhibit possible bacterial contamination choloramphenicol (50µg/ml in final concentration) in 50% (v/v) ethanol was applied to culture tubes for the 1st culturing of the isolates.

Essential Oils

Essential oils, Myrtle (*Myrtus communis*), juniper (*Juniperus communis*), Melissa (*Melissa officinalis*) and thyme (*Thymus vulgaris*) were prepared as concentrated stock solution using distilled water and filter sterilized under anaerobic conditions by passing through a 0.22 µm universal filters using syringe. They were solubilized in ethyl alcohol according to Ha et al. (2001) and added into medium under anaerobic conditions at 0.01, 0.1, 0.5 and 1mg/ml final concentrations. Fungal cultures containing essential oils in various concentrations (stated above) were examined up to 6 days using inverted microscope (Soif XDS1B) for the survival of fungi (+: survived; -: not survived). For this procedure, three tubes were examined for each essential oil concentration treatment and process was repeated twice. To determine the possible effects of the ethanol (used to dissolve essential oils) on the survival of AGF, ethanol containing tubes (without essential oil supplementation) were also examined (Ha et al., 2001).

Enzyme Sources

Fungal cells and culture supernatants from experimental cultures were harvested after 3-4 days of incubation period. Culture supernatants were centrifuged for 10min (11,000 *g* at room temperature) and the clarified enzyme solutions and cell extracts were kept at −70 ^{o}C until needed for enzyme assays, which were conducted in the presence and absence of various essential oil concentrations. The enzyme source for all enzyme reactions was a solution of the appropriate enzyme diluted with sodium phosphate buffer (pH: 6.5) at the ratio of 1:1 (v/v).

The cultures were centrifuged at 1250 *g* for 10 min for the separation of the culture medium and the fungal biomass. Cell-free supernatant was used to determine extracellular enzyme activity. Fungal biomass was washed twice with 50 mM sodium-phosphate buffer and then broken down by using ball-mill dismembranator (Retsch, Germany) and resuspended in the same buffer. Cellular debris was subsequently removed by centrifugation and the clarified extract was used as intercellular activity. Extracellular and intercellular fractions were stored at – 20 ^{o}C until required.

Enzyme Assays

The protein contents of samples were determined with the aid of the method described by Lowry et al. (1951). Xylanase and carboxymethylcellulase (CMCase) activities were determined by measuring the reducing sugars released from xylan and carboxymethylcellulose (CMC) containing media respectively according to DNS method of Miller (1959) as detailed below. The concentrations of substrate solutions in all assays were 0.5% (w/v) in 50 mM sodium phosphate buffer (pH 6.5). Reducing sugar, resulted from the enzymatic reaction, was determined by measuring the absorbance at 540 nm

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(Spectromax, UK) xylose for xylanase and glucose for cellulase activities as standard. The reaction was halted by the addition of 2 M Na_2CO_3 solution. Units of activity are defined as µmoles of product released per minute under assay conditions. All assay procedures were conducted in triplicate, repeated twice and the mean values are used.

Statistical Analysis

The significance of differences for treatment means were calculated using general linear model procedures of statistical package program (SPSS, 1985) with the variables fitted being different levels of essential oil treatments.

RESULTS

Survival of Fungal Isolates in Essential Oil Supplemented Medium

A monocentric isolate *Neocallimastix* sp. GMLF2 and an *Orpinomyces* sp. GMLF18, which has polycentric thallus characteristics, were grown on basal anaerobic medium containing either CMC or xylan as sole carbon sources. Possible effects of four essential oils (myrtle, juniper, melissa and thyme) on the survival of two fungal isolates were presented in Table 1, when culture medium of AGF contained either CMC or xylan as sole energy source. Both fungal isolates, *Neocallimastix* sp. GMLF2 and *Orpinomyces* sp. GMLF18 gave a same response to essential oil supplementation from the point of survival rate that no fungal life was observed when essential oil concentration was 1mg/ml. Same survival characteristic was recorded for these fungal isolates regardless of energy type used in culture media. Survival was totally inhibited for both fungal isolates when essential oil concentration reached to 1mg/ml when they were grown in the medium containing CMC as sole energy source. Same results were obtained when medium contained xylan and therefore, the date were illustrated in same table (**Table 1**). A notable effect was observed when essential oil of juniper was added to the growth medium at the final concentration of 0.50 mg/ml at which level no survival was observed regardless of fungal species (**Table 1**).

***Effects of Essential Oil Supplementation on Enzyme Activities of Neocallimastix* sp. GMLF2**

Possible effects of essential oils on the total (**Table 2**) and specific (**Table 3**) fibrolytic enzyme activities of monocentric *Neocallimastix* sp. GMLF2 were determined when the isolate was grown in the medium containing CMC or xylan as sole carbon source. CMCase supernatant activity of the *Neocallimastix* sp. GMLF2 was remarkably reduces ($P<0.05$) following the supplementation of essential oils (regardless of origin) and these reductions in enzyme activity were observed more significant parallel to the increase of essential oil concentration in culture medium. It should be noted that essential oil derived from thyme was the least effective on the reduction of supernatant CMCase activity of the isolate *Neocallimastix* sp. GMLF2 compare to other supplements.

Table 1. Effects of essential oils on survival of fungi cultured in the media containing CMC or xylan as sole carbon source.*

Essential oil concentration (mg/ml)	Fungal isolates							
	Neocallimastix sp. GMLF2				*Orpinomyces* sp. GMLF18			
	Myrtle EO	Juniper EO	Melissa EO	Thyme EO	Myrtle EO	Juniper EO	Melissa EO	Thyme EO
0.00	+	+	+	+	+	+	+	+
0.01	+	+	+	+	+	+	+	+
0.10	+	+	+	+	+	+	+	+
0.50	+	-	+	+	+	-	+	+
1.00	-	-	-	-	-	-	-	-

EO: Essential oil; (+): Survival of isolates were observed; (-): No isolate was survived *: Same results were recorded for both energy type (CMC or xylan).

Araştırma Makalesi/Research Article
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Table 2. Total CMCase and xylanase activities of *Neocallimastix* sp. GMLF2 growing CMC and xylan (respectively) containing culture media.

	Essential oil	Concentration of essential oil (mg/ml)				
		0.00	0.01	0.10	0.50	1.00
Supernatant CMCase (µmol/min/ml)	Myrtle	25,9±0,8[a]	20,0±0,7[b]	18,4±0,6[b]	18,2±0,7[b]	7,8±0,2[c]
	Juniper	25,9±0,8[a]	15,3±0,9[b]	12,4±0,8[b]	7,3± 0,9[c]	4,1±0,8[c]
	Melissa	25,9±0,8[a]	16,3±2,7[b]	22,9±2,1[ab]	8,0±0,4[c]	6,8±0,6[c]
	Thyme	25,9±0,8[a]	24,8±0,9[a]	23,5±1,6[a]	11,5±0,2[b]	8,8±0,9[b]
Supernatant Xylanase (µmol/min/ml)	Myrtle	137,0±4,5[a]	142,8±3,6[a]	144,0±2,6[a]	136,3±0,3[a]	27,7±2,3[b]
	Juniper	137,0±4,5[a]	128,7±0,8[a]	127,7±2,7[a]	10,4± 2,4[b]	9,1±2,6[b]
	Melissa	137,0±4,5[b]	152,6±4,6[a]	148,4±2,3[ab]	120,8±1,4[c]	25,3±1,4[d]
	Thyme	137,0±4,5[b]	148,8±1,3[a]	149,5±1,6[a]	137,3±0,9[b]	13,2±1,4[c]

A sharp reduction was recorded for total supernatant xylanase activity when juniper essential oil concentration was increased from 0.10 to 0.50 mg/ml or over in culture medium of isolate *Neocallimastix* sp. GMLF2. Moreover essential oil obtained from juniper had the most significant adverse effect on both CMCase and xylanase enzyme activities for supernatants. In contrast to the CMCase supernatant activity of the fungal isolate, thyme essential oil showed moderate effect on the total supernatant xylanase activity.

Specific CMCase and xylanase activities of the isolate GMLF2 when it was grown CMC or xylan containing media supplemented with essential oils (at various concentrations) were also determined (**Table 3**). In general, similar effects of essential oil supplementation to total activity were observed for specific activities. Remarkable effects of all essential oils were observed when the concentrations were 0.50 mg/ml or above that concentration regardless of essential oil origin.

The main threshold effect of the essential oil was determined when the 0.50 mg/ml was added into culture medium of the fungal isolates.

***Effects of Essential Oil Supplementation on Enzyme Activities of Orpinomyces sp* GMLF18**

The effects of essential oils on the total (**Table 4**) and specific (**Table 5**) fibrolytic enzyme activities of polycentric isolate *Orpinomyces* sp. GMLF18 were also determined when the isolate was grown in CMC or xylan containing medium as sole energy source.

Table 3. Spesific CMCase and xylanase activities of *Neocallimastix* sp. GMLF2 growing CMC and xylan (respectively) containing culture media.

	Essential oil	Concentration of essential oil (mg/ml)				
		0.00	0.01	0.10	0.50	1.00
Supernatant CMCase (µmol/min/mg protein)	Myrtle	261,0±8,4[a]	161,2±5,9[bc]	165,0±5,7[b]	135,7±5,4[c]	74,0±2,0[d]
	Juniper	261,0±8,4[a]	147,2±8,8[b]	116,8±7,8[b]	66,2±8,2[c]	47,6±9,9[c]
	Melissa	261,0±8,4[a]	182,4±16,7[b]	146,2±25,0[b]	57,8± 3,5[c]	61,3±5,8[c]
	Thyme	261,0±8,4[a]	176,2±6,9[b]	135,31±9,6[c]	103,3±2,6[cd]	77,3±8,4[d]
Supernatant Xylanase (µmol/min/mg protein)	Myrtle	895,8±29,4[b]	1063,4±26,8[a]	1020,3±18,4[a]	985,5±2,4[ab]	217,5±18,3[c]
	Juniper	895,8±29,4[b]	1040,8±6,9[a]	640,8±13,7[c]	88,5±20,7[d]	71,1±20,1[d]
	Melissa	895,8±29,4[b]	1176,2±35,8[a]	1083,0±16,9[a]	929,7± 10,9[b]	211,5±12,4[c]
	Thyme	895,8±29,4[c]	928,9±8,4[bc]	975,7±10,8[ab]	1003,8±7,1[a]	77,9±8,4[d]

Table 4. Total CMCase and xylanase activities of *Orpinomyces* sp. GMLF18 growing CMC and xylan (respectively) containing culture media.

	Essential oil	Concentration of essential oil (mg/ml)				
		0.00	0.01	0.10	0.50	1.00
Supernatant CMCase (µmol/min/ml)	Myrtle	23,3±0,5^{a}	21,7±1,1^{a}	15,8±0,9^{b}	12,0±1,4^{b}	2,6±0,4^{c}
	Juniper	23,3±0,5^{a}	31,6±0,8^{b}	15,4±1,0^{c}	4,9± 0,7^{d}	3,4±1,4^{d}
	Melissa	23,3±0,5^{a}	20,9±1,4^{a}	16,3±0,4^{b}	12,8±0,6^{b}	1,4±0,04^{c}
	Thyme	23,3±0,5^{a}	33,0±1,4^{b}	24,3±0,4^{a}	13,7±0,4^{c}	4,0±0,9^{d}
Supernatant Xylanase (µmol/min/ml)	Myrtle	107,3±4,9^{a}	31,6±0,3^{bc}	42,89±3,1^{b}	26,6±5,3^{c}	12,1±1,0^{d}
	Juniper	107,3±4,9^{a}	99,7±4,4^{a}	61,6±9,6^{b}	10,8± 0,8^{c}	8,0±4,1^{c}
	Melissa	107,3±4,9^{a}	48,5±4,8^{b}	31,7±2,9^{b}	11,4±4,7^{c}	3,7±2,6^{c}
	Thyme	107,3±4,9^{a}	189,8±7,7^{b}	164,7±1,9^{b}	133,8±9,3^{a}	19,1±4,4^{c}

Table 5. Spesific CMCase and xylanase activities of *Orpinomyces* sp. GMLF18 growing CMC and xylan (respectively) containing culture media.

	Essential oil	Concentration of essential oil (mg/ml)				
		0.00	0.01	0.10	0.50	1.00
Supernatant CMCase (µmol/min/mg protein)	Myrtle	161,2±3,7^{a}	172,6±8,8^{a}	104,5±6,5^{b}	78,3±9,1^{b}	23,6±4,0^{c}
	Juniper	161,2±3,7^{a}	205,6±5,2^{b}	96,3±6,8^{c}	32,9±5,1^{d}	24,8±10,6^{d}
	Melissa	161,2±3,7^{a}	140,8±9,9^{a}	103,6±3,1^{b}	92,6±4,7^{b}	10,7±0,3^{c}
	Thyme	161,2±3,7^{a}	190,0±8,4^{b}	168,3±3,0^{ab}	87,0±3,0^{c}	30,6±7,3^{d}
Supernatant Xylanase (µmol/min/mg protein)	Myrtle	660,9±30,5^{a}	207,3±2,2^{c}	319,8±23,2^{b}	205,3±41,4^{c}	106,6±9,2^{d}
	Juniper	660,9±30,5^{a}	493,3±67,3^{b}	406,3±63,5^{c}	104,7±8,2^{d}	107,5±55,3^{d}
	Melissa	660,9±30,5^{a}	398,1±38,5^{b}	346,0±32,4^{b}	89,2±37,1^{c}	28,5±19,8^{c}
	Thyme	660,9±30,5^{a}	1444,9±59,0^{b}	1169,3±13,7^{c}	935,2±65,1^{d}	150,1±35,1^{e}

The effect of Melissa essential oil on the fibrolytic enzyme activities of the polycentric isolate, *Orpinomyces* sp. GMLF18, was noticeable. The highest adverse effects were determined when Melissa concentration was increased to 1.00 mg/ml in culture medium and even enzyme activities were negligible at that concentration. The least adverse effect was recorded when thyme essential oil was supplemented to the culture medium of GMLF18 for both CMCase and xylanase activities. When essential oil concentration was increased to 1.00 mg/ml, CMCase and xylanase activities were reduced remarkably (**Table 4**).

Effect of the Melissa essential oil on the specific supernatant CMCase and xylanase activities of isolate *Orpinomyces* sp.GMLF18 was similar to the total enzyme activities of that isolate. This essential oil reduced the fibrolytic activities of the isolate more significantly compare to the other three essential oil sources. The least adverse effect was observed when culture media was supplemented with thyme essential oil for both CMCase and xylanase activities (**Table 5**).

Lower essential oil (Juniper and Thyme oils) supplementation to the culture medium containing CMC as sole carbon sources caused slight induction in CMCase activity of GMLF18. Similar results were recorded for specific xylanase activity of *Orpinomyces* sp**.**GMLF18 when the culture medium was treated with Thyme oil.

DISCUSSION

No fungal growth was observed following the supplementation of essential oils at the 1mg/ml concentration in the culture medium. Similar results were reported by Talepzadeh et al. (2012) that, growth of *Neocallimastix*-like fungal isolate was totally inhibited when it was cultured in the medium containing essential oil of *Zataria multiflora*. Furthermore Grande-Tovar et al. (2016) studied the possible effects of essential oil, extracted from *Austroeupatorium inulifolium*, on the survival of the aerobic fungal genera namely *Aspergillus* spp, *Pencillium* spp and *Fusarium* spp and they reported that fungal growth was inhibited up to 70% by this essential oil. They recorded that, *Penicillium nalgiovense* and *Aspergillus parasiticus* were more resistant to the essential oil of *Austroeupatorium inulifolium* (Grande-Tovar et al., 2016) whilst both fungal isolates were tend to be more sensitive to the Juniper essential oil in current study.

All rumen fungi, reported so far, synthesize large number of fibrolytic enzymes to degrade complex plant materials which is essential for their energy requirements. These hydrolytic enzymes, however, could

be affected (induction / reduction) by various feed materials ingested by host herbivores. In current study, relatively higher specific supernatant xylanase activity were observed particularly when 0.01mg/ml essential oil, derived from thyme, was supplemented to culture medium of GMLF2 and these results could be explained with the fact that lower concentration of thyme essential oil induced the enzyme activity. Similar results were reported by Ha et al. (2001) that lower saturated fatty acids had stimulatory effects on the cellulose digestion by *Neocallimastix frontalis*, although its higher concentrations inhibited the enzyme activity significantly.

Stimulatory effects of the lower amount of essential oils on the extracellular hydrolytic enzymes of non-rumen habitating fungi were also reported. Sub-lethal concentration of essential oil of *Austroeupatorium inulifolium*, for instance, has remarkable induction effects on the endocellulase, α-amylase, protease and pectinase enzymes' production and activity of aerobic fungal isolates namely *Penicillium, Aspergillus* and *Fusarium* (Grande-Tovar et al., 2016).

The fact that lower concentrations (sub-lethal supplementation) of essential oils inducing the enzyme production and activities may have a great potential for biotechnological applications and therefore that point must be studied in deep content. Moreover, further experimental works must conducted to understand whether action modes of these essential oils are the same for *in vitro* and *in vivo* conditions.

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