

## Research Article

# Effect of Plant Oils upon Lipase and Citric Acid Production in *Yarrowia lipolytica* Yeast

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The nonconventional yeast *Yarrowia lipolytica* degrades very efficiently hydrophobic substrates to produce organic acids, single-cell oil, lipases, and so forth. The aim of this study was to investigate the biochemical behavior and simultaneous production of valuable metabolites such as lipase, citric acid (CA), and single-cell protein (SCP) by *Yarrowia lipolytica* DSM 3286 grown on various plant oils as sole carbon source. Among tested plant oils, olive oil proved to be the best medium for lipase and CA production. The *Y. lipolytica* DSM 3286 produced  $34.6 \pm 0.1$  U/mL of lipase and also CA and SCP as by-product on olive oil medium supplemented with yeast extract. Urea, as organic nitrogen, was the best nitrogen source for CA production. The results of this study suggest that the two biotechnologically valuable products, lipase and CA, could be produced simultaneously by this strain using renewable low-cost substrates such as plant oils in one procedure.

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## 1. Introduction

The fermentation of fatty low-value renewable carbon sources aiming at the production of various added-value metabolites such as organic acids, single-cell oil, and lipases presents a noticeable interest in the sector of industrial microbiology and biotechnology [1–3]. The yeast *Yarrowia lipolytica* degrades very efficiently hydrophobic substrates such as n-alkanes, fatty acids, fats, and oils, which they have specific metabolic pathways, for production of single-cell protein, single-cell oil, organic acids, and lipases [4–7].

Lipases (EC 3.1.1.3) are among the most important classes of industrial enzymes. Lipases are used in the production of detergents, cosmetics, pharmaceuticals, flavour enhancers and foods [8, 9]. *Y. lipolytica* is one of the most extensively studied lipolytic yeasts, and it secretes lipase. The fact that lipases remain active in various organic solvents has allowed their use in the catalysis of inter- and transesterification reactions of oils and in enantioselective transesterification [10]. The *Y. lipolytica* is able to produce several lipases (extracellular, membrane-bound, and

intracellular activities), and its lipase production depends on media composition and environmental conditions [11–14].

Choupina et al. cloned and identified two lipase genes (LIP1 and LIP3) which the deduced amino acid sequences are similar to lipases from *Candida cylindracea* and *Geotrichum candidum*. Pignede et al. isolated and characterized the LIP2 gene, which encodes the extracellular lipase YLip2 (38.5 kDa). They showed that this gene is responsible for all of the extracellular lipase activity of *Y. lipolytica*. Destain et al. reported that extracellular lipase production was improved by chemical mutation and the properties of mutant lipase were the same of those of the wild type: molecular weight 38 kDa, optimum pH 7 and optimum temperature 37°C [15–17]. Subsequently Fickers et al. isolated LIP7 and LIP8 genes encoding extracellular lipases. Kamzolova et al. screened a huge number of *Y. lipolytica* strains and reported lipase activities ranging from 1.8–45.5 U/mL. Results for other wild *Candida* species showed a lipase production of the same magnitude with the present investigation (e.g., concentrations 2–15 U/mL for various carbon sources used) [2, 7].

Citric acid (CA) is the most important organic acid produced in tonnage using fermentation. Global production of CA was estimated about 1.5 million tonnes in 2004. CA has many industrial applications, for instance, as flavour, acidifying and preservative additive in the food and pharmaceutical industry, as stabiliser for vegetable oils and fats or as complex forming and bleaching component in many washing detergents [3, 18].

The *Y. lipolytica* is unique in its ability to produce and excrete into the medium a broad range of organic acids—the Krebs cycle intermediates, CA, isocitric acid (ICA) and 2-ketoglutaric acid—as well as pyruvic acid. CA production in all developed countries follows a conventional procedure which involves the use of *Aspergillus niger* (as producer) and molasses (as substrate). Using *Y. lipolytica* would have several advantages compared to the *Aspergillus*, including a wider substrate range, a smaller sensitivity to low dissolved oxygen concentrations and heavy metals, and higher product yield [1, 3, 18, 19]. Furthermore, these strains produce CA under intracellular nitrogen limitation [20, 21]. The maximum and minimum CA production values by *Y. lipolytica* strains were reported 217 g/L on petrolatum and 1.4 g/L on glycerol, respectively [1, 22].

The aim of the study was to investigate growth of *Y. lipolytica* on media composed of various plant oils utilized as the sole carbon source and effect of the nature of the (fatty) carbon source and the nitrogen source used upon the biochemical behavior and metabolites production (e.g., Organic acids and lipases) in flask-submerged fermentations.

## 2. Materials and Methods

**2.1. Microorganism.** *Y. lipolytica* DSM 3286 strain was obtained from the culture collection of the DSM, Germany.

**2.2. Growth Conditions.** *Y. lipolytica* strain conserved at 4°C on YPD-agar medium [23]. For cultivation, the strain was grown at 29°C and 200 rpm in 50 mL YPD or production media in 250 mL flasks.

**2.3. Production Media.** The composition of the basal medium for lipase production [8] with some modifications was: olive oil 10 g/L, yeast extract 2 g/L,  $\text{KH}_2\text{PO}_4$  0.5 g/L,  $\text{K}_2\text{HPO}_4$  0.5 g/L,  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  0.5 g/L,  $\text{CaCl}_2$  0.1 g/L, NaCl 0.1 g/L. Initial pH of the media was adjusted to 6. The soy bean, canola, castor, sesame, wheat bud, sweet almond, bitter almond, walnut and coconut oils (Table 1) were added to the basal lipase production medium instead of olive oil at the same volume, when the effects of carbon sources were being studied. For investigation of nitrogen sources effects, similar concentrations of mineral ( $\text{NH}_4\text{Cl}$ ,  $\text{NH}_4\text{NO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NH}_4\text{H}_2\text{PO}_4$ ) and organic (casein, urea, peptone, soy bean flour, beef extract and malt extract) nitrogen compounds were replaced instead of yeast extract. All chemicals were laboratory grade and purchased from Sigma.

**2.4. Determination of Biomass and Unconsumed Substrate.** Cells were harvested by centrifugation at 4600 g for 6 minutes and washed successively with hexane and centrifuged. Biomass was determined from the dry weight at 80°C for 24 hours [2, 24]. Unconsumed substrate fat was measured according to method used by Papanikolaou et al. [2].

**2.5. Lipase Activity Assay.** The extracellular lipase production was determined using a spectrophotometric assay method. Briefly, 100  $\mu\text{L}$  of cultivation medium supernatant was added to a solution of 0.504 mM *p*-nitrophenyl laurate (PNPL) in 50 mM phosphate buffer, pH 7.0. This solution was incubated at 37°C for 15 minutes before adding the cultivation medium. The production of *p*-nitrophenol was monitored by spectrophotometer (Uvi Light XS, France) at 410 nm during the linear period of product accumulation. One unit (U) of lipase activity is defined as the amount of enzyme that produces 1  $\mu\text{mol}$  of product per minute [23].

To confirm that the enzyme detected by the spectrophotometric method was lipase and not merely esterase, in some cases, titrimetric method was also performed using olive oil as substrate. An emulsion created by olive oil at 40% (v/v) and polyvinyl alcohol at 2% (w/v) was used as substrate for the lipase assay. The solution was emulsified with the aid of a homogeniser for 6 minutes at maximum speed, in order to obtain a satisfactory dispersion of oil particles into the aqueous phase. Then the enzyme solution (1 mL), pure or diluted, depending on the quantity of lipase, was added to 5 mL of substrate emulsion and 4 mL of 100 mM phosphate buffer, pH 8.0. Samples were incubated for 10 minutes on a shaker (150 rpm) at 37°C. The reaction was stopped by adding 15 mL ethanol or 4 mL of an acetone-ethanol (volume ratio = 50 : 50) mixture. Enzyme activity was determined by titration of the fatty acid released with 50 mM NaOH. One activity unit of lipase was defined as the amount of enzyme, which released 1  $\mu\text{mol}$  of fatty acid per minute under assay conditions [2, 7, 15, 23].

**2.6. CA and ICA Determination.** The CA and ICA production was determined by HPLC (an Inertsil ODS-3 reversed-phase column at 210 nm; 20 mM phosphoric acid was used as a mobile phase with the flow rate of 1.0 mL/min) [25].

## 3. Results and Discussion

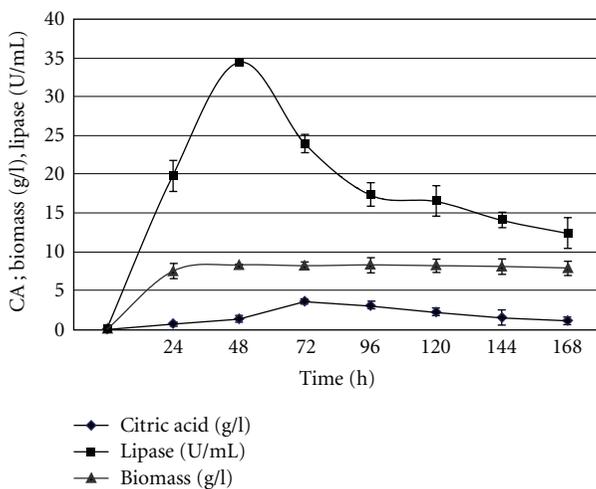
*Y. lipolytica* DSM 3286 was cultivated in different media containing various plant oils as the sole carbon source. Various mineral and organic nitrogen sources were also tested for their capacity to support the production of metabolites (lipase, CA and ICA) and biomass by this strain. Effect of plant oils and nitrogen sources upon the biochemical behavior and metabolites production of *Y. lipolytica* DSM 3286 was investigated by measuring biomass, lipase activity, CA and ICA concentration at various time points within a period of 7 days (Figure 1). For all media, the maximum lipase activity was occurred 48 hours after inoculation and the high level production of organic acids was determined after 72 hours (Figure 1).

TABLE 1: Characteristic parameters of biomass, lipase activity, and CA and ICA production by *Y. lipolytica* DSM 3286 on lipase basal medium with different plant oils.

Oil source (S)	X	$Y_{X/S}$	$Y_{L/S}$	$P_L$	$q_L$	$Q_L$	$Y_{Cit/S}$	$P_{Cit}$	$q_{Cit}$	Cit/Isocit
Wheat bud oil	1.50	0.150	0.747	0.155	0.103	155.625	0	0	0	—
Sweet almond oil	8.00	0.800	1.415	0.294	0.036	294.791	0.008	0.001	0.0001	1.00
Bitter almond oil	3.61	0.361	1.472	0.306	0.084	306.666	0.011	0.001	0.0004	0.78
Castor oil	1.79	0.179	1.245	0.259	0.144	259.375	0.023	0.003	0.0017	1.00
Sesame oil	6.96	0.696	1.846	0.384	0.055	384.583	0.065	0.009	0.0012	0.56
Soy bean oil	5.14	0.514	1.981	0.412	0.080	412.708	0.115	0.015	0.0031	0.43
Olive oil	8.30	0.830	3.460	0.720	0.086	720.833	0.360	0.050	0.0060	0.80
Canola oil	6.48	0.648	2.401	0.500	0.077	500.208	0.147	0.020	0.0031	0.54
Walnut oil	4.75	0.475	0.971	0.202	0.042	202.291	0.056	0.007	0.0016	0.63
Coconut oil	6.35	0.635	1.741	0.362	0.057	362.708	0.035	0.004	0.0007	0.66

TABLE 2: Characteristic parameters of biomass, lipase activity, and CA and ICA production by *Y. lipolytica* DSM 3286 on lipase basal medium with different nitrogen sources.

Nitrogen source	X	$Y_{X/S}$	$Y_{L/S}$	$P_L$	$q_L$	$Q_L$	$Y_{Cit/S}$	$P_{Cit}$	$q_{Cit}$	Cit/Isocit
Malt extract	5.08	0.508	0.573	0.119	0.023	119.375	0.260	0.036	0.007	1.078
Beef extract	8.48	0.848	1.244	0.259	0.030	259.166	0.126	0.017	0.002	0.782
Soy bean flour	9.54	0.954	1.763	0.367	0.038	367.291	0.310	0.043	0.004	0.914
Peptone	8.23	0.823	1.928	0.401	0.048	401.666	0.220	0.030	0.003	0.788
Casein	9.73	0.973	2.404	0.500	0.051	500.833	0.132	0.018	0.001	1.211
Yeast extract	8.20	0.820	3.470	0.722	0.088	722.916	0.362	0.050	0.006	0.828
Urea	6.66	0.666	1.683	0.350	0.052	350.625	0.393	0.054	0.008	1.301
NH <sub>4</sub> Cl	6.88	0.688	1.012	0.210	0.030	210.833	0.116	0.016	0.002	1.064
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	7.10	0.710	0.748	0.155	0.021	155.833	0.109	0.015	0.002	1.211
NH <sub>4</sub> NO <sub>3</sub>	6.99	0.699	0.682	0.142	0.020	142.083	0.110	0.015	0.002	1.222
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	5.52	0.552	0.204	0.042	0.007	42.500	0.273	0.037	0.006	1.716

FIGURE 1: A representative graph for biomass, lipase and CA production by *Yarrowia lipolytica* DSM 3286 on basal (olive oil) medium during 7 days. Each experiment was repeated at least three times (data represent means  $\pm$  SD,  $n = 3$ ).

The highest lipase activity (34.6 U/mL) and CA concentration (3.6 g/L) was achieved in olive oil medium. The lowest lipase activity (7 U/mL) and CA concentration (0 g/L)

was detected in wheat bud oil medium (Figure 2). Mineral nitrogen sources showed no significant effect on lipase production. In contrast, marked increase in lipase and CA productivity was observed upon addition of organic nitrogen sources except for malt extract (Figure 3). Urea proved to be a suitable nitrogen source for CA production as in olive medium supplemented with urea, CA concentration reached up to 3.9 g/L in contrast the lipase level was low. Then experimental data were analyzed (Tables 1 and 2). Lipase production by some plant and vegetables oils (sunflower oil, corn oil, olive oil and so on) and animal fats has been studied [2, 6, 26, 27]. CA can be produced from alkanes, vegetables oils, fats, glycerol, ethanol, molasses and starch hydrolysates [1, 4, 22, 25, 28–32]. Nevertheless, only a few studies have examined both lipase and CA productions simultaneously [7].

In this study we investigated *Y. lipolytica* lipase and CA production at the same time with various plant oils and nitrogen sources. Plant oils were applied as the sole carbon source, because *Y. lipolytica* produces bioemulsifier [33]. The *Y. lipolytica* degrade and oxidise very efficiently hydrophobic substrates, such as fats, oils, alkanes and fatty acids [2, 4]. Glucose or other carbon sources which are used as cometabolism for utilization plant oil in other studies [8, 25] were not used in this study. This makes

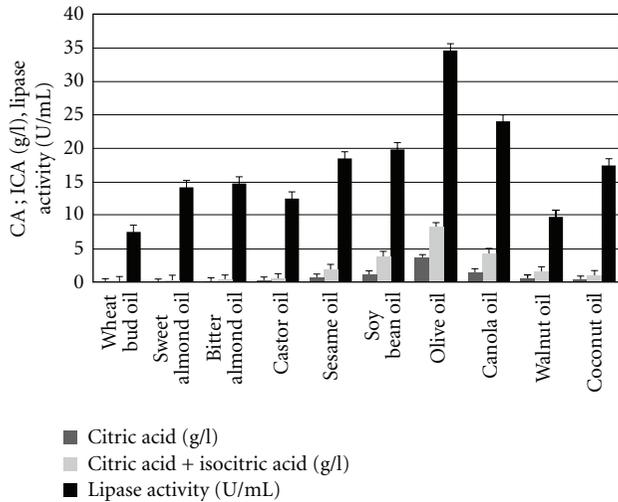


FIGURE 2: The maximum lipase activity (at 48 hours), CA+ICA and CA production (at 72 hours) by *Y. lipolytica* DSM 3286 on lipase basal medium with different plant oils; wheat bud, sweet almond, bitter almond, castor, sesame, soy bean, olive, canola, walnut, and coconut oils. (data represent means  $\pm$  SD,  $n = 3$ ).

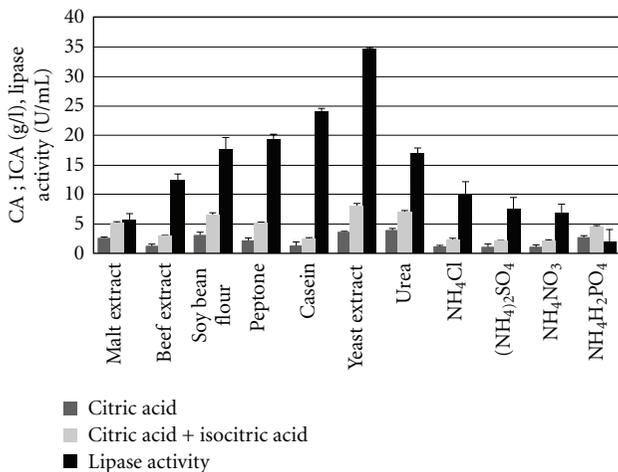


FIGURE 3: The maximum lipase activity (at 48 hours), CA+ICA and CA production (at 72 hours) by *Y. lipolytica* DSM 3286 on lipase basal medium with different organic nitrogen sources (malt extract, beef extract, soy bean flour, peptone, casein, yeast extract and urea) and mineral nitrogen sources ( $\text{NH}_4\text{Cl}$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{NH}_4\text{NO}_3$  and  $\text{NH}_4\text{H}_2\text{PO}_4$ ). (data represent means  $\pm$  SD,  $n = 3$ ).

the production more cost effective and also decreases the biomass. The highest lipase activity was detected on medium with olive oil (Figure 2). These results could be justified by the fact that *Y. lipolytica* strains display a lipase activity which acts preferentially on oleyl residues at positions 1 and 3 of the glyceride. The extracellular lipase requires oleic acid as stabilizer/activator, whereas the cell bound lipases dose not, and is different in several other properties from the extracellular enzyme [3]. It has been mentioned that olive oil has 55–83% oleic acids [34, 35]. Considering chemical compound of other plant oils, it seems that lipase production

is markedly influenced by oleic acid concentration. Canola oil, for its oleic acid content (55%), after olive oil is good candidate for lipase production. Probably the reason is that an expression system containing the *LIP2* gene expressed under the control of the oleic acid inducible *POX2* promoter [36].

Another reason is selective uptake of fatty acids by *Y. lipolytica*, because two hypotheses have been formulated to explain of the transport of hydrophobic substrate such as fatty acids into microorganisms. The hydrophobic substrate can be solubilised (or pseudosolubilised) in the presence of surface active compounds (surfactant-mediated transport) or they can adhere directly to the cell wall. In *Y. lipolytica*, evidence for both mechanisms has been described. Their presence depends on the growth phase and seems to be regulated in an oleate-responsive manner, that is, inducible by alkane and oleic acid and repressed by glucose [4].

Concerning fatty-acid uptake, investigations carried out by Kohlwein and Paltauf in *Y. (Saccharomycopsis) lipolytica* concluded that below a threshold of  $10 \mu\text{M}$ , an energy-free transporter was required, whereas above this concentration fatty acids like lauric or oleic acid diffused freely. Moreover, these authors proposed that at least two different chain-length-selective transporters are present: one carrier system is specific for  $\text{C}_{12}$  and  $\text{C}_{14}$  fatty acids, and the second for  $\text{C}_{16}$  and  $\text{C}_{18}$  such as oleic acid [37, 38].

The production of lipase was low on almond oils media, nevertheless their high level of oleic acid content (60–75%) is as well as olive oil (not showed data). The probable reason is that almond oils have antimicrobial and toxic compounds such as benzaldehyde and hydrocyanic (prussic) acid. Our results showed that organic nitrogen sources increased biomass (Casein; 9.73 g/L), lipase (yeast extract; 34.7 U/mL) and CA (Urea; 3.9 g/L) production as compared with mineral nitrogen source (Figure 3). Organic nitrogen source provides nutritional needs (amino acids, vitamins, etc.) for growth and culture supplements for extracellular lipase production [3]. Intensive CA production occurred under nitrogen limitation. Nitrogen source is limited in media with organic nitrogen source [32].

On the basis of other reports [2, 7, 8, 23], the level production of lipase ( $34.6 \pm 0.1 \text{ U/mL}$ ) by *Y. lipolytica* DSM 3286 on plant oils in flask-submerged fermentation, especially olive oil, is interesting. Therefore olive oil, for others analyzed parameters including  $Y_{X/S}$  (0.830 g/g),  $Y_{L/S}$  (3.460 U/g),  $P_L$  (0.720 U/h) and  $q_L$  (0.086 U/g.h), is a suitable carbon source for lipase production (Table 1).

Although in comparison with glucose and other carbon sources, CA production was low on oils and C/N ratio may be not suitable for CA production. In this study lipase production was prior to CA production and then CA as by-product detected on plant oils media. Altogether the *Y. lipolytica* DSM 3286 strain is essentially low CA producing yeast, even on glucose and optimized media (not showed data). Olive oil medium supplemented with urea (CA = 3.9 g/L) had best results between other organic nitrogen sources and therefore urea is suitable nitrogen source for CA production. On the basis of other reports [1, 22], amount of CA ( $3.6 \pm 0.3 \text{ g/L}$ ) as by-product in lipase

production process by *Y. lipolytica* DSM 3286 on olive oil, with  $Y_{Cit/S}$  (0.393 g/g),  $P_{Cit}$  (0.054 g/h) and  $q_{Cit}$  (0.008 g/g.h), is acceptable (Table 2).

It was interesting that maximum lipase production time (after 48 hours) was different from maximum CA production time (after 72 hours) on plant oils as sole carbon source. The maximum of the lipase activity was observed for cells of stationary growth phase (after 48 hours), and the transition of yeast cells from the exponential growth phase to the growth retardation caused by the exhaustion of nitrogen in the medium was accompanied by a decrease in the lipase activity; which is apparently due to a decrease in the rate of synthesis of lipase under conditions of nitrogen limitation [2]. The nitrogen supply was exhausted, which coincided with the maximum biomass accumulation and the beginning of CA and ICA production [7]. Thus, CA and ICA were produced principally by *Y. lipolytica* DSM 3286 on olive oil during the stationary growth phase; the total CA and ICA concentration reached maximum of 8.08 g/L at 72 hours.

In the literature, it is suggested that lower  $Y_{X/S}$  values (around 0.5–0.7 g/g) obtained from high-lipase producing wild *Y. lipolytica* strains are very satisfactory for single-cell protein fermentation from fatty substrates [2, 7]. Therefore plant oils with high content of oleic acid such as olive oil supplemented with  $(NH_4)_2SO_4$  ( $Y_{X/S} = 0.710$  g/g) and organic nitrogen, except Malt extract ( $Y_{X/S} = 0.508$  g/g) and urea ( $Y_{X/S} = 0.666$  g/g), and also sweet almond oil ( $Y_{X/S} = 0.8$  g/g) and sesame oil ( $Y_{X/S} \sim 0.7$  g/g) are very satisfactory for biomass and single-cell protein production (Tables 1 and 2).

In previous studies, investigators usually used only one or limited number of substrate and, also they tested either lipase or CA production [2, 18, 23]. One advantage of presents study is testing more than twenty carbon and nitrogen sources and measuring lipase and organic acids production simultaneously. One disadvantage of CA production is same amounts of CA and ICA production on plant oils media (Tables 1 and 2).

#### 4. Conclusion

In conclusion, our results show that lipase and CA could be produced by *Y. lipolytica* using renewable low-cost substrates such as plant oils. The maximum organic acids were produced after high level of lipase production that is an interesting relationship between lipase and organic acids (CA and ICA) production. The *Y. lipolytica* DSM 3286 strain is essentially low CA producing yeast. Our study suggests that fair amounts of CA could be produced by this yeast as by-product. Further study to improve its CA production is suggested. Furthermore, by mutagenesis and metabolic engineering methods, levels of CA and even lipase production could be increased while ICA production, which is an undesirable product, could be decreased. Manufacturing of these two important biotechnological products (lipase and CA) simultaneously during one simple procedure could be economically very valuable.

#### Abbreviations

L:	Maximum lipase activity (U/mL)
X:	Biomass (g/L)
S:	Substrate (g/L)
Cit:	Maximum concentration of citric acid (g/L)
t:	Time (h)
$Y_{X/S}$ :	Biomass yield on substrate (g/g)
$Y_{L/S}$ :	Lipase yield on substrate (U/g)
$Y_{Cit/S}$ :	Citric acid yield on substrate (g/g)
$P_L$ :	Lipase productivity; lipase on fermentation time (U/h)
$P_{Cit}$ :	Citric acid productivity; citric acid on fermentation time (g/h)
$q_L$ :	$L/X.t$ ; specific rate of lipase production (U of lipase/g of biomass.h)
$q_{Cit}$ :	$Cit/X.t$ ; specific rate of citric acid production (g of citric acid/g of biomass.h)
V:	Initial volume of liquid culture (l)
$Q_L$ :	$L/V.t$ ; lipase volumetric productivity (U/l.h)
$Q_{Cit}$ :	$Cit/V.t$ ; citric acid volumetric productivity (g/l.h)
Isocit:	Isocitric acid (g/L)
Cit/Isocit:	Citric acid and Isocitric acid ratio (g/g).

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