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Effect of Nitrogen and Carbon Sources on Lipase Production by *Penicillium aurantiogriseum*

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Summary

A wild fungal strain isolated from soybean oil and identified as *Penicillium aurantiogriseum* initially presented a volumetric lipase activity of 0.4 U/mL in submerged culture in a medium containing 0.5 % yeast extract and 1 % olive oil. Studies were undertaken to improve lipase production. The effect of nitrogen source was studied by adding casein peptone, meat peptone, yeast extract or ammonium sulfate to a medium containing potassium nitrate and other mineral salts. The best yield, of 13 U/mL after 72 h, was obtained with the medium supplemented with ammonium sulfate. With the ammonium sulfate concentration increased to double the C/N ratio from 2.5 to 5, a lipolytic activity of 18 U/mL was obtained. Olive, corn, soy and sunflower oils were tested as carbon sources in this medium, with olive oil at 1 % giving a lipolytic activity of 25 U/mL after 48 h, the highest yield obtained in this study. Enzyme production was best at 29 °C, within a range tested from 26 to 32 °C. These results are promising because this strain produces lipase in an inexpensive inorganic medium and we succeeded in increasing the lipolytic activity 62-fold over the initial values obtained with the non-optimized medium.

Key words: lipases, *Penicillium aurantiogriseum*, media optimization, lipolytic activity, inorganic nitrogen sources

Introduction

Lipases (glycerol ester hydrolases (E.C.3.1.1.3)) find applications in many areas of biotechnology due to their ability to catalyze enantioselective reactions with a wide range of substrates and their stability over wide variations of temperature and pH. Their major application in terms of quantity is as additives for laundry detergents. However, they also find applications in the food industry, in the production of aromas and the maturing of

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cheeses, in agrochemistry, in the production of insecticides and other pesticides, and in the pharmaceutical industry, in the synthesis of drugs such as naxopren and ibuprofen. Further, they can be used to modify fats and oils and in the synthesis of biosurfactants (*1–3*). New applications are being developed based on the ability of lipases to catalyze synthesis reactions in systems with very low concentrations of water. Such applications have the potential to replace chemical synthesis steps because lipases are capable of producing optically pure compounds either through synthetic reaction or via resolution of racemic mixtures (*4,5*).

With this upsurge in the development of applications of lipases, recently there has been much interest in the production of lipases by fungi, since their lipases are typically extracellular and therefore relatively easy to recover after the fermentation (*1*). Many species of the genus *Penicillium* have been noted as producers of lipases with desirable properties, which would have potential applications in a number of different areas. Lipases of *P. aurantiogriseum* (previously *P. cyclopium*) have been extensively studied. They find industrial application in the production of monoglycerides and have also been used to determine the concentration of triglycerides in blood and blood products (*6*).

Fungal lipases are typically produced in submerged fermentation processes, with the use of complex culture media that include as ingredients vitamins, amino acids and complex sources of organic compounds such as yeast extract, peptones, soy meal and corn steep liquor (*1,7–11*). For fungi of the genus *Penicillium*, better results have been obtained with organic nitrogen sources, or a combination of organic and inorganic sources, such as peptone or yeast extract with ammonium sulfate (*12*), than with inorganic compounds, such as ammonium sulfate, as the sole nitrogen source (*13,14*). Organic nitrogen sources can be costly and also cause difficulties in enzyme purification after the fermentation. As a result, despite the potential to produce lipases from *Penicillium*, the production process is often not economical. The objective of the current work was therefore to optimize lipase production by a locally isolated strain of *P. aurantiogriseum* on relatively low cost media.

Materials and Methods

Microorganism

The fungal strain was isolated from soybean oil and tested for lipase production on agar plates that contained (per liter of distilled water): agar 15 g, olive oil 10 mL, Rhodamine B 0.01 g and Tween 80 0.001 %. After 7 days of incubation at 28 °C, the plate was irradiated with UV light (350 nm), with a fluorescent halo indicating lipolytic activity. Lipase production was confirmed in a liquid medium (medium A, Table 1), as described below. The fungus was stored as a suspension of spores in glycerol 50 % at 4 °C. Prior to each fermentation it was subcultured in Czapek medium (*15*) with an additional 1 % of olive oil.

Culture media composition

The original liquid medium contained yeast extract 0.5 % and olive oil 1 %, and cultures were incubated in a shaker at 29 °C and at 120 rpm. The mineral salt solution contained (per liter of distilled water): KNO_3 2.0 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; K_2HPO_4 1.0 g; $ZnSO_4 \cdot 7H_2O$ 0.44 g; $FeSO \cdot 7H_2O$ 1.1 g and $MnSO_4 \cdot 7H_2O$ 0.2 g. In the study of the effect of nitrogen source, seven different media were compared as shown in Table 1. In this experiment the carbon source was 1 % olive oil and the media were prepared in phosphate buffer (pH=7.0, 100 mM). In the study of carbon source, either 1 % of vegetable oil (olive, soybean, corn or sunflower) or 1.8 % of anhydrous glucose was added to medium F. The C/N ratio in these media was 2.5. In the experiments of the optimum temperature, 1 % of olive oil was added to medium F. All experiments were done with duplicate flasks, with the results reported as the mean of the duplicates.

Culture conditions

Cultures were carried out in Erlenmeyer flasks (500 mL), with 125 mL of medium. The inoculum was prepared in 125 mL Erlenmeyer flasks with 25 mL of medium. The cultures were inoculated with 1 mL of spores suspension (10^8 mL^{-1}) and the flasks were agitated at 120 rpm at 29 °C. After 48 h, the whole volume was transferred into 500 mL Erlenmeyer flasks with 125 mL of liquid, using the same conditions of temperature and agitation. At time intervals, samples were withdrawn from the growth medium and then filtered through four layers of cheese-cloth in order to obtain cell-free samples.

Analytical methods

Lipase activity was determined by the *p*NPP (*p*-nitrophenyl palmitate) method (*16*). The coefficient of extinction of *p*-nitrophenol (*p*NP), $1.5 \cdot 10^4$ L/mol/cm, was determined from the absorbance measured at 410 nm of standard solutions of *p*NP. One unit was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute at 37 °C, under the conditions of the assay. Activity tests were also done with other esters of *p*NP, namely, *p*-nitrophenol acetate (*p*NPA), *p*-nitrophenyl butyrate (*p*NPB), *p*-nitrophenyl caproate (*p*NPC) and *p*-nitrophenyl caprate (*p*NPCA). The titrimetric method was also used to confirm the lipolytic activity, using triolein or tributyrin (Sigma) as the substrate (*17*). For this method, one unit of lipase activity was the amount of the enzyme that produced 1 μmol of free fatty acids per minute, at 37 °C.

Protein was determined by the method of Bradford (*18*). The fatty acid compositions of corn, soy, sunflower and olive oils were determined by gas chromatography, with a 15 % DEGS column, hexane as the solvent for the samples and nitrogen as the carrier gas. The column temperature was 180 °C.

Results

Preliminary studies

The fungal strain isolated from soybean oil presented lipolytic activity after 7 days of incubation at 28 °C on agar plates containing olive oil and Rhodamine. It

was later identified as *Penicillium aurantiogriseum* Dierckx by the Fungal Culture Collection, FIOCRUZ (Rio de Janeiro, Brazil) and deposited as IOC 4212. Lipase production was confirmed by submerged cultivation in a medium composed of yeast extract 0.5 % and olive oil 1.0 % (medium A). A maximum volumetric activity of 0.4 U/mL and a maximum specific activity of 22 U/mg were achieved after 48 h.

Presumptive identification of lipase

Given that *P. aurantiogriseum* (*P. cyclopium*) has been reported as producing three lipases, the substrate specificity was briefly investigated. With the same enzyme concentration, activities of 92 U/mL with *p*NPCA and 87 U/mL with *p*NPP were obtained while in a comparison of relative activities on triglycerides, the activity with tributyrin was 5 times greater than that with triolein. These results suggest that lipase III is the dominant lipase in the crude extract, given the facts that: firstly, neither lipase I nor lipase II hydrolyze mid-length esters of *p*-nitrophenol; and, secondly, lipase III has a 5 times greater activity on tributyrin than on triolein (*19*) whereas lipase I is only twice more active on tributyrin than on triolein (*20*). Note that lipase II is only capable of hydrolyzing mono- or diglycerides (*11*). However, since this work was done on the crude extract and not on purified fractions, the general terms lipase and lipolytic activity are used in the rest of this paper.

Effect of nitrogen source

To increase lipase production by the fungus and considering that high nitrogen concentrations are typically used for the production of fungal lipases (*21*), medium A was supplemented with various organic and inorganic nitrogen sources (Table 1). Supplementation was most effective in the case of addition of inorganic salts, with the highest lipolytic activity (12 U/mL) obtained after 72 h with medium F (Fig. 1). However, with KNO_3 as the sole source of nitrogen (medium G) the maximum lipolytic activity was relatively low (3.5 U/mL after 72 h). Since growth and lipase production did occur on this medium, KNO_3 was included in the calculation of the C/N ratio for all media. Both of the media supplemented only with organic sources (C and D) performed similarly to medium A. In medium E, which contains yeast extract in addition to ammonium sulfate, the lipase production was high (9 U/mL).

Table 1. Composition of culture medium for the study of the effect of nitrogen source

Medium	C/N Ratio	Composition (mass per volume ratio percentage)
A	14.1	yeast extract 0.5 %
B	9.1	yeast extract 0.5 % in salt solution
C	2.5	yeast extract 0.5 %, casein peptone 2.0 % in salt solution
D	2.5	yeast extract 0.5 %, meat peptone 2.0 % in salt solution
E	2.5	yeast extract 0.5 %, $(NH_4)_2SO_4$ 1.0 % in salt solution
F	2.5	$(NH_4)_2SO_4$ 1.0 % in salt solution
G	2.5	KNO_3 2.0 % in salt solution

The specific activity was highest for the medium containing only inorganic nitrogen sources (297 U/mg, medium F), followed by the medium containing the combination of inorganic salts and yeast extract (130 U/mg, medium E). The specific activities attained with the other media were relatively low (59, 10 and 25 U/mg for media A, C and D, respectively). The lower specific activity values obtained for the fungus cultivated in media containing polypeptides could be due to the release of other proteins into the medium.

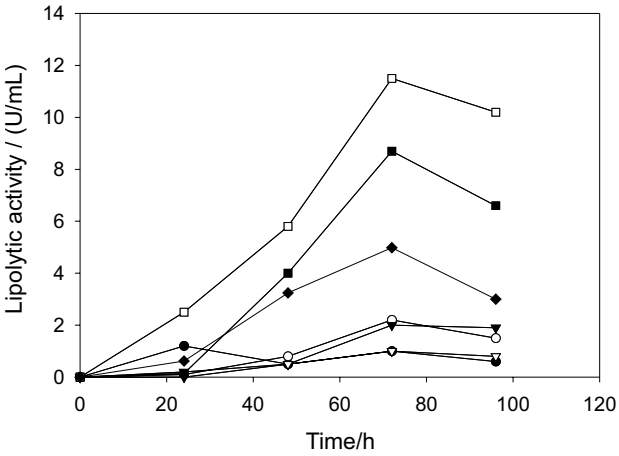

Fig. 1. Effect of nitrogen source on the production of lipases by *Penicillium aurantiogriseum* Dierckx IOC 4212. The culture media are as shown in Table 1: (●) A – yeast extract; (○) B – salt solution and yeast extract; (▼) C – salt solution, yeast extract and casein peptone; (▽) D – salt solution, yeast extract and meat peptone; (■) E – salt solution, yeast extract and ammonium sulfate, (□) F – salt solution and ammonium sulfate and (♦) G – salt solution and potassium nitrate 2 %

Given that a higher lipolytic activity was obtained in a relatively short fermentation period (72 h) in a medium containing only ammonium sulfate and potassium nitrate as nitrogen sources (medium F), this medium was used as the base for the subsequent experiments.

Effect of carbon source

Vegetable oils such as soybean, corn, sunflower, olive, palm and cottonseed oils, amongst others, are cited as inducers of lipase production, comprising, at times, the sole source of carbon in the medium (*11*,*13*,*22*–*26*). To evaluate the effect of carbon source on the production of lipase by *P. aurantiogriseum*, cultures were done with the addition of corn, soybean, sunflower and olive oils, each separately. For comparison, a culture was also grown with glucose as the sole carbon source. Considering 72 h of fermentation time, the lipolytic activities in the culture broth were approximately 5 U/mL when sunflower, corn and soybean oils were used, compared to an activity of 12.5 U/mL in the presence of olive oil (Fig. 2). Lipolytic activity was not detected when glucose was the sole carbon source, confirming that the presence of an inducer is necessary for *P. aurantiogriseum* to produce lipases (*11*). The maximum specific

lipase activities obtained in these experiments were: 192 U/mg after 48 h with olive oil; 114 U/mg after 72 h with soybean oil; 220 U/mg after 112 h with sunflower oil and 138 U/mg after 112 h with corn oil. Therefore, although the volumetric activities were quite different in the presence of the various oils, the maximum specific activity did not vary so greatly. However, the time at which this maximum occurred did vary.

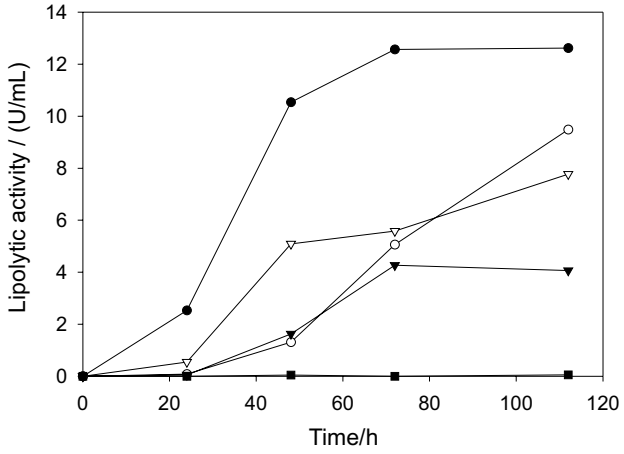

Fig. 2. Effect of carbon source on the production of lipases by *Penicillium aurantiogriseum* Dierckx IOC 4212. Inducers or carbon sources: 1 % of the following vegetable oils: olive (●), soy (▼), corn (○), sunflower (▽) and glucose (■)

Fatty acids present in the greatest proportions in these oils are oleic and linoleic acids. Triglycerides of olive oil and corn oil, which were the two best carbon sources for lipase production, contain 28 and 25 % oleic acid, respectively, while those of sunflower oil and soy oil contain 17 and 13 % oleic acid, respectively. On the other hand, sunflower, soy and corn oils have higher percentages of linoleic acid (51, 27 and 33 %, respectively), while olive oil contains only 3 % linoleic acid. Better lipase production appears therefore to be correlated with a higher content of oleic acid in the oil (*21*).

Due to the higher volumetric activity obtained with olive oil, it was maintained in the fermentation medium for the remaining experiments, but it is important to note that sunflower oil, a less expensive substrate, could also be used for industrial scale production.

Effect of the nitrogen source concentration

For fungi, relatively high nitrogen concentrations (and therefore lower C/N ratios) are typically required in order to favor the production of lipases over the production of other enzymes (*13*,*21*,*23*). To evaluate the effect of the concentration of the nitrogen source, cultures were done with increasing concentrations of ammonium sulfate while the concentration of olive oil was maintained constant, in such a manner as to give C/N ratios of 1, 2.5, 5 and 10. The maximum lipolytic activity obtained with a C/N ratio of 5 was 17 U/mL and with a C/N ratio of 2.5 it was 13 U/mL (Fig. 3). With an even higher C/N ratio of 10, lipase production was relatively poor. With a C/N ratio of 1 the amount of lipase produced was not significant and the mycelial growth in the flask was very poor.

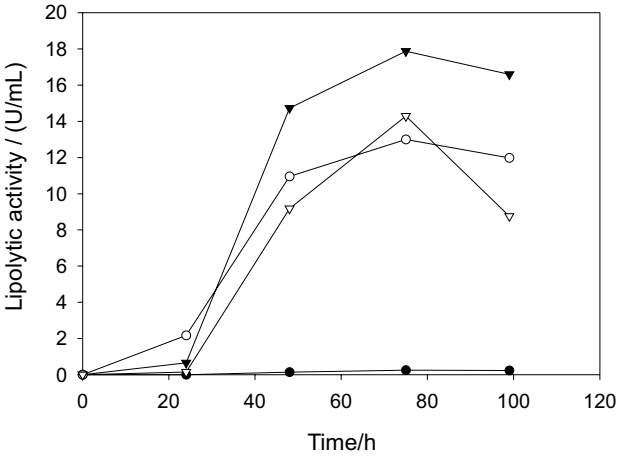

Fig. 3. Effect of the ammonium sulfate concentration on the production of lipases by *Penicillium aurantiogriseum* Dierckx IOC 4212. C/N ratios of: 1 (●); 2.5 (○); 5 (▼) and 10 (▽)

The peak volumetric activity was obtained between 72 and 96 h for all C/N ratios. The peak of the specific activity occurred after 48 h with initial C/N ratios of 2.5 and 5, and between 48 and 72 h with an initial C/N ratio of 10. The highest specific activity of 113 U/mg was obtained with a C/N ratio of 5. Reasonably high values of 103 and 83 U/mg were obtained for C/N ratios of 2.5 and 10, respectively.

Effect of the carbon source concentration

The effect of the concentration of the carbon source on lipase production was studied with the addition of different concentrations (0.5, 1, 1.5 and 2.0 %) of olive oil. The concentrations of ammonium sulfate and potassium nitrate were maintained constant. The carbon source concentration has a strong influence on the production of lipase by *P. aurantiogriseum*, as shown in Fig. 4. With an increase in olive oil concentration there was a

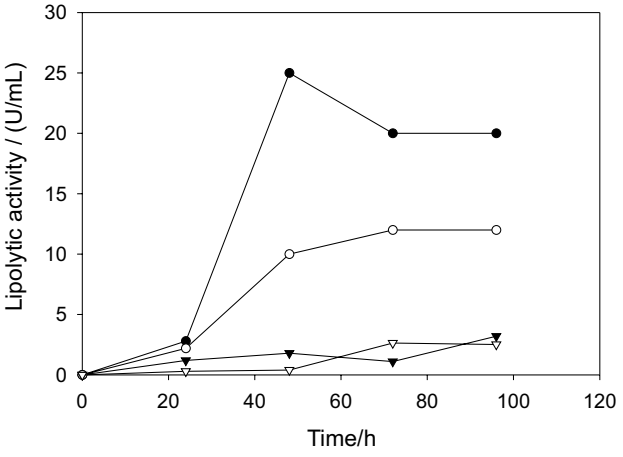

Fig. 4. Effect of the concentration of olive oil on the production of lipases by *Penicillium aurantiogriseum* Dierckx IOC 4212. Concentrations of olive oil: 0.5 % (●); 1.0 % (○); 1.5 % (▼) and 2.0 % (▽)

decrease in the peak lipolytic activity attained. Fermentations done with 1.5 and 2 % olive oil had much lower peak activities, indicating an inhibitory effect on the production of lipase by *P. aurantiogriseum*.

Effect of the incubation temperature

Cultures were incubated at 26, 29 and 32 °C (Fig. 5). At 26 and 32 °C, lipase production was one to two orders of magnitude less than the production obtained at 29 °C. These results make temperature control during the fermentation a critical factor, since relatively small variations in temperature can greatly reduce the productivity of the system.

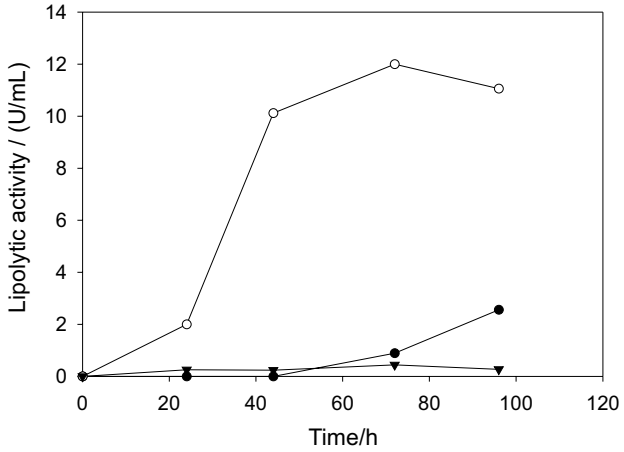

Fig. 5. Effect of temperature on the production of lipases by *Penicillium auratiogriseum* Dierckx IOC 4212. Cultivation temperatures: 26 °C (●); 29 °C (○) and 32 °C (▼)

Discussion

The current work reports on the initial characterization of the production of lipase by a strain of *Penicillium aurantiogriseum.* This is the first time that higher lipolytic activities and shorter production times have been achieved for a strain of *Pencillium* with an inorganic medium. Complex nitrogen sources, such as yeast extract, peptones, soybean meal and corn steep liquor, have traditionally been used for lipase production (*1*,*7*,*8*,*10*,*11*). In some cases amino acids and other trace nutrients have been added to these complex media or have been used in combination with inorganic salts (*9*). Chahinian *et al.* (*11*), who used corn steep liquor as the nitrogen source, reported two lipases for *P. cyclopium*, with activities of 110 U/mL (lipase I) and 10 U/mL (lipase II). However, it is difficult to compare the maximum lipolytic activity of *P. aurantiogriseum* with that obtained for *P. cyclopium* because different substrates were used to determine the lipase activity. In other studies, *P. roqueforti* produced lipolytic activities of around 10 U/mL on both bactopeptone and ammonium sulfate, but the maximum was achieved two days earlier with the bactopeptone (*27*). *P. citrinum* produced a higher volumetric yield with yeast extract, although the specific activity was greater when ammonium sulfate was the sole nitrogen source (*13*). Besides being less expensive than organic nitrogen sources, a further advantage of using inorganic nitrogen sources is that they avoid the problems with enzyme purification that can occur with more complex medium components such as yeast extract and peptones (*1*).

The type and concentration of carbon source are also important. The favorable effect of olive oil and oleic acid on lipase production has been observed by various researchers (*11*,*13*,*28*). Induction appears to be influenced not only by the lengths of fatty acids in the triglycerols, but also by the number of unsaturations. However, the manner by which these compounds influence lipase biosynthesis is not well understood (*29*). Although studies on the effect of olive oil concentration have not previously been done for *P. aurantiogriseum*, the inhibitory effect at higher concentrations has previously been noted for other species of *Penicillium*. Olive oil concentrations between 0.5 and 1 % gave maximum lipase activities of 13 U/mL for *P. restrictum* (*14*), and with *P. wortmanii*, activities of 10 and 5 U/mL were obtained with 0.5 and 1 % olive oil, respectively (*30*). The inhibition of the synthesis of lipases at higher olive oil concentrations could be due to poorer oxygen transfer into the medium. Low oxygen supplies can alter fungal metabolism and, consequently, the production of lipases (*31*).

The influence of temperature on the production of lipases by fungi has not been extensively studied but appears to be a crucial parameter. For example, for *Aspergillus niger* the optimum temperature for lipase production is 24 °C and differences as little as 1 °C can considerably decrease the yield (*29*). Cultures for production of lipases by fungi of the genus *Penicillium* are generally incubated at between 25 and 30 °C, most often at 28 °C (*11*,*13*,*14*,*27*).

Conclusion

After optimizing the media composition and culture growth conditions for the production of an extracellular lipase by *P. aurantiogriseum*, we achieved a maximum of 25 U/mL. These results are promising because this strain produces lipase in an inexpensive inorganic medium and we succeeded in increasing the maximum lipolytic activity approximately 62-fold over the initial values obtained with the non-optimized medium.

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Utjecaj dušika i ugljika na proizvodnju lipaze plijesni *Penicillium aurantiogriseum*

Sažetak

Divlji soj, izoliran iz sojinog ulja i identificiran kao *P. aurantiogriseum*, pokazivao je volumetrijsku lipaznu aktivnost od 0,4 U/mL u submerznoj kulturi s 0,5 % ekstrakta kvasca i 1 % maslinova ulja. Ispitivan je utjecaj izvora dušika dodavanjem peptona kazeina, peptona mesa, ekstrakta kvasca ili amonijeva sulfata mediju koji je već sadržavao kalijev nitrat i druge mineralne soli. Najbolje iskorištenje (od 13 U/mL, nakon 72 h) dobiveno je u kulturi s amonijevim sulfatom. Dodatkom amonijeva sulfata dvostruko je uvećan odnos C/N od 2,5 do 5, a lipolitička aktivnost iznosila 18 U/mL. Kao izvor ugljika ispitani su maslinovo, kukuruzno, sojino i suncokretovo ulje, pri čemu je dodatak od 1 % maslinova ulja omogućio najbolje iskorištenje, a lipolitička aktivnost iznosila 25 U/mL nakon 48 sati. Najbolja je proizvodnja postignuta pri 29 °C unutar ispitivana raspona od 26 do 32 °C. Rezultati ohrabruju jer ovaj soj proizvodi lipazu u jeftinom anorganskom mediju, a autori su uspjeli povećati lipolitičku aktivnost 62 puta u usporedbi s uzgojem u neoptimiranoj kulturi.