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ORIGINAL PAPER

Lipolytic activity of lipases from different strains of *Yarrowia lipolytica* in hydrolysed vegetable fats at low temperature and water activity

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Abstract

Yarrowia lipolytica is a very important yeast because many strains from this yeast are able to produce the extracelular lipases. Cold active lipase is one of the important and widely used enzymes whose spectrum of applications has widened in many industries such as in detergent formulations, food industry, leather processing, environmental bioremediations, and fine chemical synthesis as well as in pharmaceutical industries. Cold active lipases are largely distributed in microorganisms surviving at low temperatures, near 4 °C. Although a number of lipase producing sources are available, only a few bacteria and yeasts were exploited for the production of cold active lipases. Attempts have been made from time to time to isolate cold active lipases from these microorganisms having high activity at low temperatures. These lipases show great interests in different applications of food and chemistry industry. In this study, it was evaluated the lipolytic activity of lipases from different strains of Yarrowia lipolytica in the critical conditions. The aim of this research was to evaluate the ability of different Yarrowia lipolytica strains, having different origin, to grow and to produce the lipases at low temperature (4°C). 13 Lipases from Yarrowia lipolytica coded as PO1, PO11, RO3, RO15, Y10, Y22, LP PAST to 1a, LC TL TO 4b, LP TQ to 1a, LN2, 1 II YL 4, 16B and 27D, were used for enzymatic hydrolysis of two crude exotic fats, like: white palm kernel fat and shea fat. The conditions of hydrolysis was a low temperature (4°C) and low values water activity (aw 0.98 and 0.96). The lipolytic activity of lipases was evaluated by measuring the diameters of hydrolysis zone. At 4°C and aw 0.98, the Yarrowia lipolytica strains such as : RO3, 1 II YL4 and LC TL to 4b produced the cold active lipases that had the higher lipolytic activity on the palm kernel fat. In the same conditions, lipases from yeast strains like: RO3, RO15 and 1 II YL4 demonstrated a strong lipolytic activity on the shea fat. At aw 0.96, the lipase produced by the same strains of Yarrowia lipolytica shows a higher specificity of palm kernel fat and shea fat.

Keywords: *Yarrowia lipolytica*, cold active lipase, enzymes, lipolytic activity, shea fat, palm fat

1. Introduction

The rapid expansion in world production of palm oil over the last three decades has attracted the attention oils and fats industry. The palm fat (*Elaeis geuineensis*) and shea fat (*Vitellaria paradoxa*) are mature single stemmed tropical trees and belongs to palm family (*Arecaceae*). Palm kernel fat is an edible plant fat derived from the kernel of the fat palm *Elaeis guineensis*. (Poku, Kwasi, 2002) It should not be confused with the other two edible fats derived from palm fruits: coconut fat, extracted from the kernel of the coconut, and palm fat, extracted from the pulp of the palm fruit (Reeves, James B., 1979). In recent years there has been a considerable pressure by consumers to reduce or eliminate chemically synthesized additives in foods. Plants and plant products are a source of natural alternatives to improve the shelf-life and the safety of food (Lanciotti et al., 2004). The antimicrobial compounds in plant materials are commonly present in the fat fraction of leaves, flowers and flower buds, bulbs, rhizomes, fruits or other parts of the plant (Burt, 2004).

Palm kernel fat, coconut fat, and palm fat are three of the few highly saturated vegetable fats. Palm kernel fat, which is semi-solid at room temperature, is more saturated than palm fat and comparable to coconut fat. Like all vegetable fats, these three palm-derived fats do not contain cholesterol (found in unrefined animal fats). (US Federal Food, Drug & Cosmetic Act,1990) Palm fat has also been reported to be anodyne, antidotal, aphrodisiac and diuretic. It is folk remedy for headaches, pains, rheumatism, cardiovascular diseases, arterial thrombosis and an atherosclerosis (Ekpa, O.D. and Ebana, R.U.B. 1996) The palm fat is known to be effective against many forms of intestinal disorders, especially diarrhea and dysentery in infants (Honstra, G., et. al.,1986) Palm kernel fat has a fatty acid make up similar to that of coconut oil and has similar use pattern (Gerhard, R.R., Keith, D. and Ashiri, A. 1989) Palm kernel fat has similar uses to coconut fat owing to their similarity in composition (Pantzaris and Ahmad, 2004). The major fatty acids in palm kernel oil are lauric acid (C12, 48%), myristic acid (C14, 16%) and oleic acid (C18, 15%) (Pantzaris and Ahmad, 2004). Certain fatty acids (medium chain saturates) and their derivatives have adverse effects on various microorganisms (Kabara, 1978) The antimicrobial effect of fatty acids are additive and total concentration is critical for inactivating bacteria (Isaacs and Thomas, 1991).The medium chain fats in lauric oils are comparable to fats in mother's milk and have similar nutriceutical effects (Kabara, 1990).

Shea fat is a slightly yellowish or ivory-colored natural fat extracted from the nut of the African shea tree (*Vitellaria paradoxa*) by crushing, boiling and stirring. It is widely used in cosmetics as a moisturizer, salve or lotion. Shea fat is edible and may be used in food preparation. Occasionally the chocolate industry uses shea butter as a substitute for cocoa butter, although the taste is different.

Production of fatty acid and glycerol from fats and oils are important especially in oleo chemical industries. Glycerol and fatty acids are widely used as raw materials in food, cosmetics and pharmaceutical industries. (W. K.Mun, A.Rahman, 2008) Existing methods for production of fatty acid are based on chemical and physical methods at temperature and pressure.(Bahruddin Saad, Cheng Woon Linga *et all*, 2007) Hydrolysis of triglyceride to yield free fatty acids and glycerol from palm kernel fat have been studied for various parameters such as enzyme and oil loading, temperature and pressure. The use of highly active lipase from *C. rugosa* has been widely studied for the purpose of fat and oil hydrolysis.(Ting, W.J., Tung, K.Y., *et. all*, 2006) The advantages of the enzyme hydrolysis technique include the use of bio-route technology that only requires a mild temperature, simple operational procedure and low cost as well as energy consumption.

Enzymes are an essential target for the adaptation of an organism to a cold environment. The knowledge of cold adapted lipolytic enzymes in industrial applications is increasing at a rapid and exciting rate. (B. Joseph, P. W. Ramteke, *et all*., 2007) Vegetable fats are very important sources of antimicrobial fatty acids. The ability of fatty acids to interfere with bacterial growth and survival has been known for several decades. Many researchers have demonstrated the antimicrobial effect of fatty acids against Gram positive and Gram negative bacteria, fungi and protozoa, with a similar action as modern antibiotics. It was studied the physiological effect of fatty acids (Fat and FFA) derived from the hydrolysis of vegetal origin lipids which have been shown to have powerful antimicrobial activity. Preliminary studies have demonstrated the antimicrobial effect of hydrolysates obtained by enzymatic hydrolysis with lipase. In this study 13 strain of *Yarrowia lipolytica* was tested at different parameters of temperature, water activity and time, with the aim to select all the best yeast strain who are capable to produce lipases with strong lipolytic activity on the both substrates - palm kernel fat and shea fat.

The first aim of this study was to demonstrate that the different strains of *Yarrowia lipolytica* are able to grow and to produce the active lipases at the low temperature. The second aim of this study was to hydrolyze two Cameroonian crude vegetable fats: palm kernel fat and shea fat by cold lipases produced by *Yarowia lipolitica* strains at low temperature and low values of water activity.

2. Materials and Methods

2.1. Materials

The crude vegetable fats were purchased from Cameroun, Africa. The fats were obtained with traditional extraction methods. The basal media used for yeasts cultivation was Sabouraud (SAB, universal peptone, glucose and agar) from Merck KgaA Darmstad, Germany and Spirit Blue Agar (SBA) from DIFCO Laboratoires Detroit Mi, USA. The SBA was prepared at different water activities (0.98, 0.96, 0.93) with different concentrations of NaCl (Merck KgaA Darmstad, Germany). All the 13 strains of *Yarrowia lipolytica* coded *PO1, PO11, RO3, RO15, Y10, Y22, LP PAST to 1a, LC TL TO 4b, LP TQ to 1a, LN2, 1 II YL 4, 16B* and *27D* were obtained from University of Bologna, Facolta di Scienze degli Alimenti, Cesena, Italy.

2.2. Methods

2.2.1. Inoculum preparation

For inoculum preparation *Yarrowia lipolytica* strains were first cultivated in Petri dishes on Sabourand agar medium, and were incubated at 28°C for 72 hours. A small quantity of activated biomass was then inoculated in glass tubes with 8 mL Sabouraud broth. The tubes were incubated at 28°C for 72 hours. The inoculum was prepared by transfer of every 1 mL of preinoculum from incubated tubes in other new tubes with fresh Sabourand broth .

2.2.2. Medium supplemented with vegetable fats preparation

It was suspended 35 g of the powder of Spirit Blue Agar commercial medium in 1 L of purified water. After that were added the different concentrations of NaCl like 3%, 6% and 10.1%, to obtain the water activity of 0.98, 0.96 and 0.93 and mix thoroughly. Heating followed, with frequent agitation and boiled for 1 minute to completely dissolve the powder. The media was autoclaved at 121°C, for 15 minutes and after was cooled to 50-55°C. Aseptically was added 30 mL crude vegetable fats and mix thoroughly. The media was poured in Petri dishes . After that this plates were dried. (Frank and Bullerman, 1993). Tubes were incubated at 28°C for 72 hours. The number of viable cells was established by plate count method, except where viable cell numbers were less that 10^3CFU/ mL, when the MPN (Most Probable Number) technique was used.

2.2.3. Yeast cultivation and lipase activity evaluation

After drying the SBA plates with homogenized crude vegetable fats, it was put in center of plate 30 µl of every inoculums, of 10^6 CFU/ml. The yeast colony diameter and the diameter of the hydrolysis zone were measured. The evaluation of the lipase activity was determined by making the difference of these two values. The diameter of inhibition it was recorded at every 24 hours. In the same time control samples were prepared for every values of water activity and for every temperature. Samples were performed in duplicate. The incubation duration was 240 hours for every sample.

3. Results and discussions

3.1. Lipolytic activity of cold lipases from different *Yarrowia lipolytica* strains at aw 0.98 and 4°c

In this study it was observed that *Yarrowia lipolytica* strains had growth in cold conditions and at different values of water activity (0.98 and 0.96). From the beginning of the experiment until 144 hours the yeasts increased their colony diameter and after this time began to produce lipases that digest the crude vegetable fats. Figure 1 shows how all strains of *Yarowia lipolityca* work on the palm kernel fat, between 0 – 240 hours.

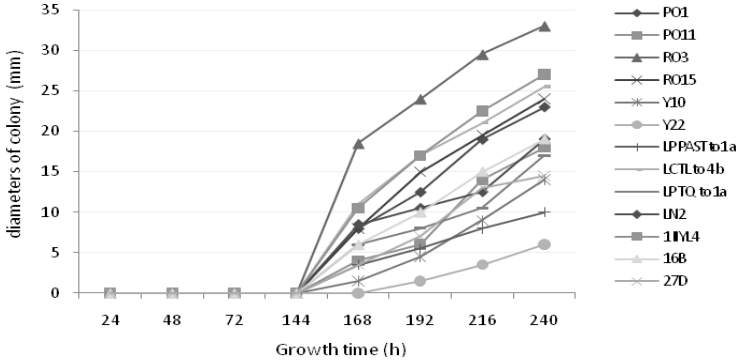

Figure 1. Lipolytic activity of lipases produced by *Yarrowia lipolytica* strains on palm kernel fat at aw 0.98

After 168 hour in the fat media the lipases were released from yeast strains and began to digest the fat according to their specificity. The best lipases that had a major diameter of hydrolysis zone were produced by strains such as: PO1, 1 II YL4, and the third one is LC TL to 4b respectively with diameters of 18.5 mm, 10.5 mm and 10.2 mm. Between 168 and 240 hours the best lipases were produced by the same yeast strains. Also all the others strains produced good diameters of hydrolysis but not in the same rates. This demonstrated that all strains were able to produce lipases with specificity on palm kernel oil at low temperature. The smallest diameter of hydrolysis zone was registered by lipase of strain Y22 and it was 2 mm after 192 hours of reaction and after 240 hours when the experiment was finished it registered a diameter of 6 mm.

In figure 2 it the shea fat was used as a substrate for the same strains of Yarrowia lipolytica and in the same conditions like the first study. Here it was observed that also that the strain RO3 is on the first place followed by the RO15 and 1 II YL4 that was the third also in the first study.

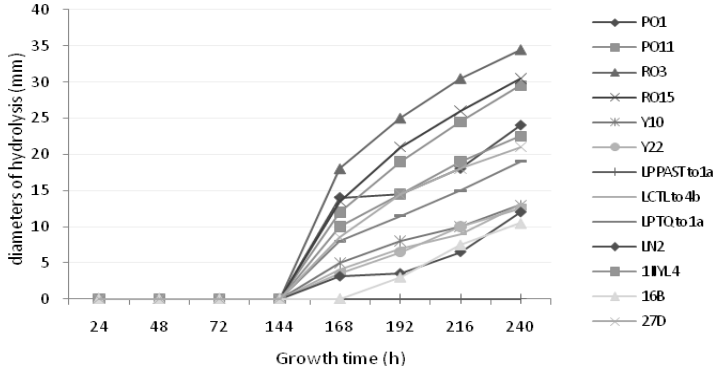

Figure 2. Lipolytic activity of lipases produced by *Yarrowia lipolytica* strains on shea fat at aw 0.98

Also here is the same general growing algorithm with two exceptions at strains PO1 and LN2, that shows a period of latency between 168 and 192 hours where the diameters remained at the same value. After 192 hours they began to produce more lipase and the diameter increased at 24 mm, respectively 12 mm until 240 hours. Also, here it was a strain that did not show a high lipase production. This was 16B and until 168 hours it did not produce any lipase, but after 192 hours the production began and at the finish of experiment it registered a diameter of 10 mm. After this experiment it was observed that at low temperature (4°C) and water activity of 0.98, in all the substrates the *Yarrowia lipolitica* strains needed a longer period of time to produce the capable enzymes to hydrolyze the vegetal fats. A very important thing was highlighted in this study regarding the *Yarrowia lipolytica* strains that are capable to grow at low temperatures and in a salty medium. The lipases produced in these conditions had a higher specificity on palm kernel and shea fat.

4.2 Lipolytic activity of cold lipases from different *Yarrowia lipolytica* strains at aw 0.96 and 4°c

In this study it was changed the value for the water activity. It was added salt in the media until reaching the value aw0.96. It was expected to have a smallest hydrolysis zone on that value of water activity but the reality was other. There were some strains that showed a capability to produce lipase starting from 144 hours until the final hours of the studies. Like the figure 3 shows, the strains labeled LPTQ to1a, RO15 and 1II YL4 had a strong lipolytic activity on palm kernel fat. Between 144 and 240 hours these three strains were producing lipase in a equal rate like the smallest water activity value (0.98) used before. The diameters recorded were in order of 15.8, 15.6 and 13 mm after 240 hours of incubation.(Figure 3) All the others strains were affected by the concentration of salt but remained also active, mostly from 216 to 240 hours. There were another 2 strains that were in a middle range of the lipolytic activity - LC TL T0 4b and RO3, that had recorded diameters of 8 respectively 6 mm after 240 hours.

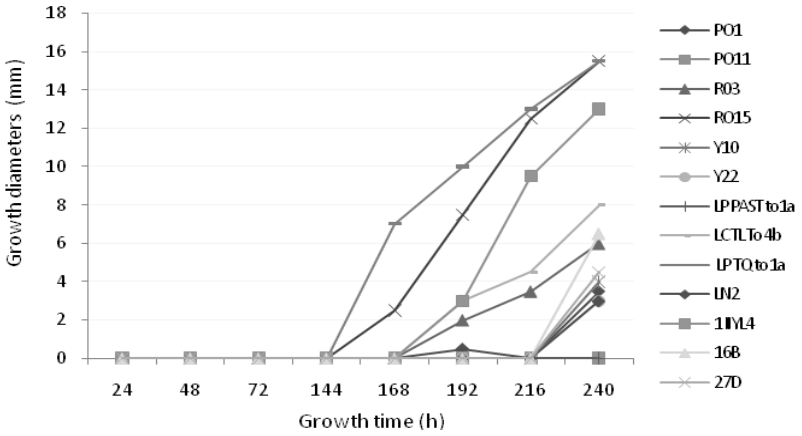

Figure 3. Lipolytic activity of lipases produced by *Yarrowia lipolytica* strains on palm kernel fat at aw 0.96

In the same conditions of low temperature and low water activity, the *Yarrowia lipolytica* strains increased after 100 hours. After 144 hours, the lipases produced by this strains, hydrolyzed the shea fat substrate (Figure 4). It was observed that the *Yarrowia lipolytica* strain coded RO15 was the best strain because it produced the most active lipase. Initially it made a hydrolysis zone with a diameter of 3 mm. After 240 hours the diameter of this area increased to a value of 17 mm. Also a strong lipolytic activity was produced by the strain PO1, with an

initial hydrolysis diameter of 6 mm. After 192 hours, this lipase had showed a hydrolysis zone with a diameter equal with lipase RO15 diameter, 7 mm respectively.

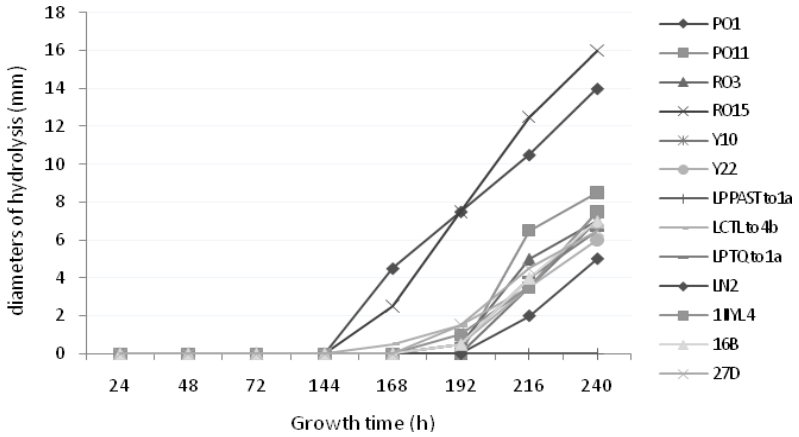

Figure 4. Lipolytic activity of lipases produced by *Yarrowia lipolytica* strains on shea fat at aw 0.96

After 240 hours, the lipase produced by *Yarrowia lipolytica* PO1 strain showed a zone of hydrolysis with a diameter of 14mm. The most resistant yeast strains were RO15 and PO1 at the temperatures of 4°C and a 6% concentration of NaCl. The lipase produced by LN2 strain had no activity in 192 hours, but until the final of this study it produced a small diameter of 6 mm.

All the lipases produced by all others strains had a similar lipolytic activity in 168 hours but they produced small diameter of no more than 2 mm but after 192 hours the diameters increased. The lipases produced by the *Yarrowia lipolytica* strains PO11, 1IIYL4, RO3, 27D and Y22 had growth on shea fat substrate and produced diameters of 6 to 8 mm. It was observed that at low temperatures and at water activities of 0.96 and 0.98 the *Yarrowia lipolytica* are capable to grow and to produce lipases with a good specificity on shea fat.

4. Conclusions

This study had demonstrated that *Yarrowia lipolytica* strains are growing at low temperatures (4°C). Regarding the water activity, it was observed that by increasing the salt concentration in medium (0.96 and 0.98) the growing of the yeast is reduced but do not disappear in time. Because of the low temperatures and the salt concentrations, the strains of *Yarrowia lipolytica* required a long period of time to grow (144-240 hours).

Among all the 13 strains of tested yeasts, the best three strains were RO3, RO15 and 1IIYL4. They had produced the most active lipases capable to hydrolyzate both substrates, palm kernel fat and shea fat. In the heavy medium conditions (4°C and 0.96 aw) only the strain RO15 presented a strong lipolytic activity. Also here was observed that the diameters of hydrolysis had decreased from 35 mm (at 0.98 aw) at 16 mm (at0.96 aw)

At the temperature of 4°C and a low concentration of salt (aw 0.98 and 0.96) the strains that showed strong activity were: RO3, RO15 and 1II YL4. They had produced enough lipase that made a good hydrolysis in time of 240 hours. That fact shows that the lipases from *Yarrowia lipolytica* are cold active lipases and they worked well at 4°C and low water activity values.

At aw 0.93 were not identified hydrolysis zones on the surfaces of analyzed substrates. Also, the presence of *Yarrowia lipolytica* colonies was not identified. Along with increasing of salt concentration, the growth of yeast was significantly reduced at 4°C, and also the lipolytic activity was reduced.

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