Lipase Production Using Microorganisms from Different Agro-Industrial By-Products

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Abstract

This work is focused on the study of different microorganisms to obtain a high level of lipase. It was selected 6 different microorganisms: Geotrichum candidum NRRLY-552, Geotrichum sp., Fusarium oxysporum and three wild yeasts from Brazil rainforest. Fermentation carried out using agro industrial by-products like corn steep liquor and protein hydrolysed in shaken flasks under different conditions according to each microorganism. The best results obtained were 17 U.cm^{-3} (Geotrichum candidum NRRLY-552) and 11 U.cm^{-3} (Geotrichum sp.) using corn steep liquor and soybean oil, 3.60 U.cm^{-3} (AAV1 wild yeast) using yeast hydrolysed and soybean oil and 3.90 U.cm^{-3} (Fusarium oxysporum) using peptone and soybean oil. This work showed that imperfect fungi Geotrichum has better potencial to produce lipase compared to other microorganisms evaluated. However, lipase from Fusarium oxysporum showed a good stability in high pH which is very interesting to new applications of lipase in alkaline conditions.

Keywords: lipase; corn steep liquor; soybean oil

1. Introduction

Fungi are microorganisms preferred as producers industrial lipases, because they usually produce extracellular enzymes, which facilitates the extraction of the fermentation medium. In addition fungi enzymes are more stable and have more diverse properties compared to lipases from other sources (Ciafardini, Zullo & Iride 2006). Although a large number of studies concerning with the application of lipase, some research groups have been investigating isolation and optimization of production of lipases from different types of microorganisms (Pastore et al. 2003).

Fungal lipase production has been widely studied, such as that obtained from fungi of the genus Geotrichum, with optimization of culture media for *Geotrichum* sp. in shaken flasks using industrial waste (Burkert et al., 2004, Maldonado et al. 2012A; Yan Yan & 2008) and in stirred reactor (Kamimura 1999); optimization of culture media for *Geotrichum candidum* in NRRLY-552 shaken flasks with synthetic medium (Loo et al. 2007, Maldonado et al. 2012B), in stirred and air-lift reactor (Burkert et al. 2005); using industrial wastes in shaken flasks , stirred and air-lift reactor (Maldonado, 2006). The lipase production by other fungi was also investigated, such as *Penicillium* (Miranda et al. 1999; Maldonado et al. 2001; Papagianni, 2013), *Fusarium* (Prazeres 2006; Pio M & 2008; Oliveira et al, 2013).

Yeasts are also widely studied to the production of different types of enzymes. Yeasts are unicellular fungal and can be found in different environments. Whereas in Brazil, with a great biodiversity, is expected that a large number of microbial species present in our country have not been described yet. Looking to tap this potential (Hernalsteens 2006) conducted a screening of different types of yeasts from samples collected in different parts of Brazil to evaluate the ability of these strains to produce enzymes. (Goldeback & Maugeri, 2013) continued these studies to verify the potential production of lipase from 44 selected yeast of the previous study.

Lipase production was studied from fungi *Geotrichum candidum* NRRLY-552, *Geotrichum* sp., *Fusarium oxysporum* and Brazilian wild yeasts. The second and third mentioned fungi were isolated by the Laboratory of Food Biochemistry (University of Campinas) and yeasts in the Laboratory of Bioprocess Engineering (University of Campinas).

2. Materials and Methods

2.1. Evaluation of lipase production by fungi *Geotrichum*

Production of lipase by *Geotrichum candidum* NRRLY-552 was studied in a previous study (Maldonado 2006), which were obtained optimum conditions for enzyme production using this microorganism. Then it was carried out an experiment using the optimized conditions: 8.0% w/v of corn steep liquor, 0.6% w/v of soybean oil, initial pH 7.0, 30°C, agitation of 250 rpm (Maldonado in 2006). The inoculum was produced using a synthetic medium with incubation time of 15 hours (Burkert, 2003) to check whether the available strain had the same characteristics as the original.

Production of lipase by *Geotrichum* sp. had been studied before obtaining optimized medium for lipase production using corn steep liquor (Burkert et al 2004). A preliminary test was carried out using fermentation medium with conditions similar to those described in the work cited above, changing the way of obtaining the inoculum. The microorganism was grown in a Petri dish containing Yeast Malt medium for 48 hours at 30 ° C, after which two circular areas of 1.0 cm in diameter were transferred to flask containing 100 mL medium of inoculum (5% w/v peptone, 0.1% w/v sodium nitrate, 0.1% w/v magnesium sulfate and 1.0% w/v soybean oil) and incubated for 15 hours. Aliquots of 10 mL of inoculum medium were transferred to flasks containing fermentation medium with 12% of corn steep liquor, 0.6% of soybean oil and an initial pH equal to 6.0 (Maldonado 2006). The fermentation was carried out by 16 hours at 30 ° C and 150 rpm and lipolytic activity and pH were evaluated during the fermentation time.

2.2. Evaluation of lipase production by Brazilian wild yeasts

Three yeasts isolated in the Laboratory of Bioprocess Engineering (University of Campinas) were selected in a previous work (Goldeback & Maugeri 2013) as yeasts with potential for production of lipases. So, it evaluated the ability of these three yeasts to produce lipase from different sources of nitrogen. The yeasts were coded AY3 (isolated from cheese), AAV1 and AC02 (isolated from the Atlantic rainforest). Fermentations were performed with three different culture media:

- Medium 1 - 5.0% w/v peptone, 0.1% w/v yeast extract, 0.1% w/v magnesium sulfate, 0.1% w/v sodium nitrate and 1.0% w/v soybean oil.
- Medium 2 - 12% w/v of yeast hydrolysate (Prodex-lac ®) and 1.0% w/v soybean oil;
- Medium 3 - 15% w/v of corn steep liquor and 1.0% w/v soybean oil.

Assays were carried out at 30°C, agitation of 150 rpm and initial pH 6,0. Inoculum was obtained from the yeast strain incubated in test tube containing 10 mL of its medium and incubated under the same fermentation conditions for 24 hours. After this period, the contents of each tube was poured into flasks containing fermentation medium and incubated for 48 hours. The lipolytic activity and pH were evaluated.

After preliminary tests, the yeast AAV1 (isolated from Atlantic rainforest) was selected for further studies. So, a Placket-Burman experimental design with 16 trials + 3 central points was made to evaluate the effect of 10 variables on the production of lipase. The variables studied were: concentration of sucrose, yeast extract, K_2HPO_4, NH_4Cl, KCI and $MgSO_4$ in the inoculum medium, inoculum time, pH, concentration of soybean oil and ratio between concentrations of yeast hydrolysate/corn steep liquor in the fermentation medium. The levels are presented on Table 1, with lipolytic activity and pH evaluated during fermentation time such as responses of the process.

2.3. Evaluation of lipase production by *Fusarium oxysporum*

In a first test for lipase production by *Fusarium oxysporum* were used four fermentation medium:

- Medium 1 - 0.06% w/v KH_2PO_4, 0.02% w/v $MgSO_4$, 0.02% w/v KCl, 0.01% w/v of $FeSO_4.7H_2O$, 1.0% w/v soybean oil (Pio & Macedo, 2008);
- Medium 2 - 5.0% w /v peptone, 0.1% w/v yeast extract, 0.1% w/v $MgSO_4$, 0.1% w/v $NaNO_3$, 1.0% w/v soybean oil (Goldeback & Maugeri, 2013);

- Medium 3 - 12% w/v of yeast hydrolysate, 1.0% w/v soybean oil.
- Medium 4 - 15% w/v of corn steep liquor, 1.0% w/v soybean oil.

All fermentation media have an initial pH adjusted to 7.2 and the fermentations were carried out at 30°C and 150 rpm for 72 hours. Inoculum was obtained from microorganism cultivated for 72 hours at 30°C in a test tube containing PDA medium (Potato Dextrose Agar). Thereafter, the spores of the microorganism were re-suspended with 5 mL of distilled water and transferred to flasks containing fermentation medium.

From the results obtained in the first test, performed by a second test to evaluate two fermentation media:

- Medium 1 - 5.0% w/v peptone, 0.1% w/v $MgSO_4$, 0.1% w/v $NaNO_3$, 1.0% w/v soybean oil;
- Medium 2 - 8% w/v of corn steep liquor, 1.0% w/v soybean oil.

In this test, the inoculum was obtained by optimized methodology (Burket, 2003) and it had the same composition as the fermentation medium. The pH of the fermentation medium was changed to 6.0 and the lipase activity was analyzed at 24, 48, 72 and 170 hours by titrimetric analysis (Kamimura et al. 1999).

3. Results and Discussion

3.1. Evaluation of lipase production by fungi *Geotrichum*

The results obtained in the experiment with optimized fermentation medium to produce lipase by *Geotrichum candidum* NRRLY-552 are shown in Figure 1, which it can be seen that all tests showed lipolytic activity very close, leading to a low standard deviation, since all tests were made in the same condition. The maximum lipase activity after 48 hours of fermentation was (17.0 ± 0.3) $U.cm^{-3}$. In previous work (Maldonado 2006) was observed the maximum production of lipase from *Geotrichum candidum* under the same conditions, between 16 and 24 $U.cm^{-3}$ after 48 hours of fermentation. The results presented in Figure 1 show that the strain used and the raw materials are suitable for the production of lipase for this microorganism.

The result obtained show the same level of enzyme activity obtained in the previous study (Maldonado et al 2012B), using synthetic medium which it was 20$U.cm^{-3}$. Although the same lipolytic activity obtained in the cost of production of lipase using corn steep liquor was 99% lower than in synthetic medium using peptone. In terms of productivity, this experiment has revealed an average value of 354 $U.cm^{-3}.h^{-1}$ in time of 48 hours of fermentation which it was so higher than 123.5 $U.cm^{-3}.h^{-1}$ found in the production of lipase of *Candida cylindracea* NRRLY-17506 (Brozzoli et al. 2009).

Results of lipase production with *Geotrichum* sp. are shown in Figure 2 and it can be seen that the lipolytic activity reached about 11 $U.cm^{-3}$ after 8 hours of fermentation. This result is lower than that obtained before - 18 $U.cm^{-3}$ on 48 hours (Burkert et al. 2004). Despite the lower results in terms of lipolytic activity, productivity was higher, because the fermentation time to obtain lipase activity was lower. In this study it was obtained a yield of 1.375 $U.cm^{-3}.h^{-1}$ as in the previous work was obtained 0.375 $U.cm^{-3}.h^{-1}$. An increase in fermentation time beyond 16 hours can lead to an increase in the production of the enzyme, according to Figure 2 there is an increase in the production of lipase from 12 to 16 hours, indicating the ability of this microorganism to continue producing enzyme under the conditions used. From these results, experimental designs were conducted to achieve a better condition for lipase production by *Geotrichum* sp. evaluating the conditions of the fermentation medium of previous work (Burkert et al. 2004) and the conditions of inoculum. The results are contained in another work (Maldonado et al. 2012 A).

3.2. Evaluation of lipase production by Brazilian wild yeasts

The results of the preliminary tests are shown in Table 2.Analyzing the responses, it appears that the production of lipase by the yeast AAV1 was two to three times higher than the other yeasts for the different sources of nitrogen used. (Goldeback & Maugeri, 2013) had found that among 44 pre-selected wild yeasts, the three yeast mentioned above were the largest producers of lipase when using peptone as nitrogen source. The above results show that these yeasts are also capable to use agro-industrial residues as source of nitrogen resulting in lipolytic activity levels compatible with those obtained with peptone and yeast extract.

Although the ability of these yeast to produce lipase, the lipolytic activity was very low for different applications (hydrolysis and etherification of vegetable oils for example). Thus, to continue the study chose to use only the yeast AAV1, which yielded better results.

Ten variables related to the inoculum medium and fermentation medium were evaluated in a Placket Burman experimental design type. Values used and answers, lipolytic activity and pH as a function of fermentation time, are contained in Table 3. The results presented in Table 3 show little difference to preliminary tests. The maximum lipase activity obtained was 3.22 $U.cm^{-3}$ after 48 hours of fermentation, showing that the different conditions evaluated did not promote significant improvement in the lipase activity of the yeast selected. The analysis of the main variables of the process after 48 hours is presented in Table 4. According to the analysis of effects it is noted that all variables in relation to the concentration of nutrients in the inoculum had no effect or had a negative effect on lipase activity within the ranges studied.

Compared with the results obtained in preliminary tests indicate it is possible to say that yeast had better adaptation and reached a higher lipolytic activity when cultivated in an inoculum medium equal to the fermentation medium, as it can be seen in Table 2. The introduction of nutrients sucrose, yeast extract and salts mentioned above were an attempt to improve the quality of the inoculum to increase the lipolytic activity of the yeast, but through the experimental design it was found that these nutrients are not needed for the production of lipase by this yeast and did not lead to an improvement in lipase activity. Inoculum time, pH and concentration of soybean oil did not show significant effect in the range evaluated

The only variable exploited that showed an interesting result of the lipase activity was the ratio between concentrations of yeast hydrolysate and corn steep liquor. Note that when substitute yeast hydrolysate to corn steep liquor there was an increase in lipase activity, suggesting that this substrate is better for the production of lipase than the yeast.

3.3. Evaluation of lipase production by *Fusarium oxysporum*

The results obtained in the first test for lipase production by *Fusarium oxysporum* are shown in Table 5. The results of lipase production by *Fusarium oxysporium* were very low, with a maximum enzymatic activity of 1.0 U. cm^{-3}. Another study using the same fungus (Prazeres,Cruz &Pastore, 2006) produced 5.0 U.cm-^{3} after 96 hours of fermentation. Since this is a fungus, the inoculation procedure adopted, made by a spore suspension obtained directly from microorganism cultured on solid medium and then transferred to the fermentation medium may have led to a worse adaptation of the fungus to the fermentation medium. This can be explained by the growth conditions which are quite different in the two types of medium. Furthermore, re-suspending the spores in distilled water and inoculating a predetermined volume of suspension can lead to large variation in the initial count of fungal cells, which leads to a significant impact on the results of the fermentation, as was previously observed on lipase production by *Geotrichum candidum* (Burkert 2003).

Thus, a second test was carried out to produce lipase by *Fusarium oxysporum* using the conditions of the inoculum modified (Burkert 2003), in that after culture the microorganism in a solid medium, a circular area of 1 cm diameter was transferred to a inoculum medium and it was incubated for 15 hours. After this time it was made the inoculation of the microorganism in the fermentation medium. The results of the second test are shown in Table 6.

It is observed by the second experiment, the change in the procedure of inoculum led to an increase in lipolytic activity. Comparing tables 5 and 6 it is possible to see that media with the same nitrogen sources showed better results in the second experiment with changes in the conditions of inoculum. With peptone as nitrogen source there was an increase of about 8 times in lipase activity after 72 hours of fermentation, which increased from 0.5 (medium 2, Table 5) to 3.9 $U.cm^{-3}$ (medium 1, Table 6). Towards corn steep liquor, the increase was about 3 times of the enzyme at the same time, now 0.7 (medium 4, Table 5) to 2.03 U. cm^{-3} (medium 2, Table 6). These results suggested that *Fusarium oxysporum* has the potential to produce higher levels of lipase, since the selected best culture conditions for this microorganism.

Another interesting result is the data of lipase activity after 170 hours of incubation. Note that the pH of the fermentation medium was above 8.0 after 72 hours and continued to rise in the process range up to 170 hours of processing, however, the lipase activity was not significantly damaged by this high pH in the medium fermentation, even after a long period of time, observing a lipolytic activity almost similar in both periods analyzed (72 and 170 hours) for a medium 1 (peptone) and an increase in lipase activity in the case medium 2 (corn steep liquor). This result shows that the lipase produced by *Fusarium oxysporum* shows much greater stability to alkaline pH when compared with *Geotrichum candidum* lipase obtained by NRRLY-552, whose half-life decreases quite sharply when this enzyme is at pH above 8.0 (Maldonado 2006).

This result indicates that *Fusarium oxysporum* lipase may have various applications because of its feature of greater stability in alkaline pH.

4. Conclusion

The experiments showed that the fungi *Geotrichum* used showed better results for lipase activity in evaluated conditions. However, the production of lipase by other microorganisms can be increased using other concentration ranges, other nutrients and growing conditions in order to increase the lipolytic activity. Furthermore, one can evaluate the specificity of lipase obtained by these microorganisms which can be applied to other processes. For example, in the case of the *Fusarium oxysporum* lipase produced has showed good stability to alkaline pH, a characteristic that can be exploited in many reaction schemes.

5. References

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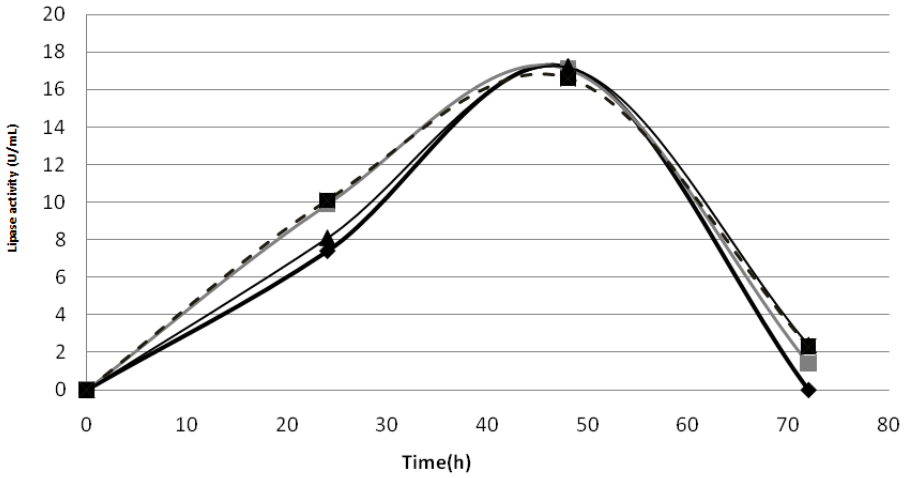

Figure 1. Lipase production from *Geotrichum candidum* NRRLY-552 with corn steep liquor (8.0% w/v), soy oil (0.6% w/v), initial pH 7.0, 30°C and 250 rpm.

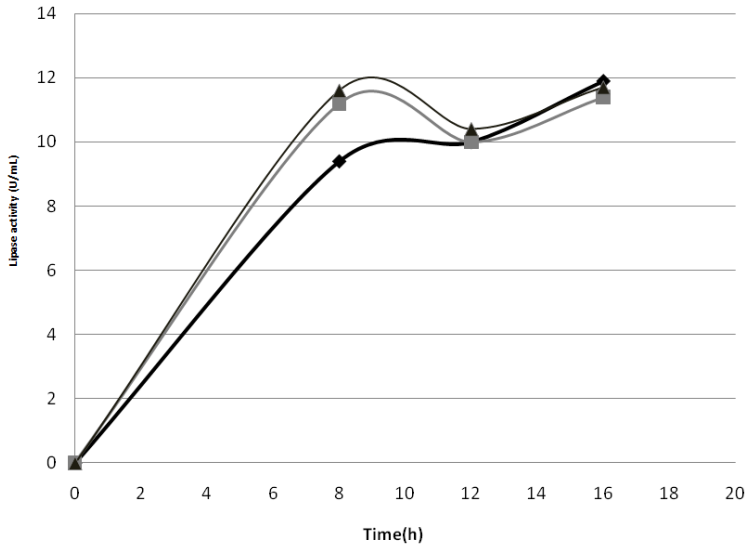

Figure 2. Lipase production by *Geotrichum* sp. with corn steep liquor (12% w/v), soy oil (0.6% w/v), initial pH 6.0, 30°C and 150 rpm.

Table 1 - Levels of the variables studied for lipase production by yeast AAV1

Variable	-1	0	+1
Sucrose (%)	10	20	30
Yeast extract (%)	2.0	5.0	8.0
K_2HPO_4 (%)	0	2.0	4.0
NH_4Cl (%)	0	1.0	2.0
KCl (%)	0	1.0	2.0
$MgSO_4$ (%)	0	0.5	1.0
Inoculum time (h)	12	18	24
Initial pH	5.0	6.0	7.0
Soybean oil (%)	0.5	1.0	1.5
Protein hydrolysate/CSL (%w/v/% w/v)	12/0	6/6	0/12

Table 2 - Lipase activity and the final pH for lipase production by wild yeasts after 24 hours fermentation

Trial	Yeast	Fermentation medium*	Lipolytic activity (U/mL)	pH
1		1	1.7 ± 0.6	7.77
2	AY3	2	1.0 ± 0.2	7.14
3		3	1.7 ± 0.3	6.50
4		1	3.5 ± 0.1	7.35
5	AAV1	2	3.6 ± 1.3	7.08
6		3	3.0 ± 0.5	6.34
7		1	1.0 ± 0.6	7.84
8	AC02	2	1.8 ± 0.9	7.27
9		3	1.6 ± 0.2	6.09

* Nitrogen source used: Medium 1 = Peptone (5%); Medium 2 = hydrolysed yeast (12%); Medium 3 = corn steep liquor (15%).

Table 3 – Lipolytic activity and pH to lipase production from Brazilian wild yeast AAV1 in shaken flasks at 30°C and 150 rpm

Trial	Sacch arose (%w/v)	Yeast extract (%w/v)	K_2HPO_4 (% w/v)	NH_4Cl (% wv)	KCl (%w/v)	$MgSO_4$ (%w/v)	Inoculum time (h)	pH	Soybean oil (%w/v)	Prodex-lac® /CSL (%/%w/v)	Lipase ($U.cm^{-3}$)		pH	
											24h	48h	24h	48h
1	30(1)	2.0(-1)	0(-1)	0(-1)	4(1)	0(-1)	12(-1)	7(1)	1.5(1)	12/0(-1)	0.61	0.18	6.74	6.90
2	30(1)	8.0(1)	0(-1)	0(-1)	0(-1)	2(1)	12(-1)	5(-1)	1.5(1)	0/12(1)	0.71	3.22	5.17	6.95
3	30(1)	8.0(1)	4(1)	0(-1)	0(-1)	0(-1)	24(1)	5(-1)	0.5(-1)	0/12(1)	0.36	1.41	5.25	8.05
4	30(1)	8.0(1)	4(1)	4(1)	0(-1)	0(-1)	12(-1)	7(1)	0.5(-1)	12/0(-1)	0.00	0.00	6.86	7.85
5	10(-1)	8.0(1)	4(1)	4(1)	4(1)	0(-1)	12(-1)	5(-1)	1.5(1)	12/0(-1)	0.08	0.00	5.15	7.83
6	30(1)	2.0(-1)	4(1)	4(1)	4(1)	2(1)	12(-1)	5(-1)	0.5(-1)	0/12(1)	0.30	0.00	5.13	7.91
7	10(-1)	8.0(1)	0(-1)	4(1)	4(1)	2(1)	24(1)	5(-1)	0.5(-1)	12/0(-1)	0.00	0.03	5.08	6.97
8	30(1)	2.0(-1)	4(1)	0(-1)	4(1)	2(1)	24(1)	7(1)	0.5(-1)	12/0(-1)	1.00	0.42	6.68	8.16
9	30(1)	8.0(1)	0(-1)	4(1)	0(-1)	2(1)	24(1)	7(1)	1.5(1)	12/0(-1)	0.38	0.22	6.61	6.97
10	10(-1)	8.0(1)	4(1)	0(-1)	4(1)	0(-1)	24(1)	7(1)	1.5(1)	0/12(1)	2.17	1.19	6.85	8.05
11	10(-1)	2.0(-1)	4(1)	4(1)	0(-1)	2(1)	12(-1)	7(1)	1.5(1)	0/12(1)	1.28	1.93	6.83	7.82
12	30(1)	2.0(-1)	0(-1)	4(1)	4(1)	0(-1)	24(1)	5(-1)	1.5(1)	0/12(1)	2.16	0.00	5.27	6.92
13	10(-1)	8.0(1)	0(-1)	0(-1)	4(1)	2(1)	12(-1)	7(1)	0.5(-1)	0/12(1)	0.83	0.80	6.73	6.93
14	10(-1)	2.0(-1)	4(1)	0(-1)	0(-1)	2(1)	24(1)	5(-1)	1.5(1)	12/0(-1)	0.00	1.98	5.58	8.23
15	10(-1)	2.0(-1)	0(-1)	4(1)	0(-1)	0(-1)	24(1)	7(1)	0.5(-1)	0/12(1)	0.00	2.92	6.81	6.90
16	10(-1)	2.0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	12(-1)	5(-1)	0.5(-1)	12/0(-1)	0.00	1.46	5.21	7.06
17	20(0)	5.0(0)	2(0)	2(0)	2(0)	1(0)	18(0)	6(0)	1.0(0)	6/6(0)	0.00	2.12	6.06	7.77
18	20(0)	5.0(0)	2(0)	2(0)	2(0)	1(0)	18(0)	6(0)	1.0(0)	6/6(0)	0.00	1.26	6.07	7.78
19	20(0))	5.0(0)	2(0)	2(0)	2(0)	1(0)	18(0)	6(0)	1.0(0)	6/6(0)	0.00	2.48	6.78	7.78

Table 4 - Main effects on the lipolytic activity of yeast wild AAV1 after 48 hours of fermentation

Variable	Effect (U/mL)	Standard error (U/mL)	t(8)	p-value
Average	1.14	0.20	5.62	<0.01*
Sucrose	-0.61	0.44	-1.38	0.21
Yeast extract	-0.25	0.44	-0.57	0.58
K_2HPO_4	-0.24	0.44	-0.54	0.61
NH_4Cl	-0.70	0.44	-1.57	0.15*
KCl	-1.32	0.44	-2.98	0.02*
$MgSO_4$	0.18	0.44	0.41	0.69
Inoculum time	0.07	0.44	0.16	0.87
pH	-0.06	0.44	-0.12	0.90
Soybean oil	0.21	0.44	0.48	0.65
Protein hydrolysate/CSL	0.90	0.44	2.03	0.08*

*Statistically significant at $p \leq 0.15$.

Table 5 - Production of lipase by *Fusarium oxysporum* with different fermentation media in shaken flasks at 30°C and 150 rpm

Fermentation medium	Lipolytic activity 24h (U/mL)	Lipolytic activity 48h (U/mL)	Lipolytic activity 72h (U/mL)	pH 24h	pH 48h	pH 72h
1	0.00	0.00	0.3 ± 0.2	6.77	7.25	7.43
2	0.76 ± 0.09	1.0 ± 0.1	0.5 ± 0.3	7.83	7.87	8.19
3	0.00	0.00	0.00	7.42	7.72	7.55
4	0.00	0.00	0.7 ± 0.3	7.14	8.16	8.21

Table 6 - Production of lipase by *Fusarium oxysporum* in synthetic and industrial media in shaken flasks at 30°C and 150 rpm

Fermentation medium	Lipolytic Activity 24h (U/mL)	Lipolytic Activity 48h (U/mL)	Lipolytic Activity 72h (U/mL)	Lipolytic Activity 170h (U/mL)	pH 24h	pH 48h	pH 72h	pH 170h
1	1.8 ± 0.6	1.6 ± 0.2	3.9 ± 0.2	3.32 ± 0.03	7.93	7.92	8.30	8.68
2	1.8 ± 0.2	0.94 ± 0.04	2.03 ± 0.08	3.38 ± 0.00	7.86	7.99	8.09	8.86

* Source of nitrogen: medium 1 = peptone, medium 2 = corn steep liquor; carbon source = soybean oil