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Detection of extracellular enzymatic activity in microorganisms isolated from waste vegetable oil contaminated soil using plate methodologies

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In the present study, from a total of 100 strains isolated from waste vegetable oil contaminated soil, 38 bacterial and 14 fungi strains that presented positive lipolytic activity were obtained by detection through Rhodamine B Agar 0.02% w/v as a screening method. Additionally, two other enzymatic activities were determined. Positive proteolytic activity was evaluated in Casein Hydrolysis Agar and chitinolytic activity was identified by change in coloration in Bromocresol Purple Agar. Using these methodologies, we were able to report 18 microorganisms with two enzymatic activities and 6 microorganisms with all three enzymatic activities, thereby establishing these techniques as suitable and fast approaches for detection and semi-quantification of extracellular enzymatic activity.

Key words: Enzyme, lipases, proteases, chitinases, rhodamine B, soil.

INTRODUCTION

The enzymatic activity of a microorganism is in many cases influenced by the environment. The evaluation at the simplest level with the diversity analysis of present enzymes and ratios between and within major elements like C, N and P provides an insight into the microbial community response to changing nutrient resources (Caldwell, 2005).

The isolation from an oil contaminated soil could give an indication of the metabolic capacity of the microorganism's adaptation to these conditions. Among the enzymes commonly identified, three enzymes, lipases, proteases and chitinases are of high importance. Lipases, belonging to the family of hydrolases, are capable of catalyzing diverse reactions like, alcoholysis, hydrolysis, esterification and transesterification (Hasan et al., 2010). Lipases have been isolated and purified from fungus, yeasts, bacteria, plants and animals (Fuji et al., 1986; Pahoja and Sethar, 2012) and for their characteristics, they are used in food and textile industry, for biodegradable polymers synthesis and biodiesel production, among others (Fuji et al., 1986; Falch, 1991; Snellman et al., 2002; Noureddini et al., 2005; Hasan et al., 2010; Sangeetha et al., 2011).

As well, proteases are thoroughly distributed in nature

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and have microbes as their main source. As one of the main industrial enzymes, they are primarily used in detergents for their remotion capacity of protein based textile stains, with additional applications in leather, food, pharmaceutical industry and bioremediation processes (Anwar and Sleemuddin, 1997; Vijayalakshmi et al., 2011; Banerjee et al., 1999; Vishwanatha et al., 2010).

Finally, chitinolytic activity was determined considering that chitinases are found in bacteria, fungus, insects, plants and animals (Chernin et al., 1997; Gooday, 1990) and have a wide range of biotechnological applications, especially in chitin oligosaccharides and N-acetyl-D-glucosamine production (Pichyangkura et al., 2002) as well as bioconversion of chitin wastes to unicellular proteins (Vyas and Deshpande, 1991).

The aim of this study was to determine enzymatic activities of three of the most important enzymes found on bacterial and fungal strains using fast and reliable plate methodologies that allowed to process, identify and provide semi-quantification of extracellular enzymatic activity.

MATERIALS AND METHODS

Sampling

The samples were taken from waste cooking oil contaminated soil using a clean spatula at 5 cm of depth following a 10 km straight line with sampling every 2 km. The samples were placed in plastic bags, then transported to the laboratory and kept at room temperature.

Isolation and conservation

The samples were processed through serial dilution in 0.85% sterile saline solution, taking 1 g of the collected soil from each location. Nutritive Agar (NA) was used for bacterial isolation, sterilized and poured in Petri dishes to solidify. Single streak inoculation was performed with incubation at 37°C for 120 h. Potato dextrose agar (PDA) was used for fungi isolation and prepared by dissolving 39 g in 1000 ml of distilled water. Additionally, PDA with chlorampenicol at 1% (PDAC) was prepared and poured in Petri dishes to solidify. The incubation was performed for 21 days at 25°C. In addition submerged fermentations were prepared with nutritive broth supplemented at 1% with vegetable oils, including olive (OLI) (Cárdenas et al., 2001), canola (CAN) and waste vegetable oil (WVO), respectively. These remained in agitation for 96 h at 25°C and 150 rpm for latter inoculation through serial dilution in PDA, NA and PDAC. All media were sterilized in autoclave at 121°C for 15 min. Once morphological characteristics were determined, the pure strains were conserved in 20% glycerol at -20°C.

Sample and assay preparation

After a pre-culture of 24 h, all the bacterial suspensions (0.85%) saline were adjusted to 1 in the McFarland scale and centrifuged at 10,000 rpm, 4°C for 20 min and the supernatant was recovered (Rajeswari et al., 2011; Vijayalakshmi et al., 2011). Primary lipolytic activity selection of the bacterial strains was made by fluorescence intensity through simple streak in plates with Rhodamine B Agar. Semiquantification of lipases, protease and chitinase potential was determined using bacterial solutions adjusted to 1 on the McFarland scale, inoculating 10 µl of supernatant in 3 mm holes in each of the selective mediums and incubated for 48 h. For fungal strains, 1 cm sterile paper circles were placed on the center of Petri dishes adding 2 µl of fungal solution adjusted to 1 x 10^{-6} conidias/ml and incubated at room temperature for 144 h.

Lipolytic activity

Due to the fact that the soil from where the samples were taken was contaminated with waste vegetable oil, the first screening applied to the microorganisms isolated was the determination of lipolytic activity. Base media contained 1.25 g of yeast extract, 4.5 g of nutritive broth and 10 g of bacteriological agar in 450 ml of distilled water. The lipoidal emulsion was prepared with 200 ul of Tween 80, 30 ml of olive oil and 50 ml of water adjusted to pH 7. Both were sterilized at 121°C for 15 min separately. The dye was prepared aseptically adding 50 mg of Rhodamine B to 50 ml of sterile water and adding 20 ml of dye solution to lipoidal emulsion and mixing vigorously. For a final volume of 500 ml of Rhodamine B Agar at 0.02% w/v, 50 ml of dye and lipoidal emulsion were added to base media (Alken-Murray). All samples were analyzed under UV light at 350 nm. Together with the presence of fluorescence for identification of positive lipolytic activity, a selection criteria of fluorescence intensity for bacterial strains was followed (Rabbani et al., 2013; Carissimi et al., 2007) with 3 categories (1, 2 or 3), selecting those that coincide with category 3 from a mean of 6 repetitions.

Proteolytic activity

Casein hydrolysis agar containing 1 g of KH_2PO_4, 0.5 g of KCl, 0.2 g of $MgSO_4.7H_2O$, 0.1 g of $CaCl_2.2H_2O$, 25 ml with 15% of powdered skim milk, 10 g of glucose and 12 g of agar in 1 L of distilled H_2O was prepared. The milk powdered preparation was done by adding 3.75 g of powdered skim milk to 25 ml of distilled water mixed to homogenize in a creamy texture before adding it to the media; once added, pH was adjusted to 5.4 and sterilized at 121°C for 10 min (Mata Villegas, 2008).

Chitinolytic activity

Bromocresol purple agar was prepared containing 4.5 g of colloidal chitin, 0.3 g of $MgSO_4 \cdot 7H_2O$, 3 g of $(NH_4)SO_4$, 2 g of KH_2PO_4, 1 g of monohydrate citric acid, 15 g of agar, 0.15 g of Bromocresol Purple and 200 ul of Tween 80, pH was adjusted to 4.7 and sterilized at standard conditions (Lunge and Patil, 2012).

Enzymatic rate determination

For proteases and chitinases found in fungi, an enzymatic rate determination was used, with the formula:

$$\text{Enzymatic Activity index} = \frac{\text{Colony diameter + halo diameter}}{\text{Colony diameter}}$$

Statistical analysis

The obtained results were analyzed using analysis of variance (One-way ANOVA) with statistical significance of $p<0.05$, followed by a post hoc Tukey test.

Table 1A. Effect of the diverse inductors on the isolation of positive bacterial strains for lipolytic activity.

Source	Bacterial strains	Lipases	Positive strains (%)
Direct Isolation	26	12	46.2
Induction with OLI	21	12	57.1
Induction with WVO	16	10	62.5
Induction with CAN	11	4	36.4
Total	74	38	51.4

Table 1B. Additional enzymatic activities determined for bacterial strains.

Source	Chitinases	Positive strains[1] (%)	Proteases	Positive strains[1] (%)
Direct isolation	0	0.0	2	7.7
Induction with OLI	2	9.5	3	14.3
Induction with WVO	3	18.8	0	0.0
Induction with CAN	0	0.0	2	18.2
Total	5	6.8	7	9.5

[1]= Percentage calculated from the total of isolated strains.

RESULTS AND DISCUSSION

Initially, 74 Gram negative bacterial strains were obtained from which, 38 were positive for lipase production by fluorescence observation under UV light at 350 nm (Table 1A) and it was observed that the best inductor for lipases was WVO with 62.5% of positives strains.

To this primary screening, chitinases and proteases identification was added (Table 1B), obtaining 5 positive strains by observation of a coloration change from yellow to purple and 7 positive strains through hydrolysis halo observation, respectively (Figure 1).

For fungi, 26 strains were obtained from which, 14 presented lipolytic activity and where induction with olive oil (Table 2A) was the most succesfull with 100% of the strains positive. Furthermore, 15 strains presented chitinolytic activity (Table 2B) shown by coloration change and 11 strains presented proteolytic activity shown by hydrolysis halo (Figure 2).

Lipases

Different techniques have been used for lipase detection, including those with Tween 80 and tributyrin as substrate in solid media (Cárdenas et al., 2001; Sierra, 1957); these involve the development of clear areas around the colonies as an enzymatic activity indicator. However, tributyrin shows activity for esterases as well as lipases (Kim et al., 2001), which makes it a non-specific method (Kumar et al., 2012).

Another technique, using a chromogenic substrate Rhodamine B, has an action mechanism where the union with fatty acids and mono- and diglycerids has proved to be a fast and specific method for analysis which is insensitive to pH changes (Kouker and Jaeger, 1987; Hou and Johnston, 1992; Willerding et al., 2011; Rabbani et al., 2013), reason why it was chosen for the lipolytic activity detection of the worked samples. Following the procedure of fluorescence intensity, only the strains with category 3 were selected to proceed to inoculation of the bacterial supernatant in Rhodamine B agar Petri dishes in order to semi-quantify extracellular lipase activity.

The results were reviewed after 48 h of incubation, time reported to allow better contact between substrate and strains (Feng et al., 2010; Boonmahome, 2013) and shown in Table 3, in which strain 12 presented the highest enzymatic activity with a halo diameter of 10.7 mm followed by strain 38 with 10.6 mm, both considered as good producers according to the criteria established by Hou and Johnston (1992). From the results of the statistical analysis ($p<0.05$), strains 12, 38, 35, 30 and 31 were selected for further study and given that from this analysis the selected strains were part of the same group, from spectrophotometric quantification, we were able to discern and stablish significant difference between them (data not shown).

For the determination of fungal strains with lipolytic activity (Figure 3) fluorescence intensity was not included because based on the visual analysis performed, differences within strains were not detected. We focused on the ability of the fungi to grow in a solid media that contained olive oil (Savitha et al., 2007). They were observed at 144 h of incubation, obtaining the results shown in Table 3. This technique was considered useful as reference, emphasizing that the growth of the fungi in

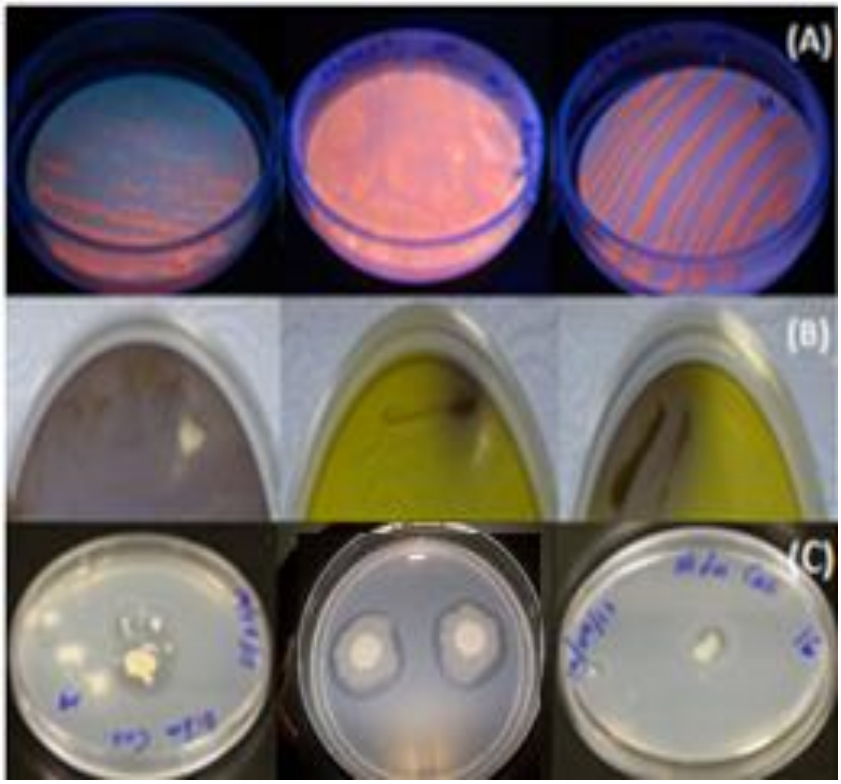

Figure 1. A) Positive bacterial strains for lipolytic activity observed under UV light at 350 nm. B) Positive bacterial strains with chitinolytic activity in bromocresol purple agar. C) Positive bacterial strains with proteolytic activity in casein hydrolysis agar.

Table 2A. Effect of the diverse inductors on the isolation of positive fungal strains for lipolytic activity.

Source	Fungal strains	Lipases	Positive strains (%)
Direct isolation	17	6	35.3
Induction with OLI	3	3	100.0
Induction with WVO	0	0	0.0
Induction with CAN	6	5	83.3
Total	26	14	53.8

Table 2B. Additional enzymatic activities determined for fungal strains.

Source	Chitinases	Positive strains[1] (%)	Proteases	Positive strains[1] (%)
Direct Isolation	7	35.5	7	41.2
Induction with OLI	3	100.0	1	33.3
Induction with WVO	0	0.0	0	0.0
Induction with CAN	5	83.3	3	50.0
Total	15	57.7	11	42.3

[1]= Percentage calculated from the total of isolated strains

Rhodamine B helps to determine which strains had a higher development using olive oil as a substrate (Rifaat et al., 2010; Rajeswari et al., 2011) and to categorize in a semiquantitative way, the strains that presented a higher

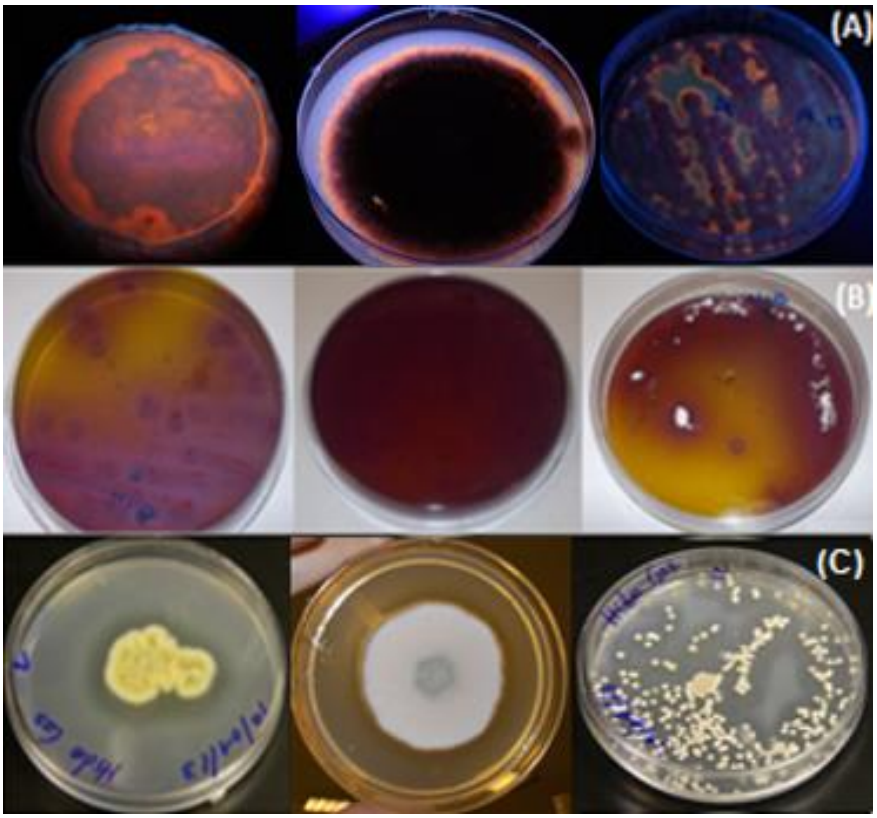

Figure 2. Positive fungal strains for: A) lipolytic activity; B) chitinolytic activity; C) proteolytic activity.

Table 3. Halo size for lipolytic activity.

A. Halo size for lipolytic activity of bacterial strains									
Strain	2^{ab}	5^{bc}	11^{a}	12^{c}	30^{abc}	31^{abc}	35^{abc}	36^{ab}	38^{c}
Halo (mm)	3.8	8.8	2.0	10.7	7.5	6.8	4.6	4.0	10.6

B. Halo size for lipolytic activity of fungal strains											
Strain	1^{ab}	2^{ab}	3^{ab}	4^{ab}	11^{ab}	18^{ab}	19^{ab}	22^{a}	23^{ab}	24^{b}	26^{a}
Halo (mm)	30.7	28.7	24.3	25.0	39.3	40.5	30.2	32.0	39.4	38.7	31.0

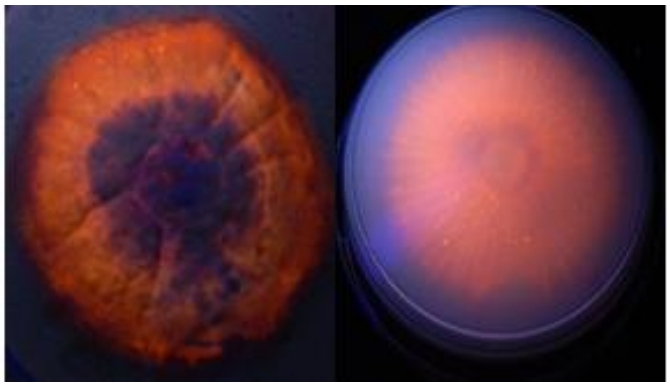

Figure 3. Fungal strains with lipolytic activity.

lipolytic potential in the incubation time stablished. Based on this, strain 18 had the highest growth with a 40.5 mm of maximum halo.

Proteases

The results obtained for bacterial proteases (Table 4) showed strain 18 as the main producer with a maximum hydrolysis halo of 25.0 mm after 48 h of incubation. This showed a similar activity when compared with the highest reported by Sánchez et al. (2004), differing in time of incubation, an important role as some strains require more time (up to 96 h) to be able to show their highest

Table 4. Halo size for proteolytic activity.

Strain	3[c]	8[a]	14[c]	15c	18[d]	22b[c]	24[ab]
Halo (mm)	17.5	8.3	19.0	17.8	25.0	15.0	6.7

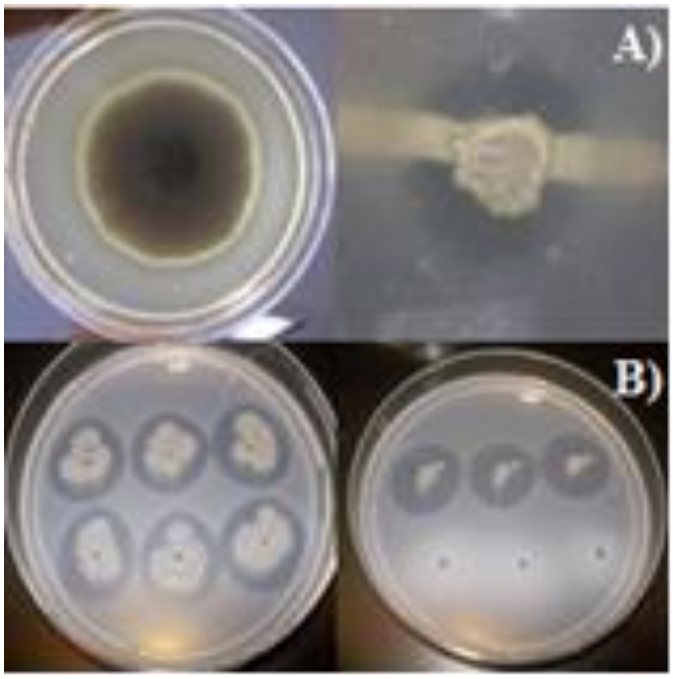

Figure 4. A) Strains with hydrolysis halos for fungal proteolytic activity. B) Strains with hydrolysis halo for bacterial proteolytic activity.

Table 5. Halo size for proteolytic activity of fungal strains proteolytic and proteolytic enzymatic rate.

Strain	1[ab]	2[a]	3[a]	4[a]	5[c]	6[ab]	10[bc]	18[a]	24[a]	25[ab]	26[d]
Halo (mm)	2.0	1.2	1.0	1.0	6.7	3.0	5.2	1.5	1.0	2.3	13.3
P.E.R.[1]	2.06	2.04	2.03	2.03	2.31	2.14	2.18	2.03	2.03	2.06	2.75

[1] P.E.R. = Protease enzymatic rate.

potential (Sánchez et al., 2004; Rodas Junco et al., 2009). Proteases, a group of enzymes whose function is peptide chain hydrolysis of proteins to polypeptide or free amino acids (Alnahdi, 2012), were tested in accordance with their function in skimmed milk agar which contains casein, (Santhi, 2014). This approach allowed us to observe the breaking of links resulting from the enzimatic activity as a halo around the colony (Figure 4).

Within fungal strains that showed protease activity (Table 5), it can be observed that strain 26 presented the highest activity with an enzymatic rate of 2.75 equivalent to 13.3 mm. Comparing the activity obtained from actinomycetes and microorganisms isolated from tropical soil, a superior behaviour of our isolated strains can be observed (Rodas Junco et al., 2009), which indicates a wide variety of protease producing microorganisms. When contrasting our results with those obtained by several authors, it can be observed that the difference in locations from which the samples were gotten is very wide and therefore the microorganisms present diverse adaptations to these particular environments giving an insight into the plasticity of this enzymes. It is also noticiable that considering the differences in the percentage of skim milk used in this assays and therefore

Table 6. Halo size for bacterial chitinolytic activity.

Strain	20[ab]	22[a]	30[a]	31[bc]	34[c]
Halo (mm)	19.0	15.0	12.0	50.0	50.0

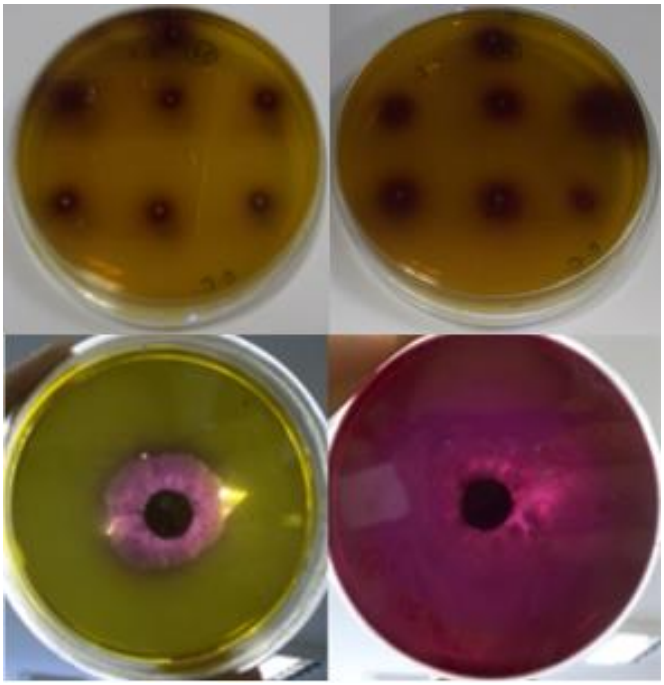

Figure 5. A) Bacterial strains with chitinolytic activity. B) Fungal strains with chitinolytic activity.

the amount of casein added to the media, ranging from 1 to 15%, it can be observed that even at 1%, this is a suitable and sensible assay that requires a minimmun amount of substrate added to the media (Kamat et al., 2008; Ahmad et al., 2014).

Chitinases

For bacterial chitinases (Table 6), strain 31 and strain 34 showed chitinolytic potential according to the criteria of El-Tarabily et al. (2000) and Rodas Junco et al. (2009) with halos up to 50 mm in 48 h of incubation (Figure 5) which also correspondent to reports made by Kuddus and Ahmad (2013).

Chitinases are found in an extensive variety of organisms in which fullfill different functions, specifically in fungi where they have autolytic, nutritional and morphogenetic roles (Patil et al., 2000). To determine the activity of chitinolytic enzymes, one of the most common procedures consist of visualization of the breake of chitine to N-acetiglucosamine. In this study, bromocresol purple in a media supplied with colloidal chitine was used

Table 7. Halo size for chitinolytic activity of fungal strains and chitinolytic enzymatic rate.

Strain	Halo (mm)	C.E.R.
1[ab]	8.7	2.39
2[a]	7.3	2.30
3[ab]	7.0	2.50
5[a]	6.7	2.22
6[a]	4.0	2.19
10[ab]	9.2	2.49
11[a]	3.8	2.24
18[a]	6.8	2.21
19[a]	1.5	2.14
22[b]	15.3	3.65
23[a]	5.0	2.17
24[a]	5.0	2.16
25[a]	4.7	2.16
26[a]	5.6	2.29

[1]C.E.R= Chitinolytic enzymatic rate.

through which the change in pH of acid to basic facilitates the detection with coloration turning from yellow to purple (Agrawal and Kotasthane, 2012; Lunge and Patil, 2012). Given that chitinases have different roles in fungi and bacteria, they serve nutrition and diverse functions such as parasitism (Patil et al., 2000; Ahmad et al., 2014); it was expected, for the nature of the sample, to find this enzymes.

The results obtained for fungal chitinases showed strain 22 with high production based on enzymatic rate and halo size (Table 7) according to the results obtained by Agrawal and Kotasthane (2012) that classified different groups (1 = no chitinase activity; 2 = low chitinase activity; 3 = medium chitinase activity and 4 = high chitinase activity).

Conclusion

From a total of 100 strains isolated from waste vegetable oil contaminated soil, 52 positive isolates for lipolytic, proteolytic or chitinolytic activity were found which were categorized based on their capacity to grow in solid media supplmented with sutiable substrates. With this results, these useful and time-saving techniques are highlighted for detection and categorization of enzymatic activities, reporting 18 microorganisms that present at least two enzymatic activities and 6 with all (three) enzymatic activities.

Conflict of interests

The authors declare that there is no conflict of interest.

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