

EVALUATION OF LIPOLYTIC INDUCTION OF ENZYMES FROM
Pseudomonas aeruginosa ISOLATED FROM AFRICAN PALM FRUIT (*Elaeis*
guineensis)

EVALUACIÓN DE LA INDUCCIÓN DE ENZIMAS LIPOLÍTICAS A PARTIR DE
UNA *Pseudomonas aeruginosa* AISLADA DEL FRUTO DE PALMA AFRICANA (*Elaeis*
guineensis)

ABSTRACT

Gram negative microorganisms with lipolytic activity isolated from palm fruit, identified as *Pseudomonas aeruginosa* were evaluated for lipase induction. Glucose was used as carbon source, it was determined that concentrations of 3 and 5% have a biomass yield of 72,13 and 44,59% respectively. The enzyme induction was performed with 3% of glucose at three times (0, 11 and 18 hours of fermentation), using as inducers palm oil, tween 20 and palm oil emulsion:tween 20. The enzyme activity was assessed by titration of fatty acids and hydrolysis of *p*-nitrophenylpalmitate. The highest lipolytic activity was achieved with emulsion at 11 hours of fermentation, with 3,805 $\mu\text{moles/min}$ for medium MM and 4,855 $\mu\text{moles/min}$ for medium MME. Hydrolysis of 100 μM *p*-nitrophenylpalmitate showed that at 300 minutes of reaction are released 100 μM of *p*-nitrophenol in the MM medium and 102 μM for the MME medium. The protein was quantified by Modified Bradford, reaching

values of 81.71 $\mu\text{g/mL}$ for MME medium and 72.82 $\mu\text{g/mL}$ for MM medium. The results of this study contribute to the biotechnological application as a catalyst in the production of biodiesel and / or oleochemicals.

Keywords: lipolytic activity, fatty acids, enzyme induction, hydrolysis.

RESUMEN

Microorganismos Gram negativos con actividad lipolítica aislados del fruto de palma africana, identificados como *Pseudomona aeruginosa* se evaluaron para la inducción de la lipasa. Se utilizó glucosa como fuente de carbono, determinándose que concentraciones de 3 y 5% presentan un rendimiento biomasa/sustrato de 72.13 y 44.59%, respectivamente. Con glucosa al 3% se realizó inducción enzimática en tres tiempos (0, 11 y 18 horas de fermentación), utilizando como inductores aceite de palma, tween 20 y emulsión de aceite de palma:tween 20. La actividad enzimática se evaluó por titulación de ácidos grasos e hidrólisis de *p*-nitrofenilpalmitato. La mayor actividad lipolítica se logró con emulsión a las 11 horas de fermentación, con un máximo de 3.805 $\mu\text{moles/min}$ para el medio MM y 4.855 $\mu\text{moles/min}$ para el medio MME. La hidrólisis de 100 μM de *p*-nitrofenilpalmitato evidenció que a 300 minutos de reacción se liberan 100 μM de *p*-nitrofenol en el medio MM y 102 μM con el medio MME. La proteína se cuantificó por Bradford Modificado logrando valores de 81.71 $\mu\text{g/mL}$ para el medio MME y 72.82 $\mu\text{g/mL}$ para el medio MM. Los resultados de este estudio contribuyen en la aplicación biotecnológica como catalizador en la producción del biodiesel y/o oleoquímicos.

Palabras claves: actividad lipolítica, ácidos grasos, inducción enzimática, hidrólisis.

INTRODUCTION

Lipases are lipolytic enzymes, classified as carboxylic ester hydrolases (EC. 3.1.1.3), that break ester bounds of acylglycerides by adding a water molecule generating free fatty acids and glycerol. Lipases are versatile and interesting in biotechnology because they could catalyze a wide range of lipids hydrolysis reactions. In organic solvents medium, enzymes make synthesis or groups exchange reactions (transesterification and interesterification) within different molecules as lipids, carbohydrates and amino acids (1, 2).

Applications of lipases are many and varied. Originally lipases were used for fats and oils hydrolysis, but they are also capable of carrying out the reverse process, that is to said, synthesize ester bonds. Due to its stereospecificity, the biotechnological potential of lipases is huge and arises great interest of the food, chemical, pharmaceutical, agricultural, medical or cosmetic industry, as well of others (1, 3, 4).

Some bacteria produce and excrete lipase that can catalyze the hydrolysis and synthesis of long chain acylglycerol, which can be produced with high regioselectivity and enantioselectivity (5). The production of microbial lipolytic enzymes is influenced by nutritional and physical-chemical factors. Some studies aim to determine the optimal

conditions for enzyme production for different types of microorganisms (6, 7, 8). Regarding to carbon sources, there has been usage of vegetable oils such as soybean, corn, olive, sunflower, etc. at different concentrations, as well as non-metabolizable polysaccharides, carbohydrates, and others (1, 6). There is also a great variety of nitrogen sources. The most commonly used are peptone, yeast extract, corn liquor, ammonium sulfate, ammonium nitrate. They have been used in different concentrations and combinations (6, 9).

The presence of lipids in culture medium like: butter, olive oil, canola oil, fish oil, can influence in the lipolytic activity and production of microbial lipases (10, 11). As general behaviour, lipolytic activity (intracellular and extracellular) grows as the lipids concentration increases, but at elevated levels of them in the culture medium, it may turn out toxic (5).

Because lipolytic enzymes hold industrial process importance it is necessary to carry out studies where parameters for improve their yield can be determined. For example, improvement of carbon concentration and carbon sources for growing microorganisms, types of inductors that would increase the production level of enzyme, and types of substrates.

Moreover, the massive growth of palm cultivation in Colombia has been given by two reasons mainly: employment generation in many rural areas, and the release of the country of energy pressure. Palm oil represents a potential alternative for renewable fuel production

such as biodiesel, and the figuring in basic oleochemicals market. The national government has turned its attention to increase area of palm oil crop (12). According to Acosta (13) in the short term, the goal in the Colombian territory is to achieve coverage of 100% in fuels such as bio-gasoline and biodiesel. In this way, microbial enzymes isolated from palm fruit are used in oleochemical processes. This type of project generates a positive contribution to the national economy. Therefore, in this project a bacterium with lipolytic activity isolated from mature palm oil fruit by Peña (14) was used. A bacterium was evaluated with inducer agents, in three induction times, for the production of lipolytic enzymes.

MATERIALS AND METHODS

Microorganism and culture medium

Gram negative microorganisms whit lipolytic activity isolated from palm fruit oil by Peña (14) were identified as *Pseudomonas aeruginosa*. Two methods of fermentation were used: MM medium consisting of $(\text{NH}_4)_2\text{SO}_4$ (0.5% w/v), casein peptone (1.0% w/v), K_2HPO_4 (0.5% w/v), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1% w/v); and the medium MME that is the same medium MM with the addition of yeast extract (0.5% w/v). The conditions fermentation were 37°C, 200 rpm, 24 hours, with 1% (v/v) of pre-inoculum.

Effect of carbon source

The carbon source used was glucose at concentrations of 3%, 5%, 10%, 15% and 20% (w/v).

Inducers agents

As inducers agents of lipolytic enzyme were used palm oil, tween 20 and palm oil with tween 20 at a ratio 4,8:1 v/v (oil:emulsion). The concentration of inducer agents was 0.3% (w/v) in separate tests. The inductors were added to the fermentation medium (the better result of biomass) in three different induction times. The induction time was determined from growth kinetics, corresponding to the initial stage of fermentation (0 h), exponential phase (11 h) and stationary phase (18 h), respectively.

Lipolytic activity determination

• Titration method

The percentage of acidity for each of the inducer agents was determined at 24 hours of fermentation. Acidity percentage was calculated by titration with NaOH (0.1 N) in an automatic titrator Methrom.

• Colorimetric method

Enzymatic activity was determined from the supernatant for the three inductors by a spectrophotometric method; this method is based on the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP). *p*-NPP was used as substrate in a concentration of 100 µM

and the reaction was carried out 37°C, 100 rpm for 15 minutes. The release of *p*-nitrophenol (*p*-NP) by hydrolysis is determined at 405 nm.

Enzymatic hydrolysis

The enzymatic hydrolysis was carried out with the supernatant of inductor that showed the highest activity. The reaction with *p*-NPP was carried out for 300 minutes, 37°C, 100 rpm, the release of *p*-NP was determined at 405 nm.

Determination of protein

The amount of protein in the supernatant was determined using the modified Bradford method using like standard bovine serum albumin (15).

RESULTS AND DISCUSSION

Under the fermentation conditions employed, the growth of bacteria was determined by different concentrations of carbon source. Thus, it was observed that there was cell growth inhibition when the glucose concentration was the highest. This result is similar with a study performed by Ito *et al.*, (16), where they determined the effect of glucose and ammonium on cell growth inhibition of *Pseudomonas aeruginosa* LST-03, and also determined that high concentrations of both glucose and ammonium in the fermentation medium inhibited growth of bacteria.

In another study, by Takac and Marul (17), they also were in accord in pointing out that high concentrations of glucose suppressed the lipolytic activity. Beyenal *et al.* (18) and Gupta *et al.* (10) mention that glucose in small amounts was necessary for the growth of microorganisms begin and thus release the lipase to the medium.

The biomass/substrate yield was determined with 3% and 5% of glucose and the performance obtained for both glucose concentrations was 72.13% and 44.59% respectively. Figure 1 shows the growth curve for each of the concentrations of glucose. For this reason, in this project only the lowest concentration of glucose was used for further testing.

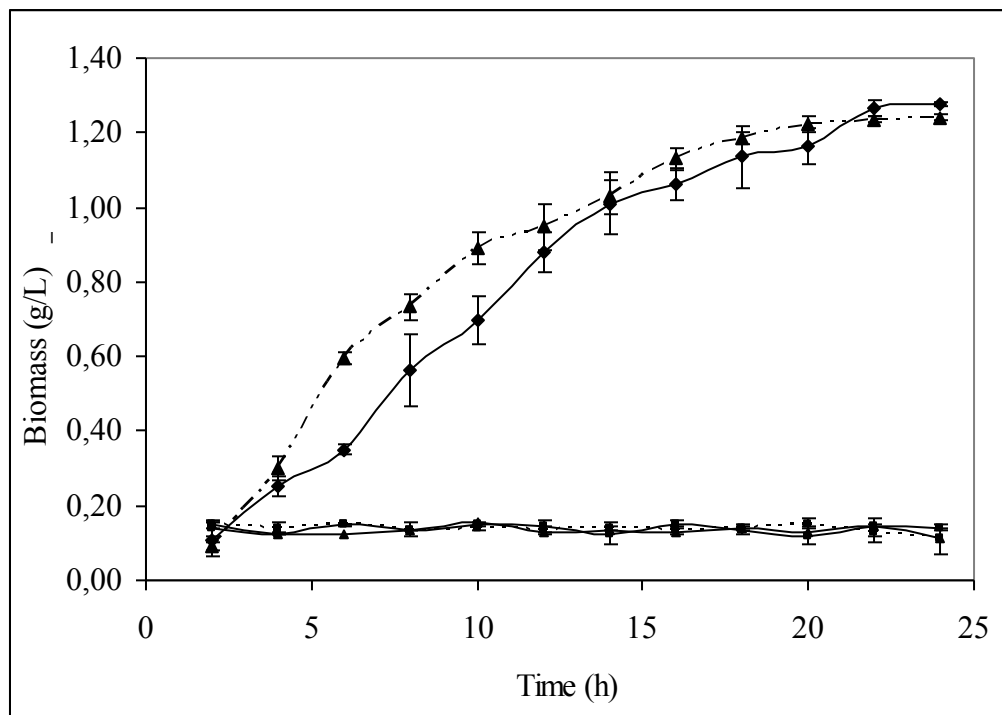


Figure 1. Growth of *P. aeruginosa* in different concentrations of glucose 3% (-▲-), 5% (—◆—), 10% (- - - - -), 15% (—■—) and 20% (—◆—). Source: Author

The lipolytic activity was calculated with glucose 3% by titration method in the medium at the end of fermentation, achieving a value of 0.01874 $\mu\text{moles/min}$. A significant difference was observed when comparing this last valued with data obtained by Morales and Munoz (19), they studied the effect of carbon source with crude palm oil to 3% and 5%, with the same microorganism, and likewise the lipolytic activity was assessed by the same titration method with values of 0.0010 and 0.0011 $\mu\text{moles/min}$, respectively.

When *P. aeruginosa* T1 is grown in the presence of glucose as carbon source, there are low levels of lipolytic activity. According to a study by Hasanuzzaman *et al.* (20) the adding of inducers to the medium increased production of the enzyme and activity above basal level. Rigo *et al.* (4), Deive *et al.* (11), Hasanuzzaman *et al.* (20) and Kumar *et al.* (21) stated that adding an inducer to the culture medium is an important parameter to improve production. Deive *et al.* (11) also coincide in pointing out that time for the addition of inducer agents must be evaluated, since the growth of microorganisms depends of that time.

Based on the mentioned it can be noted that the lipolytic activity levels were relatively low, three inducers were added to the fermentation medium at three different times using glucose 3% as carbon source. The results are shown in Figure 2.

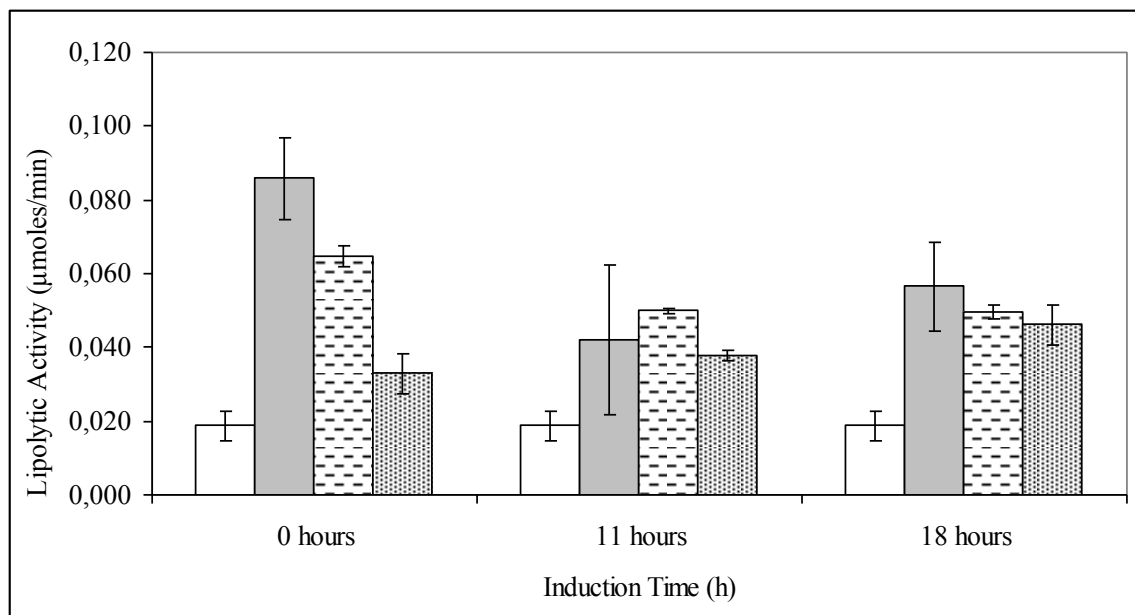


Figure 2. Lipase activity was determined by titration method at the end of fermentation (24 h) at three times of addition of inducer. Inducers agents: (■) Palm oil, (≡) Emulsion (●) Tween 20, (□) Control sample. Source: Author

The highest percentage of lipolytic activity was observed with palm oil added to the medium when the fermentation begins. The results of palm oil added at 11 and 18 hours of the fermentation were lower than the first case. This would indicate that there is release of fatty acids to the medium (enzyme is present), but the bacteria do not consume it when the carbon source tends to dry up. This is the case for emulsion because the behavior is the same, that is, one might conclude that microorganisms can grow at the interface generated by it, therefore, hydrolyzing bounds but not consuming them.

Tween 20, as inducer agent, showed induction of the enzyme because the lipolytic activity is higher than the result presented by the control sample, also could be observed that the

behavior is opposite as to the other inducers. Thus, when Tween 20 was added at 18 hours it shows higher fatty acids percentage. That is, fatty acids are present in the environment and they are also consumed when the carbon source is exhausted.

Lipase activity of the fermentation medium, by titration method, represents an indirect analyses method. For this reason, fatty acids of the inducers agents are necessary to be estimated before starting the fermentation since they contain free fatty acids which could be influencing the determination of lipolytic activity at the end of fermentation. Therefore, the results can not be reliable when the inductor is palm oil or emulsion (palm oil:Tween 20).

Because of this fact, the enzymatic activity was accomplished by the colorimetric method. Figure 3 shows the results on the hydrolysis of *p*-NPP. The inducers agents were used at different times on the fermentation medium. The fermentation was taken up to 24 hours.

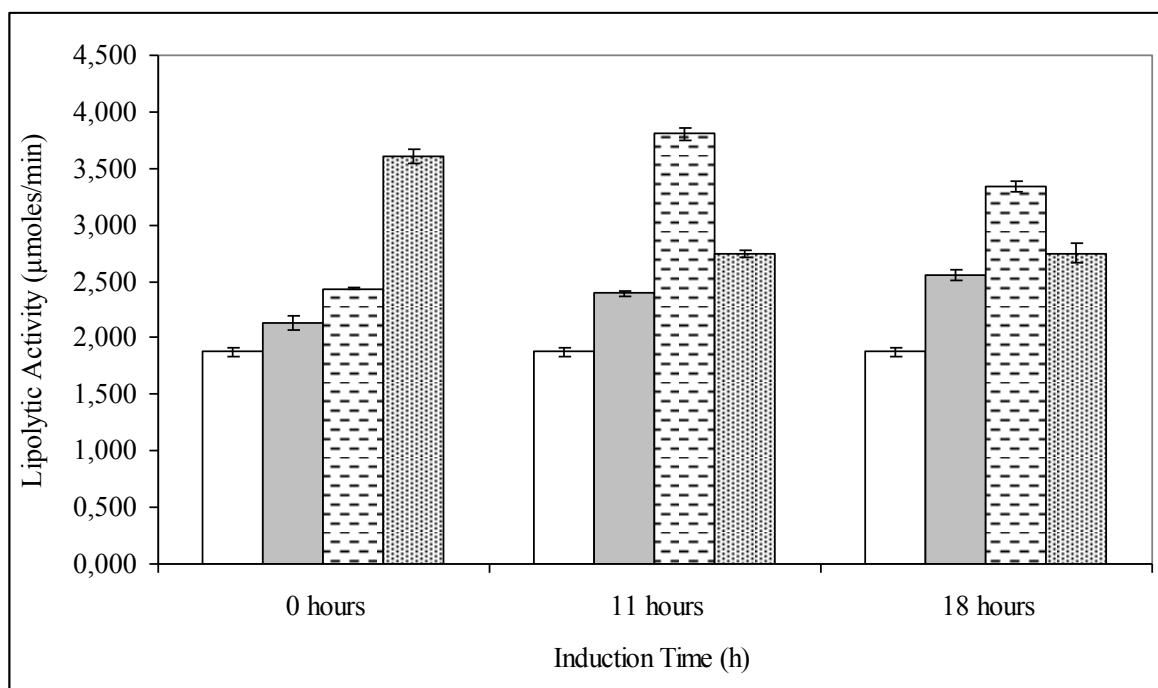


Figure 3. Hydrolysis of *p*-NPP. The inducer agents were added in three times of the fermentation. Reaction time: 15 minutes. Inducers agents: (□) control sample, (■) palm oil (≡) Emulsion (●) Tween 20. Source: Author

The control sample showed lipolytic activity in the fermentation medium that is to say, without inducer agent is possible to detect enzymatic activity. This lipolytic activity is known as basal level of the enzyme. Therefore, glucose as carbon source does not repress the release of the enzyme.

Inducers agents in the fermentation medium stimulated the release of enzymes, because all media containing inducers agents present values over 1,875 $\mu\text{moles/min}$ which is the value for the medium without inducers agents (control sample). That is, the induce agents in the fermentation medium released more enzyme as specified, for the titration method.

Palm oil as inductor verified enzyme induction, since the value of lipolytic enzyme, at three times of inducer addition, is greater than the control sample. The best result was obtained when the inductor was added at 18 hours of fermentation in the stationary phase, indicating that the enzyme hydrolyzes the *p*-nitrophenylpalmitate bonds, but also the bacteria starts to consume the products of hydrolysis when the initial carbon source begins to run low.

With Tween 20 as inductor a value of 3,608 $\mu\text{moles/min}$ was obtained, when this inductor was added to begin fermentation, hence suggesting that the bacteria used Tween 20 as an inductor to manage the release of more enzymes. With the values at 11 and 18 hours of inducer addition, it can be assumed that the bacteria are not consuming the products of hydrolysis as carbon source. Shabtai and Daya-Mishneh (22) argue that the addition of Tween 20 to the medium promotes the dissociation of the enzyme in the cell wall, thus in the supernatant there can be found enzyme. The production of the enzyme increases because the basal level facilitates the release of it in the medium.

When Tween 20 was used, the result was higher than that when palm oil was used because bacteria apparently could hydrolyze Tween 20 bonds easier. Tween 20 has a linear chain in contrast to palm oil, since this one it has a chain that is composed of three fatty acids. According to Castro et al (23) the presence of Tween 20 facilitates the incorporation of nutrients into the cell membrane favoring the release of enzyme and the hydrolysis.

Favorable levels of lipolytic enzyme production were found with emulsion (palm oil:Tween

20) as inductor agent. The emulsion was added at 11 hours of growth during the exponential phase, and the obtained value was 3,805 $\mu\text{mol} / \text{min}$. According Illanes *et al.* (5) and Reis *et al.* (24), lipases catalyze the hydrolysis of substrates when they are in micelles, small aggregates or particles in emulsion, because the place where lipolysis occurs requires at least two phases and a larger contact area. On the other hand, Bañó *et al.* (25) and Reis *et al.* (26) argue that using an emulsion facilitates the interfacial activation of lipases features, as the emulsion facilitates substrate access to enzyme active site.

We can use this fact to conclude that, the emulsion is the best inducer agent compared with the other two, because the increased activity may be due to the presence of more interfacial area to facilitate the interaction of enzyme with substrate. Another possibility could be that the highest enzyme released is due to the combined effect between emulsion and Tween 20. Moreover, Tween 20 also acts separately and facilitates the release of more enzymes.

According to previous results, the emulsion showed the best results, for this reason, the kinetic of enzymatic hydrolysis was determined with this emulsion as shown in Figure 4. The emulsion (palm oil:Tween 20) was added to the culture medium at different inducing times.

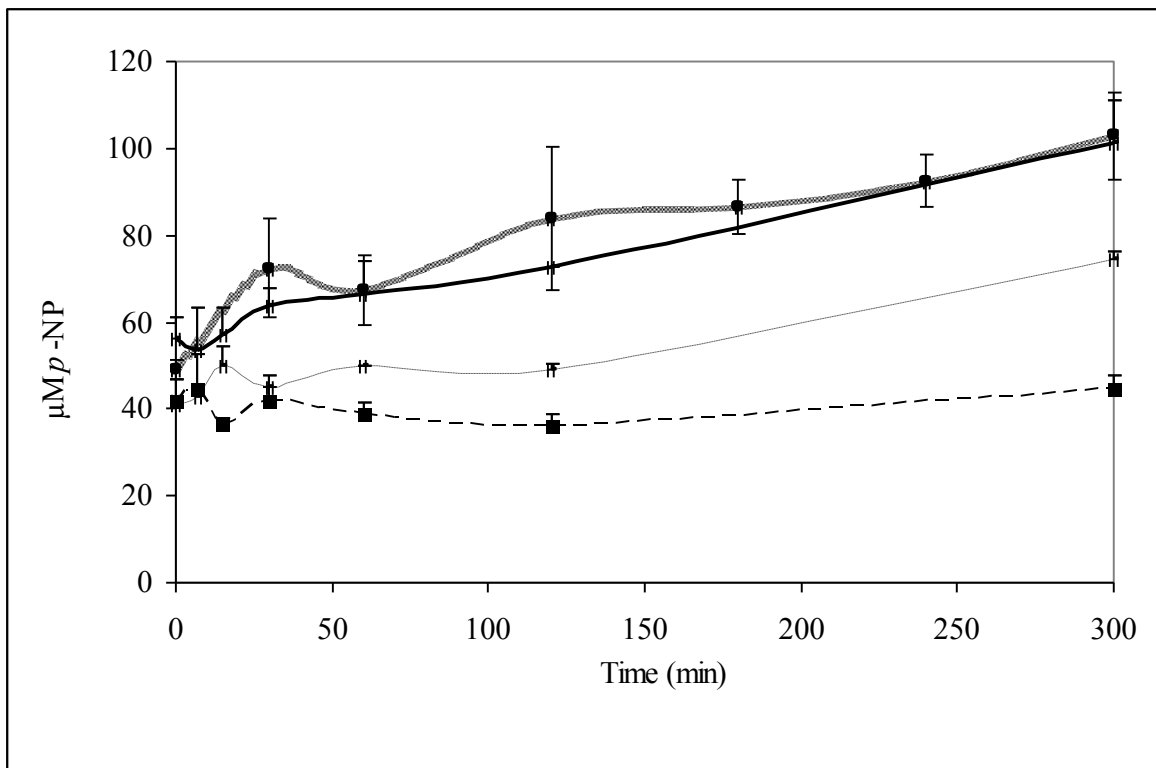


Figure 4. Kinetics of enzymatic hydrolysis of emulsion as inducer agent. Added at 0 hours (—■—), 11 hours (——), 18 hours (-----) of MM fermentation medium and emulsion in MME fermentation medium at 11 hours (.....◆.....). Source: Author

The release of 100 μM of $p\text{-NP}$ was achieved at 300 minutes of reaction with $p\text{-NPP}$ when it was added at 11 hours of fermentation, that is to say, when the bacteria were in the exponential phase it was found a greater rate of reaction.

The reaction kinetic curve presented by the control sample shows that the addition of emulsion as inducer increases the reaction rate of hydrolysis when the inducer is added at

0, 11 or 18 hours to the fermentation medium. In MME medium it was determined that at 300 minutes of reaction there were released 102 μM p-NP, when the inductor was added at 11 hours of fermentation.

Emulsion as an inducer agent, in fermentation media (MME and MM), increases the kinetic results when it is added at 11 hours fermentation. The amount of extracellular proteins in the supernatant was determined at 24 hours of fermentation for each medium by modified Bradford. In this way, the values obtained were 81.71 $\mu\text{g/mL}$ for medium MME and 72.82 $\mu\text{g/mL}$ for medium MM. These results suggest that the addition of yeast extract to the fermentation medium increases the amount of protein compared with the medium containing only glucose.

CONCLUSIONS

The acidity percentage that was obtained in the fermentation medium after 24 hours of growth can be used as indicative of the presence of the enzyme. According to the results it can be stated, that the effect of adding inductors to the fermentation medium increases the levels of enzyme production. Similarly, it was observed that using glucose as carbon source it does not suppress the basal level release of the enzyme in the fermentation medium. Values achieved with the titration method show that palm oil has the best result, but it must taken into account that this value may be due to the presence of other fatty acids at fermentation initiation.

Hydrolysis of *p*-NPP as substrate was determined by the release of *p*-nitrophenol, acting as an indicator of the presence the enzyme in the medium after 15 minutes of reaction. The reaction showed that an inducing agent stimulated the release of the enzyme into the medium, and the best yield was obtained with emulsion (palm oil:Tween 20) as inducer. The emulsion was added at 11 hours of fermentation medium and the value was 3,805 $\mu\text{moles/min}$ for MM medium. By using enriched culture medium with yeast extract (MME) to determine the presence of the enzyme, the determined value was 4,855 $\mu\text{moles/min}$.

A preliminary kinetic study of enzymatic hydrolysis was performed for the best inducer agent (emulsion) found. The reactions conditions were: 100 μM of *p*-NPP, 300 minutes of reaction, and the inductor was added at 11 hours of fermentation medium. The result for MM medium was 100 μM of *p*-NP. For the MME medium, the result was 102 μM of *p*-NP. The kinetic of enzymatic hydrolysis for the control sample has lower values than those achieved when the emulsion was used as an inducer in the release of the enzyme.

The amount of extracellular proteins was determined in the supernatant after 24 hours of fermentation for each medium. In this way were obtained a quantity of protein with a value of 81.71 $\mu\text{g/mL}$ for medium MME and a value of 72.82 $\mu\text{g/mL}$ for medium MM. These results suggest that the addition of yeast extract fermentation medium increases the amount of protein compared with the medium containing only glucose.

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