Screening of Lipases Producing Potential by *Aspergillus welwitschiae* Strains

Seleção do potencial de produção de lipases por linhagens de *Aspergillus welwitschiae*

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RESUMO  
A produção de lipases por *Aspergillus welwitschiae* apresenta vantagens quanto ao custo, rapidez do processo e segurança da linhagem utilizada. Aliado à utilização de uma linhagem potencial estão os fatores abióticos, que proporcionam boa produção de lipases. Neste estudo, 24 linhagens de *A. welwitschiae* foram selecionadas quanto à atividade lipolítica com Tween-20 como substrato. Todas as linhagens de *A. welwitschiae* foram produtoras de lipases. As linhagens UEL As 2.14 e UEL As 15.262 se destacaram quanto a produção de enzimas, pois desenvolveram o maior halo lipolítico. Ambas as linhagens foram selecionadas para caracterizar a melhor produção de enzimas lipolíticas em relação à temperatura e pH. A linhagem UEL As 2.14 apresentou maior produção de lipases a 42 °C, pH 7.0, enquanto que UEL As 15.262 a maior produção de lipases ocorreu a 40 °C, pH 9.0. Utilizando as melhores condições para ambas as linhagens, e meio líquido com Tween-20, foi analisado a produção de lipases por 24h, 48h e 72h. A maior produção de lipases foi obtida em 24 h para ambas as linhagens. UEL As 2.14 e UEL As 15.262 são boas candidatas para aplicações na indústria de alimentos, destacando a linhagem UEL As 15.262 cujas lipases são produzidas sob condições alcalinas.

Palavras-chave: lipases, *Aspergillus welwitschiae*, seleção, Tween-20, temperatura, pH.

ABSTRACT  
The lipases production for *Aspergillus welwitschiae* conferring advantages in terms of cost, speed of the process and safety of the strain used. Allied to the use of a potential strains are abiotic factors, provide good lipase production. In this study, 24 *A. welwitschiae* strains were screened for lipolytic activity towards Tween-20. All *A. welwitschiae* strains were producers of lipases. The UEL As 2.14 and UEL As 15.262 strains were outstanding in their enzyme production as they developed the largest lipolytic halo. Both strains were selected for to characterize the best lipolytic enzyme production in relation to temperature and pH. The UEL As 2.14 strain had the highest lipase production at 42 °C, pH 7.0, while the UEL As 15.262 strain had the highest lipase production at 40 °C, pH 9.0. Using the best conditions for both strains, and Tween-20 liquid medium, we verified the lipase production for 24 h, 48 h and 72 h. And in this case, the highest lipase production occurred at 24 h for two strains. So, the UEL As 2.14 and UEL As 15.262 strains is a good candidates for applications i.e. food industry, highlighting UEL As 15.262 strain which lipases producing under alkaline conditions.

Keywords: lipases, *Aspergillus welwitschiae*, screening, Tween-20, temperature, pH.
INTRODUCTION

Lipases are enzymes that have versatility of applications in different industrial areas such as biotechnology, detergent, bioremediation, food processes, among others. And due to its wide utility, there is still interest in new microbial lipases. Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) are part of a group of hydrolytic enzymes responsible for catalyzing the hydrolysis of esters formed by glycerol and fatty acids (Sharma et al., 2001; Treichel et al., 2010).

Preference is given to obtaining fungal lipases over bacterial lipases. Fungi are good lipases producers and fungal lipases are inexpensive and have the advantage of producing large amounts of extracellular lipases, making extraction and purification relatively easy (Colen et al., 2006; Aravindan et al., 2007).

However, the high lipases production is dependent on abiotic factors such as temperature, pH and substrate (Diaz et al., 2006; Kaushik et al., 2006; Lin et al., 2006; Treichel et al., 2010) and biotic factors such as fungal species and / or strains (Thomas et al., 1973).

Members of Aspergillus section Nigri has been used in various biotechnological processes for the large scale production of industrial enzymes. Some Aspergillus species have been prominent over time as A.niger most commonly used in industrial processes, in which several strains have been described as potential extracellular lipases producers (Mahadik et al., 2002; Falony et al., 2006; Mhetras et al., 2009). Morphologically identical to A. niger, A. welwitschiae, previously called A. awamori is still poorly reported for the potential extracellular lipases production (Basheer et al., 2011; Xia et al., 2011).

Most studies have reported that lipases producing for Aspergillus strains are more active in high temperature and acid conditions (Cihangir and Sarikaya, 2004; Mhetras et al., 2009). However, Aspergillus strains that alkaline lipases producing are more important commercially (Chen et al., 1998; Gulati et al., 1999; Xia et al., 2011).

On the other hand, some species of Aspergillus section Nigri have been identified as potential producers of ochratoxin A and fumonisin B2 (Abarca et al., 1994; Frisvad et al., 2007), which makes the use of such strains impossible, especially in the food industry. Thus, A. welwitschiae strains which do not produce mycotoxins and are good lipases producing are very important for various industrial applications.

In this sense, in this study, we report the screening of Aspergillus welwitschiae strains, regarding the potential production of extracellular lipases. Two strains appeared promising lipases producing with higher lipases production at high temperature and alkaline and neutral pH in 24 h.
2 MATERIAL AND METHODS

2.1 SAMPLING

A total of 24 strains of *Aspergillus welwitschiae* was used in this study. The strains were previously isolated from garlic marketed in Brazil and identified by Vanzela et al. (2020). All strains do not produce ochratoxin A and fumonisin B2 (Vanzela et al. 2020).

2.2 SCREENING OF LIPOLYTIC ASPERGILLUS WELWITSCHIAE STRAINS

The 24 strains of *Aspergillus welwitschiae* were evaluated for lipases producing on Tween-20 (polyoxyethylene sorbitan monolaurate) agar medium, prepared according to Sierra (1957). For Tween-20 agar was use Tween-20 10mL/L, peptone 10g/L, NaCl 5g/L, CaCl₂.H₂O 0.1g/L and agar 15g/L, pH 6.0. The *Aspergillus welwitschiae* strains were inoculated at the center of the Petri dishes containing the solid medium and the plates were incubated at 28 °C for 4 days, following incubated at 4 °C for 12 hours, for the formation of the enzymatic degradation halo. The crystalline opaque halo around the growing fungal colonies was characteristic of lipolytic activity.

The Enzyme Index (EI) was expressed by relationship between the diameter of the enzymatic activity halo plus colony growth and the colony growth diameter (Hankin and Anagnostakis, 1975). All assays were performed in triplicate. All data were evaluated by Tukey's test using the software R (R Core Team, 2016).

2.3 EVALUATED OF TEMPERATURE AND PH FOR LIPASES PRODUCTION FOR ASPERGILLUS WELWITSCHIAE

After screening of lipolytic *A. welwitschiae* strains with significant EI were evaluated for extracellular lipases production under different temperatures and pHs.

Under the same conditions described in 2.2 item, the selected strains (significant EI) were evaluated for lipases production at temperatures of 25 °C, 28 °C, 30 °C, 32 °C, 35 °C, 37 °C, 40 °C and 42 °C. With the identification of the best temperature for lipases production, the selected strains were evaluated at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0.

2.4 LIPASE PRODUCTION BY ASPERGILLUS WELWITSCHIAE UNDER SUBMERGED FERMENTATION

A total of 10⁶ conidias/mL of both *Aspergillus welwitschiae* strains were grown in 250 mL Erlenmeyer flasks containing 75 mL of Tween-20 medium. The flasks were incubated at 40 °C or 42 °C at 115 rpm for 24 h, 48 h and 72 h. Every 24 hours one vial was removed and the mycelium collected by filtration and the drying fungal biomass was weight. The extracellular fluid (crude
extract) was used for the quantification of the enzyme. All fermentation runs and lipase assays were performed in triplicate. The results represent the mean ± SD.

2.5 QUANTIFICATION OF LIPASES PRODUCED BY ASPERGILLUS WELWITSCHIAE UNDER SUBMERGED FERMENTATION

Lipase activity was assayed quantitatively against p-nitrophenyl palmitate (pNPP) as substrate (Winkler and Stuckmann, 1979) pNPP (30 mg) dissolved in 10 mL propanol was emulsified in 90 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 207 mg of sodium deoxycholate and 100 mg of gum arabic as emulsifying agents. An aliquot (2.9 mL) of freshly prepared pNPP substrate solution was pre-warmed at 37 °C, then 0.1 mL of crude extract enzyme solution (diluted where necessary) added, and the mixture incubated for 15 min at 37 °C. The absorbance was measured spectrophotometrically at A410nm, and the amount of p-nitrophenol released determined from a standard p-nitrophenol calibration curve. One unit (U) of lipase activity was set as the amount of enzyme that releases 1 μmol of p-nitrophenol per minute.

3 RESULTS

3.1 SCREENING OF LIPOLYTIC ACTIVITY AND EVALUATED OF TEMPERATURE AND PH FOR LIPASES PRODUCTION FOR ASPERGILLUS WELWITSCHIAE STRAINS

In the screening all 24 Aspergillus welwitschiae strains used in this study were lipases producing in Tween 20 agar. The range of EI corresponding to lipases production was 1.5 to 2.18. A total of four A. welwitschiae strains showed significant EI (UEL As 2.14, UEL As 15.262, UEL As 28.430 and, UEL As 11.228). The UEL As 2.14 and UEL As 15.262 strains were selecting, due to the higher EI (2.18 and 2.09, respectively) for further analysis (Table 01).

Lipase production by both A. welwitschiae strains (UEL As 2.14 and UEL As 15.262) in the temperature range 25 °C to 42 °C showed significant differences. Higher lipase production (higher EI) was observed with increasing temperature. The UEL As 2.14 strain at 42 °C produced EI - 3.15 while UEL As 15.262 at 40 °C produced EI - 2.38 (Table 02). Regarding the diameter of A. welwitschiae strains colonies, there was decrease in the diameter of the colonies as the temperature increased, compared to the initial analysis temperature (28 °C).

Using the selected temperatures for higher lipase production by A. welwitschiae strains, we selecting which pH is appropriate for the highest production of these enzymes in Tweem-20 agar. The highest lipase production by UEL As 2.14 and UEL As 15.262 strains was observed at neutral and alkaline pHs, specifically pH 7.0 (42 °C) and 9.0 (40 °C), respectively. The highest EI was observed by UEL As 15.262 with EI - 6.93 at pH 9.0, followed by UEL As 2.14 with EI - 3.95 at pH
7.0. Regarding the diameter of *A. welwitschiae* colonies, a decrease in diameter was observed as the pH became more alkaline.

3.2 QUANTIFICATION OF LIPASE PRODUCTION POTENTIAL BY ASPERGILLUS WELWITSCHIAE AT SELECTED TEMPERATURE AND PH

After evaluating the best temperature and pH for lipase production by UEL As 2.14 and UEL As 15.262 strains, we verified lipase production by both strains under the same conditions for 24 h, 48 h and 72 h. The highest lipase production occurred at 24 h, decreasing at 48 h and 72 h. The UEL As 2.14 strain in 24 h (42 °C, pH 7.0) produced 2.35 U/mL, however, the highest lipase production (4.16 U/mL) occurred by UEL As 15.262 strain in 24 h (40 °C, pH 9.0) (Table 03). At 48 h and 72 h there was a decrease in lipase production by UEL As 2.14 by 55.3% and 28.9%, respectively. While, there was a decrease of 55.3% and 40.6% of lipase production by UEL As 15.262 at 48 and 72 h respectively.

4 DISCUSSION

Lipases are widely produced among plants, animals and microorganisms, mainly fungi are used in various applications such as textile, food, detergent production (Sharma et al., 2001; Iftikhar and Hussain, 2002; Singh et al., 2012; Sena et al., 2006; Salihu et al., 2016). The production of lipases by microorganisms and specifically by fungi has some advantages over plants and animals, such as easy handling, high yield with low cost. Among the fungal species that stand out for production are *Aspergillus welwitschiae* belonging to *Aspergillus* section Nigri, is widely used in industrial processes. A lipase of the species *A. awamori* currently called *A. welwitschiae* has been described with potential application in bioremediation (Basheer et al. 2011).

On the other hand, fungal lipase production is dependent on some parameters such as fungal strain (Thomas et al. 1973). The screening “Agar-Plate Assay” (Sierra, 1957) on Tween-20 substrate in which demonstrated clear zone around the fungal colonies indicates lipase production. All *A. welwitschiae* strains previously characterized for non-production potential of ochratoxin A and fumonisin B2, showed lipolytic activity by Agar-Plate assay using Tween-20 agar. In others studies, different fungal species were also screened for lipase production using Tween-20 as substrate (Alves et al. 2002; Salihu et al. 2011; Venkatesagowda et al. 2012).

Although all *A. welwitschiae* strains produced extracellular lipases, there was variability between strains in lipase production (EI 1.5 - 2.18). The industrial demand for new sources of lipases is large and the variability of lipases production stimulates of the screening new safe strains. The high...
variability of lipase production is observed in several studies that screen for lipase producing strains (Rapp and Backhaus, 1992; Alves et al. 2002; Falony et al. 2006; Katogán et al. 2014).

Other important factors that influencing lipase production are abiotic parameters such as substrate, temperature and pH (Bankole and Joda, 2004; Bhattacharya and Raha, 2004; Negedu et al., 2011; Colla et al. 2015). So, the UEL As 2.14 and UEL As 15.262 strains of A. welwitschiae were selected with higher lipase production potential which showed higher EI (2.14 and 2.09, respectively). Appropriate abiotic parameters along with favorable genetic traits are known to provide higher lipase activity and it has been reported that between temperature and pH, the best combination for both selected strains (UEL As 2.14 and UEL As 15.262) was high temperature and neutral and alkaline pH. These data are agree with other authors that too found lipase production by fungal strains at high temperatures (37 °C to 65 °C) and neutral to alkaline pHs (7.0 to 8.5) (Chen et al. 1998; Gulati et al, 1999; Freire et al. 1997; Benjamin and Pandey, 2001; Diaz et al. 2006; Shangguan et al. 2011).

So, using the both strains in the conditions selecting (pH and temperature), the lipolytic activity was assessed quantitatively (U/mL), using the same liquid culture medium with Tween-20 as substrate for 24 h, 48 h and 72 h. The extracellular fluid was used for assayed against pNP-palmitate as substrate. Both strains confirmed lipase production for all hours assayed, although the levels of lipase activity varied from both strains.

For both strains the high lipase activity was observed in the 24 hours, and there was a decrease in 48 h and 72 h. Although there was a decrease in lipase production at 48 h and 72 h in both strains, the decrease in lipases production was smaller for UEL As 15.262 (55.3% and 40.6%; 48 h - 72 h), whose best lipases production occurs under alkaline conditions in relation to UEL As 2.14 (55.3% and 28.9%; 48 h – 72 h). This fact is considered of great relevance as most Aspergillus strains produce more active lipases at temperature range of 40 °C -50 °C and acid or neutral pHs 4.0 - 7.0 (Chen et al., 1998; Gulati et al., 1999; Freire et al. 1997; Benjamin and Pandey, 2001; Diaz et al. 2006; Shangguan et al. 2011).

5 CONCLUSION

All A. welwitschiae strains produced extracellular lipases in Tween-20 substrate, and there were high lipases producing for the UEL As 2.14 and UEL As 15.262. The evaluated of both strains in different conditions of temperature and pH showed that UEL As 2.14 is a good active lipase producing at 42 °C, pH 7.0 and UEL As 15.262 at 40 °C, pH 9.0. The two strains is a candidate for industrial applications, especially UEL As 15.262 which lipases producing under alkaline conditions.
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DISCLOSURE STATEMENT

The author reports no conflicts of interest in this work.

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Table 01. Screening of extracellular lipase production potential by *A. welwitschiae* strains isolated from garlic.

| Strain         | Enzymatic Index* (EI) | Strain         | Enzymatic Index* (EI) |
|----------------|-----------------------|----------------|-----------------------|
| UEL As 2.14    | 2.18 ± 0.02a          | UEL As 28.411 | 1.69 ± 0.11fghij     |
| UEL As 15.262  | 2.09 ± 0.06ab         | UEL As 1.05   | 1.68 ± 0.03fghij     |
| UEL As 28.430  | 2.03 ± 0.03abc        | UEL As 20.290 | 1.67 ± 0.05fghij     |
| UEL As 11.228  | 2.02 ± 0.09abc        | UEL As 28.425 | 1.65 ± 0.00fghij     |
| UEL As 25.348  | 1.96 ± 0.04abcd       | UEL As 27.405 | 1.65 ± 0.03fghij     |
| UEL As 7.182   | 1.91 ± 0.03bcd        | UEL As 28.422 | 1.65 ± 0.01fghij     |
| UEL As 6.136   | 1.85 ± 0.04def        | UEL As 24.323 | 1.63 ± 0.05fghij     |
| UEL As 11.225  | 1.82 ± 0.07defg       | UEL As 34.462 | 1.62 ± 0.07fghij     |
| UEL As 26.365  | 1.80 ± 0.08efgh       | UEL As 7.200  | 1.62 ± 0.01fghij     |
| UEL As 27.397  | 1.79 ± 0.18defgh      | UEL As 32.459 | 1.58 ± 0.02fghij     |
| UEL As 29.432  | 1.74 ± 0.03defgh      | UEL As 12.247 | 1.55 ± 0.04fghij     |
| UEL As 12.233  | 1.72 ± 0.01fghij      | UEL As 6.144  | 1.50 ± 0.04fghij     |

*EI means performed in experimental triplicate at 28 °C for 4 days. Distinct letters indicate significant differences at 5% significance level according to the Tukey test. In bold are the strains selected for having the highest values of EI.

Table 02. Evaluation of the best temperature and pH for the potential production of extracellular lipases by *A. welwitschiae* strains.

| Strains | Temperature (°C) | Enzymatic Index (EI) | Selected temperature (°C) | pH   | Enzymatic Index (EI) | Selected temperature and pH | Enzymatic Index (EI) |
|---------|------------------|----------------------|---------------------------|------|----------------------|-----------------------------|----------------------|
| UEL As 2.14 | 25               | 1.52                 | 5.5                       | 1.29 |                      |                             |                      |
|          | 28               | 2.18                 | 6.0                       | 1.32 |                      |                             |                      |
|          | 30               | 1.57                 | 6.5                       | 1.56 |                      |                             |                      |
|          | 32               | 1.76                 | 7.0                       | 3.95 | 42 °C, 7.0           | 3.95                       |                      |
|          | 35               | 1.87                 | 7.5                       | 2.45 |                      |                             |                      |
|          | 37               | 2.36                 | 8.0                       | 2.95 |                      |                             |                      |
|          | 40               | 2.79                 | 8.5                       | 3.81 |                      |                             |                      |
|          | **42**           | **3.15**             | **9.0**                   | 2.17 |                      |                             |                      |
|          | 25               | 1.91                 | 5.5                       | 2.16 |                      |                             |                      |
|          | 28               | 2.1                  | 6.0                       | 2.65 |                      |                             |                      |
|          | 30               | 1.62                 | 6.5                       | 2.02 |                      |                             |                      |
|          | 32               | 1.76                 | 7.0                       | 2.08 | 40 °C, 9.0           | **6.93**                   |                      |
|          | 35               | 1.74                 | 7.5                       | 2.61 |                      |                             |                      |
|          | 37               | 1.65                 | 8.0                       | 2.42 |                      |                             |                      |
|          | **40**           | **2.38**             | **9.0**                   | 2.83 |                      | **6.93**                   |                      |
|          | 42               | 2.33                 | 9.0                       | 2.17 |                      |                             |                      |

The best conditions for lipase production for each strain are shown in bold.

Table 03. Evaluation of extracellular lipase production potential by *A. welwitschiae* strains in Tween-20 liquid culture medium under previously selected conditions.

| Strains | pH | Temperature (°C) | Time (Hours) | Lipolytic activity (U/mL) |
|---------|----|------------------|--------------|----------------------------|
| UEL As 2.14 | 7  | 42               | 24           | 2.357                      |
|          |    |                  | 48           | 1.302                      |
|          |    |                  | 72           | 0.68                       |
| UEL As 15.262 | 24 | 40               | 4.162        |
|          | 9  |                  | 48           | 2.3                        |
|          |    |                  | 72           | 1.692                      |

Bold is the highest lipolytic activity for each *A. welwitschiae* strains in hours.