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Bioprospecting of lipolytic microorganisms obtained from industrial effluents

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ABSTRACT

The lipases have ability to catalyze diverse reactions and are important in different biotechnological applications. The aim of this work was to isolate and characterize microorganisms that produce lipases, from different food industry effluents localized in Pelotas, RS/Brazil. Bacteria were identified using Gram stain and biochemical tests (Vitek 2®). Fungi were identified according to macro and micromorphology characteristics. The extracellular lipase production was evaluated using the Rhodamine B test and the enzymatic activity by titration. Twenty-one bacteria were isolated and identified as *Klebsiella pneumoniae* ssp. *pneumoniae, Serratia marcescens, Enterobacter aerogenes, Raoultella ornithinolytica* and *Raoultella planticola*. Were characterized isolated filamentous fungi by the following genera: *Alternaria* sp., *Fusarium* sp., *Geotrichum* sp., *Gliocladium* sp., *Mucor* sp., *Paecilomyces* sp. and *Trichoderma* sp. Extracellular lipase production was observed in 71.43% of the bacteria and 57.14% of the fungi. The bacterium that presented better promising enzymatic activity was *E. aerogenes* (1.54 U/ml) however between fungi there was not significant difference between the four isolates. This study indicated that microorganisms lipase producers are present in the industrial effluents, as well as these enzymes have potential of biodegradation of lipid compounds.

Key words: effluent, enzyme activity, extracellular lipase production, lipase enzyme, lipolytic microorganism.

INTRODUCTION

The lipase enzymes are classified as hydrolases (triacylglycerol hydrolases) that catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, free fatty acids and glycerol (Almeida et al. 2013). Besides the hydrolysis, they can also catalyze esterification and interesterification reactions (acidolysis, alcoholysis and transesterification) and aminolysis (Carvalho et al. 2003, Patil et al. 2011). The large catalytic ability of enzymes allows their use in many biotechnological and industrial applications, such as food and dairy products, detergents, textile, cosmetics, pharmaceutical field, papermaking, bio surfactants and biopolymers synthesis, as well as in wastewater treatment (Sharma et al. 2001, Jaeger

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and Eggert 2002, Mendes et al. 2005, Verma et al. 2012).

The microbial lipases are very important due to their potential use in industrial applications (Anbu et al. 2011). The large applicability in this area is due to certain properties such as stability, selectivity, large substrate specificity and because they do not need cofactors (Castro et al. 2004), beyond different catalytic activities, genetic manipulation facility and the faster growth of microorganisms in low cost culture media (Hasan et al. 2006). Many microorganisms are lipase producers as well as the main part of commercial lipases is from microbial origin (Veerabagu et al. 2013). In this context, the lack of knowledge of this microbial diversity in effluents motivates to interest in the microorganisms bioprospecting, because they may show biotechnological application potential in different areas. In the environmental area, they can be promising in technologies used in biological systems for the environment treatment with the purpose of eliminating pollutants (Peixoto et al. 2008).

The oily effluents can cause environmental pollution, if not treated appropriately, and they cause different operational problems in wastewater treatment systems, resulting in inefficient processes of treatment (Leal et al. 2002, Sugimori et al. 2002, De Oliveira 2014). The biological treatment, which aim is the removal of fat, oils and greases has been efficient, in addition to improve the operational conditions (Mendes et al. 2005, Gopinath et al. 2013, Odeyemi et al. 2013).

Considering the existing environmental microbial diversity and the various microorganisms capable of producing lipases enzymes, it is highlighted the importance of these industrial effluents for biodegradation of lipid compounds. Therefore, the aim of this present study was to isolate and identify microorganisms that produce these enzymes in industrial effluents, just like evaluation of production extracellular of the lipase and capacity of hydrolysis produced by microorganisms.

MATERIALS AND METHODS

Samples Origin and Processing

Effluents samples were collected in three slaughterhouses and in one dairy products industry, localized in the city of Pelotas, state of Rio Grande do Sul, Brazil. The treatment of these effluents in the dairy products industry is realized through the reactor UASB (Upflow Anaerobic Sludge Blanket) and aerated biological reactor. The effluent treatment in the slaughterhouses (1) and (2) is performed by settling tank, and anaerobic and facultative ponds; in slaughterhouse (3), treatment is performed by settling pond, and by aerobic and facultative ponds. The samples collecting was performed using sterile glass bottles that they were carried to the Environmental Microbiology Laboratory in the Biology Institute at the Federal University of Pelotas (UFPEL).

The samples were submitted to the enrichment process, using 10 ml from sample in 50 ml of liquid minimal media ($g.l^{-1}$: 1 g NaCl, 5 g $(NH_4)_2SO_4$, 6.2 g Na_2HPO_4, 0.9 g KH_2PO_4, 0.2 $MgSO_4.7H_2O$; adapted from Sugimori et al. 2002). To culture media was added 1 ml of micronutrients solution (Chernicharo 1997) and 5 ml of olive oil, and then they were incubated at 30°C for 120 hours on a rotary shaker (120 rpm) (Burkert et al. 2004). From the enrichment medium, were taken 100 µl to inoculate dishes containing solid minimum media with an addition of 0.1% of Nistatina fungicide (EMS – 100.000 U.I ml^{-1}) and one emulsion addition (5% of olive oil and 1% of Tween 80). The dishes for fungi growth did not receive addition of fungicide. The inoculated material was spread in dishes and then was incubated at 30°C for 72 to 96 hours (adapted from Sugimori et al. 2002). Subsequently was observed the appearance of the growth of different morphological types of bacterial

and fungal colonies, which were then replicated in new dishes in order to obtain purified colonies.

Microorganism's Identification

The obtained bacteria were characterized using the Gram stain (Silva Filho and Oliveira 2007) and then identified with biochemical tests using automatic system Vitek 2®. The isolated filamentous fungi were visualized as to macromorphology, including texture, topography, color and micromorphology, using lactophenol cotton blue, after incubating them for seven days at 30ºC in microcultive of PDA agar, between slide and coverslip.

Test of Rhodamine B

The isolated microorganisms were evaluated according to extracellular lipolytic enzymes production, using the Rhodamine B test (adapted from Lima et al. 2004, Moura et al. 2013). The extracellular lipases production is observed when liberated fatty acids and glycerol form a fluorescent complex with Rhodamine B, visualized under UV light. Microorganisms were inoculated in solid minimal media, grown in 1% of Rhodamine B solution (0.1% m/v), at pH final of 7.0.

The bacteria inoculation was made by streaking, and the dishes were incubated at 30ºC for 48 hours, while the filamentous fungi were seeded in the center of dishes and incubated at 30ºC for 168 hours. After that, dishes were visualized at dark chamber with ultraviolet light (UV) 365 nm, and were considered as positives the samples that showed halos of fluorescent orange colored around the microbial colonies.

Microbial Lipase Hydrolytic Activity

The lipase enzyme produced by the isolated microorganisms was evaluated for ability to hydrolyze the substrate (olive oil) by titration of fatty acids liberated from the degradation of triacylglycerol (adapted from Burkert et al. 2004).

In the inoculum preparation, bacteria were enriched in 30 ml of BHI medium and cells were recovered by centrifugation using a refrigerated microcentrifuge (washing: 9000 rpm for 15 min at 4°C). The cells were standardized in sterile saline (0. 85%) with an optic density of 1.0 (108 UFC/ml), and fungi were adjusted from 0.15 to 0.17 (0.4 x 10^4 a 5 x 10^4 UFC/ml) in spectrophotometer (540 nm). After the inoculum preparation, 500 µL of the content were inoculated in 30 ml of liquid minimum medium (added of olive oil) and incubated at 30ºC under stirring at 120 rpm. Bacteria were incubated for 24 hours and the fungi during 72 hours. Enzymatic extract of microorganisms was obtained by centrifugation of 10 ml of the incubated material.

For lipolytic enzymes activity evaluation an emulsion of olive oil with gum arabic at 7% (25% of olive oil and 75% of gum arabic) was used. For each isolate, a mixture having 5 ml of emulsion and 2 ml of phosphate buffer (10 mM – pH 7.0) was prepared. To this mixture was added 1 ml of enzymatic extract of each microorganism, and then this mixture was incubated at 37ºC on a rotary shaker (120 rpm), for 30 minutes.

The hydrolyze reaction was discontinued by addition of 10 ml of mixture of acetone and ethanol (1:1 v/v), and fatty acids titrated with a NaOH 0.05 M solution, using phenolphthalein as indicator. The blanks received enzymatic extract after incubation. Next was added a mixture of acetone:ethanol and finally blanks were titrated with NaOH solution.

The enzymatic activity (U/ml) was calculated according to following equation:

$$\text{Activity} = \frac{Va - Vb \times M \times 1000}{t \times Vc}$$

Where: Va corresponds to volume (ml) of NaOH solution used in the sample titration; Vb is the volume (ml) of NaOH solution used in the blank titration; Vc corresponds to volume (ml) of sample used in the reaction; M corresponds to molar

concentration of NaOH solution; t corresponds to the reaction time in minutes; and 1000 is constant dilution rate.

Enzymatic activity unit is defined as enzyme quantity that liberates 1 μmol of fatty acid per minute of reaction. Activities are expressed in U/ml, being 1 U/ml=1 μmoles/ml.min. The samples were evaluated with four repetitions and data was submitted to variance analysis and averages were compared by the Duncan test ($P<0.05$).

RESULTS AND DISCUSSION

Twenty-one bacteria and seven filamentous fungi were selected and then isolated from effluent samples of five industries in the region of Pelotas, RS, Brazil. The bacteria identified through the automatic system Vitek 2®, belong to five different species. Meanwhile the filamentous fungi were evaluated for macro and micromorphology and characterized in seven genera.

Among the twenty-one bacteria, 13 (61.90%) were identified as the following: *Klebsiella pneumoniae* ssp. *pneumoniae*. The other bacteria were characterized as the following: *Serratia marcescens* (two isolates), *Enterobacter aerogenes* (two isolates), *Raoultella ornithinolytica* (two isolates) and *Raoultella planticola* (two isolates). The identification of isolated bacteria from effluents and the production of extracellular lipases enzymes evaluated by Rhodamine B test are presented in Table I.

The isolation of these different species in industrial effluents indicates that diverse microorganisms with potential of enzyme lipase production can survive in the physical-chemical and environmental conditions of these effluents. Studies have been performed in order to improve the production of this enzyme by microorganisms growth in the most diverse culture media and in different environment conditions, in addition to the isolation of these microorganisms from diverse places.

Odeyemi et al. (2013) identified 32 lipolytic bacteria, among them *Klebsiella* and *Serratia*, isolated from wastewater of a restaurant. According to the authors, the bacteria isolated from effluents enriched with foodborne oil feature a new resource to obtain environmental enzymes, and with possibilities of commercial and environmental applications in domestic environment. Lin et al. (2012) also identified many strains of *Klebsiella* spp., besides four strains of *Enterobacter* spp., between others species, from fifty-four lipase producer bacteria. These bacteria were isolated from 198 samples obtained from different places (ground, wastewater and polluted water).

The species *R. planticola*, isolated from effluent in our study, was also isolated from ground by Sugimori et al. (2013), showing potential lipase production. The bacterium was efficient for treatment of the effluent at the restaurant, consuming 70% of lipids, in 48 hours. Many bacterial species with lipases enzyme production ability were characterized, including the ones that were isolated in our study (Sharma et al. 2001, Tebaldi et al. 2008, Messias et al. 2011, Ivanova et al. 2013).

The isolation of new microorganisms with enzyme potential production is very important because they present a wide applicability and are efficient for bioremediation processes of contaminated places and due to its efficient treatment in effluent with a high content of oils and fats (Gopinath et al. 2013).

Data in Table I showed that 71.43% of isolated bacteria presented extracellular lipase production, evidenced by fluorescent orange halo formation around colonies, demonstrating promising results for further applications. The extracellular enzyme production is a positive factor when there is an intention to explore biotechnologically microorganisms. In this way, the importance of

TABLE I
Origin, identification and extracellular lipase production from bacteria obtained from effluents of dairy products and slaughterhouses industries in Pelotas/RS, Brazil.

ID	Origin	Bacterial species	Extracellular Lipase
11	DP	*Klebsiella pneumoniae* ssp. *pneumoniae*	+
13	DP	*K. pneumoniae* ssp. *pneumoniae*	+
14	DP	*K. pneumoniae* ssp. *pneumoniae*	-
15	DP	*K. pneumoniae* ssp. *pneumoniae*	-
16	DP	*K. pneumoniae* ssp. *pneumoniae*	+
17	DP	*K. pneumoniae* ssp. *pneumoniae*	-
18	DP	*K. pneumoniae* ssp. *pneumoniae*	+
19	DP	*K. pneumoniae* ssp. *pneumoniae*	-
20	DP	*K. pneumoniae* ssp. *pneumoniae*	+
25	SH1	*K. pneumoniae* ssp. *pneumoniae*	-
27	DP	*K. pneumoniae* ssp. *pneumoniae*	-
29	DP	*K. pneumoniae* ssp. *pneumoniae*	+
30	DP	*K. pneumoniae* ssp. *pneumoniae*	+
10	DP	*Serratia marcescens*	+
12	DP	*S. marcescens*	+
21	DP	*Enterobacter aerogenes*	+
23	DP	*E. aerogenes*	+
22	DP	*Raoultella ornithinolytica*	+
24	SH1	*R. ornithinolytica*	+
26	SH1	*Raoultella planticola*	+
28	DP	*R. planticola*	+

DP: dairy products industry, SH: slaughterhouse, +: positive reaction, - : negative reaction.

lipolytic production in an extracellular form is due to facility to obtain enzymes and lower cost for isolation (Saxena et al. 2003, Martins et al. 2008).

Several factors can influence the extracellular microbial production, mainly because of carbon source availability, physical-chemical factors (temperature, pH and oxygen), agitation during growth process of microorganisms in culture medium and incubation time (Colla et al. 2012, Kumar et al. 2012, Veerapagu et al. 2013). Enzymatic production in extracellular way consists in a mechanism of adaptation to extreme conditions of environment, as well as favoring its trophic niche. Therefore, the ability of microorganisms of produce extracellular enzymes is of great importance for their survival (Gopinath et al. 2005).

Concerning quantification of enzymatic activity was observed significant variations between bacterial species, from the averages resulting of the titration, analyzed by Duncan test (Figure 1). The bacteria presented negative results for extracellular lipase production (Rhodamine B test) were not evaluated for enzymatic activity.

As from results obtained in the evaluation of the triacylglycerols hydrolysis ability, was confirmed that between the 15 evaluated bacteria,

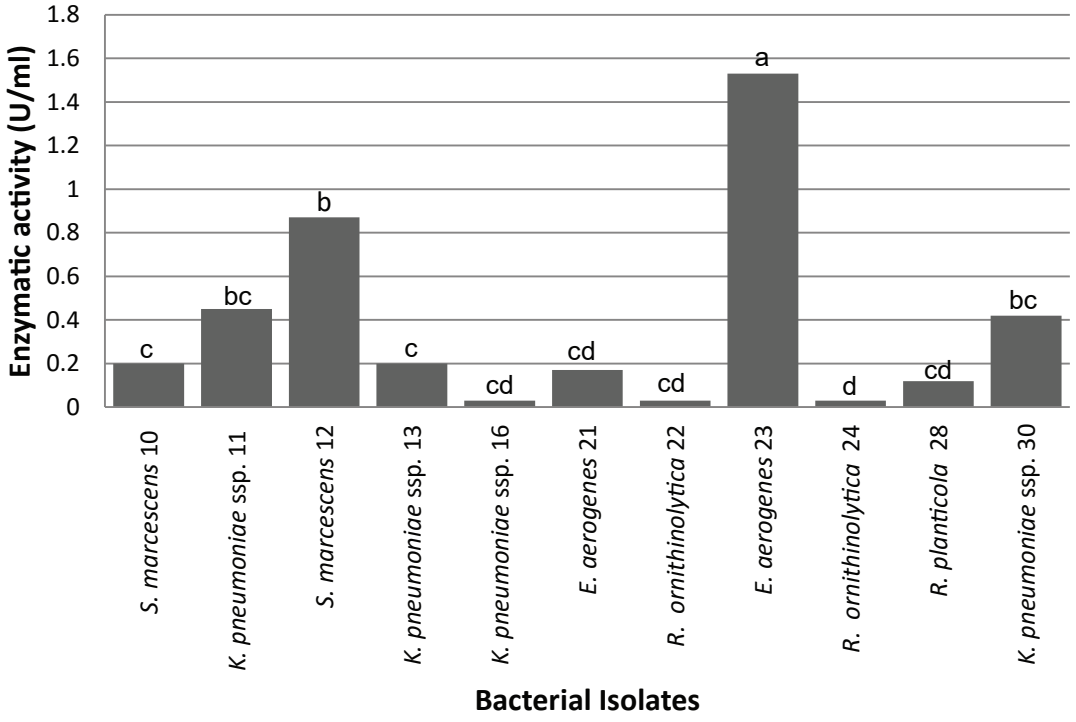

Figure 1 - Extracellular lipase enzymatic activity of bacteria obtained from effluents of dairy products and slaughterhouses industries located in Pelotas/RS, Brazil.

*[a,b,c,d] Different letters indicate statistically significant difference according to the Duncan test ($P<0.05$).

E. aerogenes (23) presented the highest efficiency, indicating a higher quantity of free fatty acids (1.54 U/ml) in the medium. However, the bacterial isolates 11, 12 and 30, did not present significant differences between them, as well as the isolates 10, 13, 16, 21, 22 and 28.

The hydrolysis evaluation of olive oil using the enzymatic extract from the isolates 18, 20, 26 and 29 did not present a significant result, and was observed negative values for the lipase activity. This result indicates that the reaction of olive oil hydrolysis did not occur or was not efficient during the incubation time.

There are diverse substances with enzymatic inhibition potential that can associate to the active site or another site of enzyme, resulting in a speed decrease or paralyzing the reaction. Temperature can influence enzymatic reaction speed because high temperatures can result in enzyme denaturalization (Champe et al. 2006). In this experiment, it is considered that the temperature was not unsatisfactory once other microorganisms presented positive and satisfactory values. Lipases enzymes also can catalyze various reactions, like as the esterification, which is an inverse reaction to hydrolysis. Therefore, during incubation time another reaction could be catalyzed, resulting in reduction of free fatty acids in reaction medium.

Zaki and Saeed (2012) evaluated the enzymatic activity of 12 strains of *Serratia marcescens*. The lipase originated by the strain that presented best enzymatic activity was assessed for degradation of triacylglycerol in various vegetable fats (soy, coconut, sesame, olive and sunflower). The analysis in spectrophotometer showed an enzymatic activity of 112 U/ml, when olive oil was used as substrate.

However, a higher activity was observed when was used sesame oil (122 U/ml). In this present study, we observed an inferior activity in both *S. marcescens* strains that can be related to different variables. The enzyme used by Zaki and Saeed was extracted and purified, and the evaluation of enzymatic activity was realized by spectrophotometry. However, in our study was used enzymatic extract produced by *S. marcescens* and determination of activity was realized by titration of free fatty acids in reaction medium.

Kumari et al. (2009) observed lipolytic activity in *Enterobacter aerogenes* (27.25 U/ml) in culture medium containing 3% of coconut oil, incubated for 60 hours. The highest enzymatic activity, when compared to results of our study, can be related to type of carbon source, seeing that we used olive oil. The incubation time can also be increase lipase production, which in our study was of 24 hours. In addition, another factor whether intrinsic or environmental, are important in enzymatic production.

According to Immanuel et al. (2008), higher activity of enzymes is related with triglycerides, which are important substrates in lipase enzyme production and may present function of inducing or inhibiting. Studies show that substrates like the olive oil increase the lipase production, presenting inductive function in enzymatic production (Rajendran et al. 2007, Tavares et al. 2011).

Others factors interfere in enzymatic production are medium conditions like temperature, pH and agitation (Willerding et al. 2011, Thakur et al. 2014). In this present study, once the bacteria have been isolated from industrial effluents, we opted to define typical conditions of environmental microorganisms, and so the incubation temperature for isolation and production of enzyme was 30ºC.

The isolate filamentous fungi from effluents were identified as the following genera: *Alternaria* sp., *Fusarium* sp., *Geotrichum* sp., *Gliocladium* sp., *Mucor* sp., *Paecilomyces* sp., and *Trichoderma* sp.. Extracellular lipase production was observed in 57.14% of isolated fungi, presenting positive results in the Rhodamine B test the following fungi: *Fusarium* sp., *Geotrichum* sp., *Gliocladium* sp. and *Mucor* sp. Table II presents extracellular lipolytic production by the filamentous fungi.

The production of lipases enzymes by fungal microorganisms is reported in literature, including the isolated fungi in this study (Gunasekaran and Das 2005, Nagy et al. 2006, Fernandes et al. 2012, Gopinath et al. 2013).

TABLE II
Lipase enzymatic activity of filamentous fungi obtained from effluents of dairy products and slaughterhouses industries located in Pelotas/RS, Brazil.

Origin	Fungal Species	Extracellular Lipase
SH3	*Alternaria* sp.	-
SH2	*Fusarium* sp.	+
SH3	*Geotrichum* sp.	+
DP	*Gliocladium* sp.	+
SH3	*Mucor* sp.	+
SH3	*Paecilomyces* sp.	-
SH3	*Trichoderma* sp.	-

DP: dairy products industry, SH: slaughterhouse, +: positive reaction, - : negative reaction.

Mukunda et al. (2012) isolated more than 200 fungi from ground and evaluated the ability of production of various hydrolytic enzymes, including lipases. The fungi genera *Trichoderma*, *Fusarium*, *Pacillomyces*, *Gliocladium*, *Alternaria* and *Mucor* did not present extracellular lipase production, seeing that they did not present the transparent halo around the colonies, using Tween as the substrate. In our study, the species *Fusarium* sp., *Gliocladium* sp. and *Mucor* sp. produced extracellular lipase, which is an important factor for further studies and applications in treatment systems.

Concerning lipase enzyme activity in the olive oil degradation, the fungi did not present significate differences according to the Duncan test. Figure 2 shows the enzymatic activity of fungi that presented positive values for the Rhodamine B test.

Nwuche and Ogbonna (2011) isolated 12 fungi from the industry effluent of palm oil, including the genera *Aspergillus, Penicillium, Trichoderma* and *Mucor*. Using similar methodology to our study, *Mucor* sp. presented enzymatic activity of 5.72 U/ml, in culture medium containing olive oil. This higher activity can be due to longer incubation time (72 hours) or being in different species, when compared to our study. Through the spectrophotometry, the activity for *Mucor griseocyanus* lipolytic varied between 0.04 to 0.1 U/ml, using different substrates such as olive oil, coconut oil, sunflower oil, among others (Armas et al. 2008). Another specie, *Mucor geophillus,* presented maximum activity of 44.56 U/ml by the titration method, having as substrate the olive oil (Naqvi et al. 2011). Incubation time (72 hours) was the same for both species.

Burkert et al. (2004), with *Geotrichum* sp., obtained enzymatic activity of 17 U/ml, after 50 hours of incubation, meanwhile Carvalho et al. (2005), observed 12.8 U/ml, with *Geotrichum candidum*, after 72 hours. Both studies used olive oil as carbon source and quantified the fatty acids by titration. The superior enzymatic activity of the species in these studies may be because the species have isolated in this study are different, as well as the incubation time of our work was only 48 hours.

According to Odeyemi et al. (2013), the biologic oily effluents treatment, had been the most efficient, with degradation of lipids compounds in miscible molecules. According to Gopinath et al. (2013), domestic and industrial residuals may have

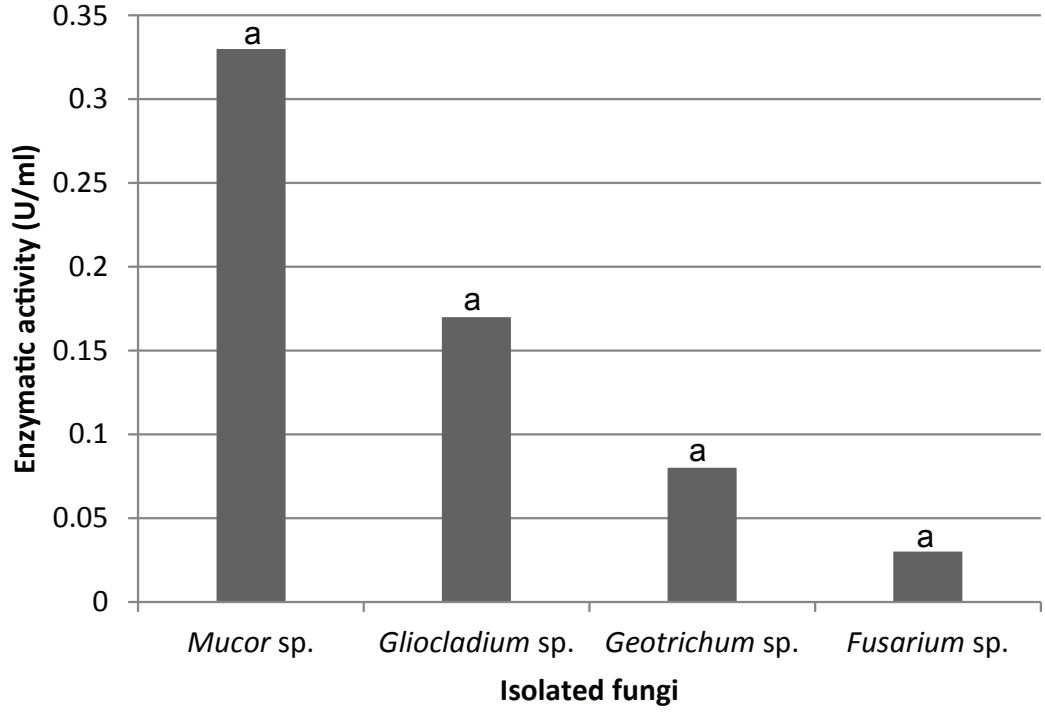

Figure 2 - Lipase enzymatic activity of filamentous fungi obtained from effluents of dairy products and slaughterhouses industries located in Pelotas/RS, Brazil.
*[a] Letter 'a' indicates no statistically significant difference according to the Duncan test ($P<0.05$).

fungi with higher potential degradation of fatty and oils, eliminating wastes and can produce beneficent substances due to its activity.

Metabolism of microorganisms shows the ability of waste elimination and the production of useful substances. Therefore, biodegradation consists in an important process to minimize the environment oil pollution, reducing the environmental impacts (Gopinath et al. 2013). In this way, diverse microorganisms may be efficient in the treatment of contaminated areas through their biotechnological applications.

CONCLUSIONS

This study demonstrated that there are microorganisms that produce lipases enzymes in industrial effluents, with ability of extracellular lipase production, a positive factor to obtaining enzymes and their use in future applications. The produced lipases presented biodegradation of lipid compound, being effective in effluents from treatment systems in various sectors.

RESUMO

As lipases têm a capacidade de catalisar diversas reações e são importantes em diferentes aplicações biotecnológicas. Este trabalho teve como objetivo isolar e caracterizar microrganismos produtores de lipases, provenientes de diferentes efluentes de indústrias alimentícias da região de Pelotas, RS. As bactérias foram identificadas quanto à coloração de Gram e a provas bioquímicas (Vitek 2®). Os fungos foram identificados quanto às características de macro e micromorfologia. A produção de lipase extracelular foi avaliada pelo teste rodamina B e a atividade enzimática, por titulação. Foram isoladas 21 bactérias, identificadas como *Klebsiella pneumoniae* ssp. *pneumoniae, Serratia marcescens, Enterobacter aerogenes, Raoultella ornithinolytica* e *Raoultella planticola.* Os fungos filamentosos isolados foram caracterizados aos gêneros *Alternaria* sp., *Fusarium* sp., *Geotrichum* sp., *Gliocladium* sp., *Mucor* sp., *Paecilomyces* sp. e *Trichoderma* sp.. A produção de lipase extracelular foi observada em 71,43% das bactérias e 57,14% dos fungos. A bactéria que apresentou atividade enzimática mais promissora foi a *E. aerogenes* (1,54 U/ml), entretanto, entre os fungos, não houve diferença significativa entre os quatro isolados. O estudo revela que assim como microrganismos produtores de lipases estão presentes em efluentes industriais, estas enzimas apresentam potencial de biodegradação de compostos lipídicos.

Palavras-chave: efluente, atividade enzimática, produção de lipase extracelular, enzima lipase, microrganismo lipolítico.

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