A novel finding that *Streptomyces clavuligerus* can produce the antibiotic clavulanic acid using olive oil as a sole carbon source.

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Running title: Olive oil as C-source for *S. clavuligerus*

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Abstract

Aims: This study aims to establish whether commercially available food oils can be used by *Streptomyces clavuligerus* as sole carbon sources for growth and clavulanic acid production.

Methods and results: Batch cultures in bioreactors showed that *Strep. clavuligerus* growth and clavulanic acid yields in a P-limited medium containing 0.6% (v/v) olive oil were respectively 2.5- and 2.6-fold higher than in a glycerolcontaining medium used as control. Glycerol- and olive oil-grown cells present different macromolecular composition, particularly lipid and protein content.

Conclusions: *Streptomyces clavuligerus* uses olive oil as the sole carbon and energy source for growth and clavulanic acid production. Yields and production rates in olive oil are comparable to those reported for oil-containing complex media. Differences in yields and in the macromolecular composition indicate that different metabolic pathways convert substrate into product.

Significance and impact of the study: This is the first report of oils being used as the sole carbon source by *Strep. clavuligerus*. Apart from economic benefits, interesting questions are raised about *Strep. clavuligerus* physiology. Defined culture media allow physiological studies to be performed in the absence of interference by other compounds. Understanding how *Strep. clavuligerus* catabolises oils may have an economic impact in clavulanic acid production.

Keywords: Clavulanic acid; Olive oil; glycerol; *Streptomyces clavuligerus;* lipids; macromolecular composition; carbon source

1. Introduction

Streptomyces clavuligerus synthesises clavulanic acid, a β-lactamase inhibitor that prevents the development of resistance to β-lactam antibiotics by pathogenic bacteria. Although the actinomycetes catabolise a wide variety of sugars, *S. clavuligerus* is not able to utilize simple carbohydrates such as glucose and other hexoses, pentoses, deoxysugars, sugaralcohols or disaccharides as a result of an impaired glucose uptake mechanism (Garcia-Dominguez et al., 1989). Thus, several typical industrial culture medium components, such as starch and other carbohydrates, are not viable for the synthesis of clavulanic acid in *S. clavuligerus*. Apart from the evident commercial importance, this metabolic anomaly raises interesting questions regarding the physiology of the species.

A commonly used carbon source for the synthesis of clavulanic acid in *S. clavuligerus* is glycerol, with arginine generally used as a nitrogen source (Aharonowitz and Demain, 1978; Mayer and Deckwer, 1996; Chen et al., 2003; Teodoro et al., 2006). The utilization of glycerol by *S. clavuligerus* raises the possibility that these bacteria could also metabolize triglycerides, in the form of food-based oils, as these can be broken down by lipases to their glycerol and fatty acid components. The utilization of oils is important for the microbial production of secondary metabolites due to their stimulation of bacterial growth and product synthesis, anti-foaming properties, and also to their higher energy content as compared to carbohydrates on a weight by weight basis (Cavanagh et al., 1994; Large et al., 1998), but only a few studies have focused on the use of oils as a carbon and energy source for antibiotic production by *S. clavuligerus*. In general, these investigations have used oils in supplementation to other carbon sources, while

also using complex culture media containing other substrates such as flour, peptones and protein hydrolysates (Lee and Ho, 1996; Large et al., 1998; Hamedi et al., 2002; Peacock et al., 2003; Choi and Cho, 2004; Maranesi et al., 2005; Ortiz et al., 2007).

The use of simple, chemically defined media generally facilitates the investigation of the physiology and biochemistry of secondary metabolite production by a microorganism. In this paper we present a quantitative analysis of the use of commercially available food oils as sole carbon sources supporting clavulanic acid production and bacterial growth in *S. clavuligerus* cultures. Our analysis shows that the levels of clavulanic acid production reached with olive oil are as high as with glycerol, the most commonly used carbon source, and antibiotic yields are comparable to those reported for complex culture media.

2. Materials and Methods

2.1. Strain. The strain used was *S. clavuligerus* ATCC 27064 NRRL 35851.

2.2 Culture media. Cultures were performed using the following media:

- SV2 medium, containing (per l): Glycerol, 15 g; Glucose, 15 g; Soya peptone, 15 g; $CaCO_3$, 1 g. The pH was adjusted to 7 and the media autoclaved at 121°C for 15 min.

- Chemically defined P-limited medium, containing (per l): NH_4Cl, 7 g; KH_2PO_4, 0.25 g; MOPS, 21 g. The pH was adjusted to pH 6.8 and the medium autoclaved at 121°C for 15 min. After sterilization, trace elements were added to the medium (10 ml l^{-1}). The trace elements stock solution contained (per l): $MgSO_4.7H_20$, 25 g; $FeSO_4.7H_2O$, 2.5 g; CoCl, 0.055 g; $CuCl_2$ 0.053 g, $CaCl_2.2H_2O$, 1.38 g; $ZnCl_2$, 1.04 g; $MnCl_2$, 0.62 g; Na_2MoO_4, 0.03 g (Bushell et al., 2006).

Glycerol (0.6% v/v) was added the P-limited medium before sterilization, while in the oil-containing experiments 0.6% (v/v) of oil was added to the autoclaved medium by sterile filtration through 0.2 μm hydrophobic Minisart SRP25 filters (Sartorius, U.K.).

2.2. Inoculum preparation. A spore suspension (1 ml; stored at -80°C) was added to a 250 ml baffled flask containing 50 ml of SV2 medium and incubated at 135 rpm for 24 h at 30°C. 500 μl of this culture were added to 25 ml P-limited medium, containing 0.6% glycerol, and incubated for 48 h under the same conditions. This two-step pre-culture method ensured minimal carry over of rich nutrients in the production flasks. The inoculum reached approximately OD_{600nm} = 1.0. All cultures

were regularly examined by microscopy for disperse growth and contamination.

2.3. Shake flask cultures. 2.5 ml of the pre-culture mentioned above were added to flasks with 50 ml P-limited medium containing either 0.6% glycerol or 0.6% oil (v/v; sunflower, soybean, flaxseed, rapeseed or olive oil). The flasks were incubated as above for 216 h and 1 ml samples were taken every 24 h. All cultures were performed in triplicate.

2.4. Bioreactor batch cultures. 1.6 l P-limited medium (containing 0.6% (v/v) olive oil or glycerol) was inoculated (5% v/v) with medium from the shake flask cultures in a 2 l bioreactor (Adaptive Biosystems, U.K.). Temperature was maintained at 30°C, pH at 6.8, agitation rate at 800 rpm and aeration rate at 0.23 l min^{-1}. Cultures were incubated for 96 h and samples were collected every 12 h.

2.5. Analytical techniques

2.5.1. Biomass. In shake flask experiments, 1 ml samples were placed in pre-weighted microfuge tubes and centrifuged at 16000xg for 10 min. Pellets from the oil-containing cultures were washed twice with distilled water to remove lipid remnant. In the bioreactor cultures, the biomass dry weight was determined by filtering 5 ml samples through 0.45 μm pre-dried, pre-weighed nitrocellulose membranes (Millipore, U.K.), rinsing three times with reverse osmosis water, and microwaving twice at 650 W for 5 min (Bushell et al., 2006). The membranes were allowed to cool in a desiccator and the weight determined. In cases where a rapid estimation of growth was required, pellets were re-suspended in 1 ml of distilled

water and the optical density (600 nm) of the suspension recorded. Due to the filamentous growth showed by the microorganism, these measurements were used only as rough estimations, and were always followed by biomass dry weight determinations.

2.5.2. Clavulanic acid. Clavulanic acid was measured according to the method of Bird et al. (1982) that involves the reaction of clavulanic acid with imidazole (at 30°C for 12 min) followed by spectrophotometric measurement of the resulting conjugate at 312 nm.

2.5.3. Macromolecular composition of bacterial biomass. Biomass pellets were washed twice with chilled distilled water and freeze-dried overnight. Total protein was measured following the method described by Hartree (1972); total lipids were determined as described by Izard and Limberger (2003); glycogen was determined following the method described by Parrou and Francois (1997); total carbohydrate was measured as described by Dubois et al. (1956).

2.5.4. Triglycerides, glycerol and unesterified fatty acids. Triglycerides were measured after treatment of the culture supernatant samples with lipase and esterase (100 U ml^{-1}; at 37ºC for 2 h) and measurement of the glycerol produced. A glycerol assay kit (Megazyme, Ireland) was used for determining glycerol. Unesterified fatty acid levels were determined with a Free Fatty Acids Half-Micro Test Kit (Roche, Germany).

3. Results

3.1. Oils support bacterial growth and clavulanic acid production. The ability of *S. clavuligerus* to grow and produce clavulanic acid in different oil-containing media was tested in exploratory shake-flask cultures and compared to media with glycerol as the carbon source. Only two out of five tested oils supported good production of clavulanic acid. The highest clavulanic acid concentration was observed in medium containing olive oil, which yielded 47 mg l^{-1} of clavulanic acid, nearly 2-fold higher than the clavulanic acid concentration measured in the glycerol medium, namely 25 mg l^{-1} (Fig. 1). Culture medium containing sunflower oil produced 18 mg l^{-1}, whereas media containing soybean, flaxseed or rapeseed oil did not support clavulanic acid production at any measurable level (data not shown). Biomass reached an OD_{600nm} of 0.6 in olive oil, sunflower oil and glycerol cultures, but the maximum values were reached at different time points with each substrate (at 72 h, 144 h and 48 h, respectively) (Fig. 1).

3.2. Bioreactor cultures. In bioreactors the kinetics of bacterial growth and clavulanic acid production showed similar profiles with glycerol- and olive oil-containing media (Fig. 2). The maximum concentration of biomass was reached at approximately 80 h, although the maximum level of clavulanic acid production with olive oil was delayed by approximately 10 h as compared to glycerol. Glycerol was depleted by 96 h in the control culture, but in the olive oil culture only 2.4 g l^{-1} of triglycerides (~25%) was consumed over 96 h, remaining constant thereafter (Fig. 2).

Biomass and clavulanic acid yields (product per substrate, $g.g^{-1}$) were 2.5- and 2.6-fold higher, respectively, with olive oil than with glycerol (Table 1). To account for

the different carbon content of the substrates yields have also been expressed in terms of mol carbon used and produced, with similar trends observed (Table 1). The rates of production of biomass and clavulanic acid were calculated by curve fitting. The maximum calculated biomass production rates were 61 ± 0.3 mg l^{-1} h^{-1} for glycerol and 58 ± 0.4 mg l^{-1} h^{-1} for olive oil, both observed at 60 h of culture. The maximum specific growth rates calculated were 0.06 h^{-1} in both media. The calculated clavulanic acid production rates were 3.7 ± 0.47 mg l^{-1} h^{-1} for glycerol and 1.4 ± 0.20 mg l^{-1} h^{-1} for olive oil, observed at 73 h. The maximum specific production rates of the antibiotic were calculated as 1.5 mg g^{-1} h^{-1} and 0.7 mg g^{-1} h^{-1} for glycerol and olive oil, respectively.

3.3. Changes in macromolecular composition of the bacteria. The macromolecular composition of *S. clavuligerus* incubated with glycerol as the carbon source differs from that of cells incubated with olive oil (Table 2). At 48 h the carbohydrate content was higher in the glycerol grown cells, but at 72 and 96 h similar values were observed in both media. In contrast, cellular lipids show much higher levels in olive oil-containing medium at 48 and 72 h, compared to the glycerol medium. Protein concentrations showed a steady increase between 48 and 96 h, with the olive oil cultures showing slightly higher concentration than the glycerol culture. Levels of glycogen remained relatively constant with glycerol, whereas a small increase is observed with olive oil.

3.4. Unesterifed fatty acids. The changes in growth parameters, clavulanic acid production and macromolecular composition point to differences in the metabolic pathways utilised when *S. clavuligerus* is cultured with either glycerol or olive oil.

Decreases in olive oil levels in the supernatant (Fig. 2), indicate that this substrate is probably hydrolysed by extracellular lipases, releasing glycerol and unesterified fatty acids as potential carbon sources. In the glycerol cultures no fatty acids were detected, whereas in the olive oil cultures the levels of unesterified fatty acids were low at less than 80 mg l^{-1}, but still higher than levels in the medium of the glycerol grown cells (Fig. 2).

4. Discussion

In this study we clearly demonstrate that *S. clavuligerus* can utilise olive oil as a carbon source with clavulanic acid yields and growth parameters similar to those obtained with glycerol (Fig. 1; Table 1). To our knowledge this is the first study to use a food-based oil as a sole carbon source for the production of clavulanic acid in *S. clavuligerus* and this finding is potentially of major economic significance for the pharmaceutical industry, as vegetable oils are much cheaper than carbohydrate substrates (Large et al., 1998). Though previous studies have demonstrated that oils, including olive oil, can be used for antibiotic production in bacteria, these studies have consistently used complex culture media containing other carbon sources (Lee and Ho, 1996; Large et al., 1998; Hamedi et al., 2002; Peacock et al., 2003; Choi and Cho, 2004; Maranesi et al., 2005; Ortiz et al., 2007). Even though the fatty acid composition of olive oil changes within a certain range, the oil can be considered a chemically defined component.

One of the most important parameters to assess the suitability of a culture medium is the product yield. As product yields are dependent on the metabolic pathways involved in the conversion of substrate into product (Westerhof and Kholodenko, 2004), different values are to be expected when diverse carbon sources are used. Interestingly, almost 75% of the olive oil initially added to the medium remained after 24 h, which is consistent with a medium not limited in the carbon source. The clavulanic acid yields in this study (Table 1) are in agreement with previous work using oils in complex culture media (Large et al., 1998; Ortiz et al., 2007), even though the yields due to oils in these studies are probably an over-estimation due to the presence of other carbon sources. The high biomass yield

observed with olive oil is in accordance with the yields observed in other studies using substrates with a high degree of reduction, such as hydrocarbons (Roels, 1983; von Stockar et al., 2006). We hypothesize that the triglycerides in olive oil are hydrolyzed by lipases produced by the bacteria (both extracellular and membrane bound lipases have been reported for *Streptomyces*; Large et al. 1999; Valdez et al. 1999), to release glycerol and unesterified fatty acids into the culture medium that can then be taken up by the bacteria. The slight delay in growth observed with the cells grown in olive oil (Fig. 1) hints at an adaptive process, e.g. to increase lipase production.

Glycerol can be used as a substrate in the glycolysis and gluconeogenesis pathways, whereas fatty acids, which are more energy-dense than glycerol (per gram of substrate), are utilised in the β-oxidation pathway. *Streptomyces* have been previously shown to use fatty acids as an energy source through intracellular hydrolysis by the β-oxidation pathway (Banchio and Gramajo, 1997). Interestingly, the observation that cells grown in olive oil have a higher lipid content than the glycerol grown cells (48 h) (Table 2), in combination with the decrease in triglyceride levels in the culture medium (Fig. 2), indicate that the fatty acids from olive oil have been taken up by the bacteria, a suggestion also supported by the low levels of unesterified fatty acids measured in the culture medium (Fig.2). The subsequent decrease of the cellular lipid levels at 72 and 96 h of culture suggests that the fatty acids are metabolised by the cells. The rapid initial increase in lipid content of bacteria grown in olive oil, as compared to glycerol (Table 2), implies that adaptation to lipid uptake is faster than for fatty acid utilization. The lipid content of olive oil grown cells (Table 2) is similar to that found in *Streptomyces lividans* grown in triolein

(Peacock et al., 2003), while the macromolecular composition values observed here are in accordance to those found in studies with *S. clavuligerus* and related genera (Davidson, 1992; Shabab et al., 1996; Roubos, 2002; Jacques, 2004).

In conclusion, our results show that *S. clavuligerus* is able to grow and produce clavulanic acid in media containing olive oil as the sole carbon and energy source. Even though the clavulanic acid concentrations reached were lower than those normally desirable in industrial processes, the antibiotic yields and production rates are comparable to those observed previously in studies using oils in combination with complex media. The use of oils has a potential economic benefit for production of antibiotics in large-scale bioprocesses, if media are supplemented with the adequate nitrogen sources. The use of defined media has the additional benefit that physiological studies can be performed in the absence of interference by other compounds.

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References

Aharonowitz, Y., Demain, A.L. (1978) Carbon catabolite regulation of cephalosporin production in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. **14**, 159-164.

Banchio, C., Gramajo, H.C. (1997) Medium- and long-chain fatty acid uptake and utilization by *Streptomyces coelicolor* A3(2): first characterization of a gram-positive bacterial system. Microbiology **143**, 2439-2447.

Bird, A.E., Bellis, J.M., Gasson, B.C. (1982) Spectrophotometric assay of clavulanic acid by reaction with imidazole. Analyst **107**, 1241-1245.

Bushell, M.E., Kirk, S., Zhao, H., Avignone-Rossa, C.A. (2006) Manipulation of the physiology of clavulanic acid biosynthesis with the aid of metabolic flux analysis. Enzyme Microb. Technol. **39**, 149-157.

Cavanagh, M.E., Ison, A.P., Lilly, M.D. Carley-Smith, S.W., Edwards, J. (1994) The utilization of lipids during *Streptomyces* fermentations. Trans. Inst. Chem. Eng. **72**, 14-16.

Chen, K.C., Lin, H.Y., Wu, J.Y., Hwang, S.C. (2003) Enhancement of clavulanic acid production in *Streptomyces clavuligerus* with ornithine feeding. Enzyme Microb. Technol. **32**, 152-156.

Choi, D., Cho, K. (2004) Effect of carbon source consumption rate on lincomycin

production from *Streptomyces lincolnensis*. J. Microbiol. Biotechnol. **14**, 532-539.

Davidson, A. (1992) Quantitative microbial physiology of *Streptomyces coelicolor* A3 (2). PhD Thesis, University of Glasgow, U.K.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. (1956) Colorimetric Method for Determination of Sugars and Related Substances. Anal. Chem. **28**, 350-356.

Garcia-Dominguez, M., Martin, J.F., Liras, P. (1989) Characterization of sugar uptake in wild-type *Streptomyces clavuligerus*, which is impaired in glucose uptake, and in a glucose-utilizing mutant. J. Bacteriol. **171**, 6808-6814.

Hamedi, J., Malekzadeh, F., Niknam, V. (2002) Improved production of erythromycin by *Saccharopolyspora erythraea* by various plant oils. Biotechnol. Lett. **24**, 697-700

Hartree, E.F. (1972) Determination of Protein: a Modification of the Lowry Method that Gives a Linear Photometric Response. Anal. Biochem. **48**, 422-427.

Harwood, J. and Aparicio, R. (2000) Handbook of olive oil: analysis and properties. Aspen Publishers Inc., Gaithersburg, Maryland USA.

Izard, J., Limberger, R.J. (2003) Rapid screening method for quantitation of bacterial cell lipids from whole cells. J. Microbiol. Methods **55**, 411-418.

Jacques, C. (2004) Modelling of metabolic pathways for S*accharopolyspora erythraea* using flux balance analysis. PhD Thesis, University College London, U.K.

Large, K.P., Ison, A.P., Williams, D.J. (1998) The effect of agitation rate on lipid utilisation and clavulanic acid production in *Streptomyces clavuligerus*. J. Biotechnol. **63**, 111-119.

Lee, P.C., Ho, C.C. (1996) Production of clavulanic acid and cephamycin C by *Streptomyces clavuligerus* in palm-oil medium. World J. Microbiol. Biotechnol. **12**, 73-75.

Maranesi, G.L., Baptista-Neto, A., Hokka, C.O., Badino, A.C. (2005) Utilisation of vegetable oil in the production of clavulanic acid by *Streptomyces clavuligerus* ATCC 27064. World J. Microbiol. Biotechnol. **21**, 509-514.

Mayer, A.F., Deckwer, W.D. (1996) Simultaneous production and decomposition of clavulanic acid during *Streptomyces clavuligerus* cultivations. Appl. Microbiol. Biotechnol. **45**, 41-46.

Ortiz, S.C.A., Hokka, C.O., Badino A.C. (2007) Utilization of soybean derivatives on clavulanic acid production by *Streptomyces clavuligerus*. Enzyme Microb. Technol. **40**,1071-1077.

Parrou, J.L., Francois, J. (1997) A Simplified Procedure for a Rapid and Reliable Assay of both Glycogen and Trehalose in Whole Yeast Cells. Anal. Biochem. **248**,186-188.

Peacock, L., Ward, J., Ratledge, C., Dickinson, F.M., & Ison, A. (2003) How *Streptomyces lividans* uses oils and sugars as mixed substrates. Enzyme Microb.Technol. **32**, 157-166.

Roels, J.A. (1983) Energetics and Kinetics in Biotechnology. Elsevier Science & Technology, Amsterdam, The Netherlands.

Roubos, J.A. (2002) Bioprocess modelling and optimization; fed-batch clavulanic acid production by *Streptomyces.* PhD Thesis, Delft University of Technology, The Netherlands.

Shahab, N., Flett, F., Oliver, S.G., Butler, P.R. (1996) Growth rate control of protein and nucleic acid content in *Streptomyces coelicolor* A3(2) and *Escherichia coli* B/r. Microbiology. **142**, 1927-1935.

Teodoro, J.C., Baptista-Neto, A., Cruz-Hernández, I.L., Hokka, C.O. and Badino, A.C. (2006). Influence of feeding conditions on clavulanic acid production in fed-batch cultivation with medium containing glycerol. Appl. Microbiol. Biotechnol. **72**, 450 – 455.

Valdéz, F, González-Cerón, G., Kieser, H.M. and Servín-González, L. (1999). The *Streptomyces coelicolor* A3(2) lipAR operon encodes an extracellular lipase and a new type of transcriptional regulator. Microbiol. **145**, 2365 – 2374.

von Stockar, U., Maskow, T., Liu, J., Marison, I.W., Patiño R. (2006) Thermodynamics of microbial growth and metabolism: An analysis of the current situation. J. Biotechnol. **121**, 517-533.

Westerhof, H. and Kholodenko, B. (2004) Metabolic Engineering. In: Metabolic Engineering in the Post-Genomic Era, eds. Westerhof, H. and Kholodenko, B., Horizon Bioscience, Norfolk, U.K.

Table 1: Yields for biomass and clavulanic acid production in *S. clavuligerus* cultures grown with glycerol or olive oil as the carbon source

	Glycerol	Olive oil	Glycerol	Olive oil
	Yield	Yield*	Yield	Yield*
	g g^{-1}	g g^{-1}	mol mol^{-1}	mol mol^{-1}
Biomass	0.27	0.69	0.30	0.40
Clavulanic acid	0.01	0.02	0.01	0.01

*Corrected for the glycerol supplied by triglycerides

The fatty acid content of olive oil was estimated to be: 13% palmitic acid, 3% stearic acid, 71% oleic acid, 10% linoleic acid and 1% linolenic acid (% w/w, based on Harwood and Aparicio, 2000). Product yield is defined as the amount of product formed per amount of substrate consumed.

Table 2: Macromolecular composition of *S. clavuligerus* biomass collected from cultures with glycerol or olive oil as the carbon source

	Glycerol			Olive oil		
	Sample time (h)			Sample time (h)		
	48	72	96	48	72	96
Carbohydrate (µg/mg biomass)	57 (56, 57)	49 (48, 49)	30 (22, 38)	27 (26, 28)	48 (47, 49)	26 (16, 35)
Lipids (µg/mg biomass)	16 (16, 15)	29 (28, 30)	19 (15, 24)	107 (103, 110)	71 (70, 71)	24 (14, 33)
Protein (µg/mg biomass)	338 (321, 355)	357 (353, 360)	494 (453, 535)	389 (379, 399)	411 (388, 433)	530 (479, 580)
Glycogen (µg/mg biomass)	263 (261, 265)	189 (188, 189)	344 (248, 440)	194 (191, 197)	283 (280, 286)	301 (285, 316)

The results are expressed as average of two biological replicates, shown in parenthesis.

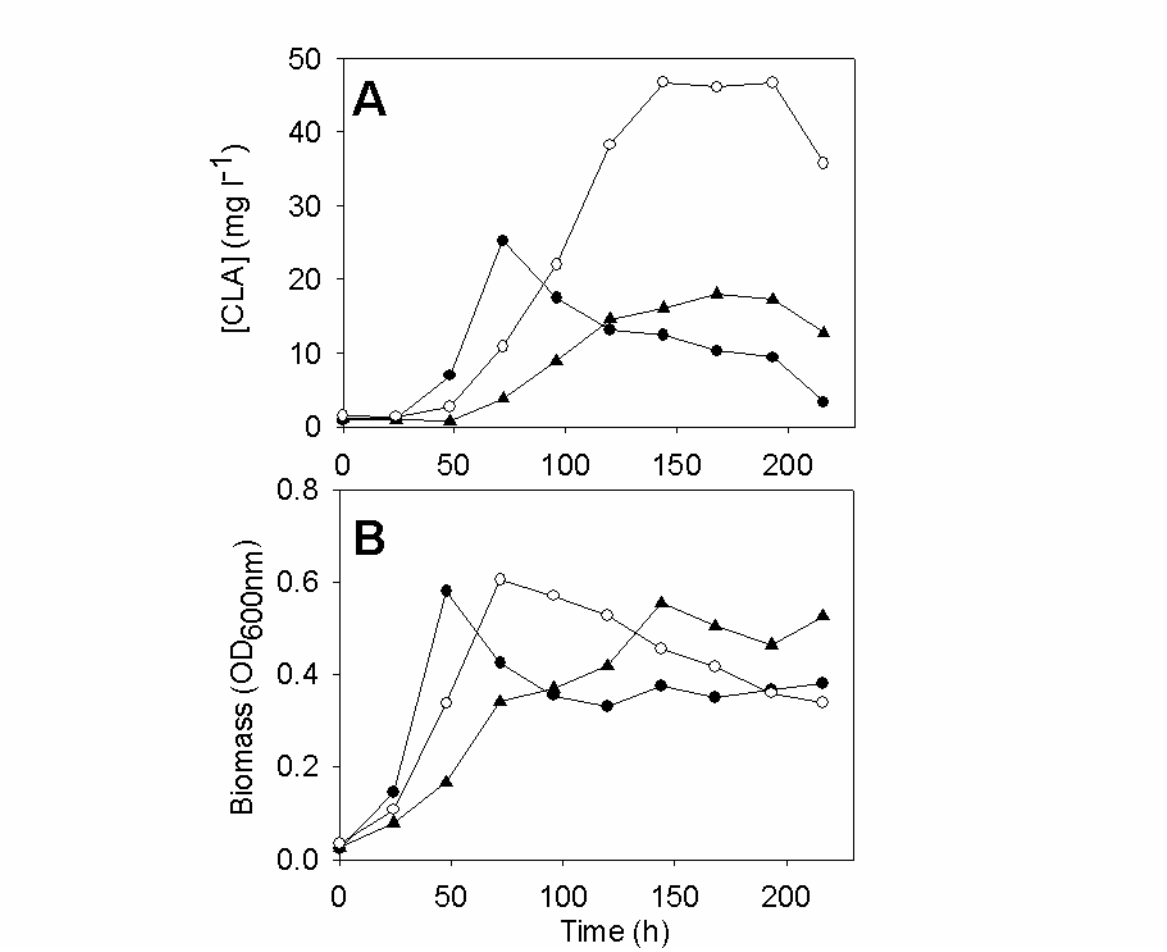

Figure 1. Clavulanic acid (CLA) production (**A**) and growth (**B**) in *S. clavuligerus* flask cultures with glycerol (●), olive oil (○) or sunflower oil (▲). The results are shown as the average of three biological replicates for glycerol and sunflower oil cultures (n=3) and of four biological replicates for the olive oil cultures (n=4).

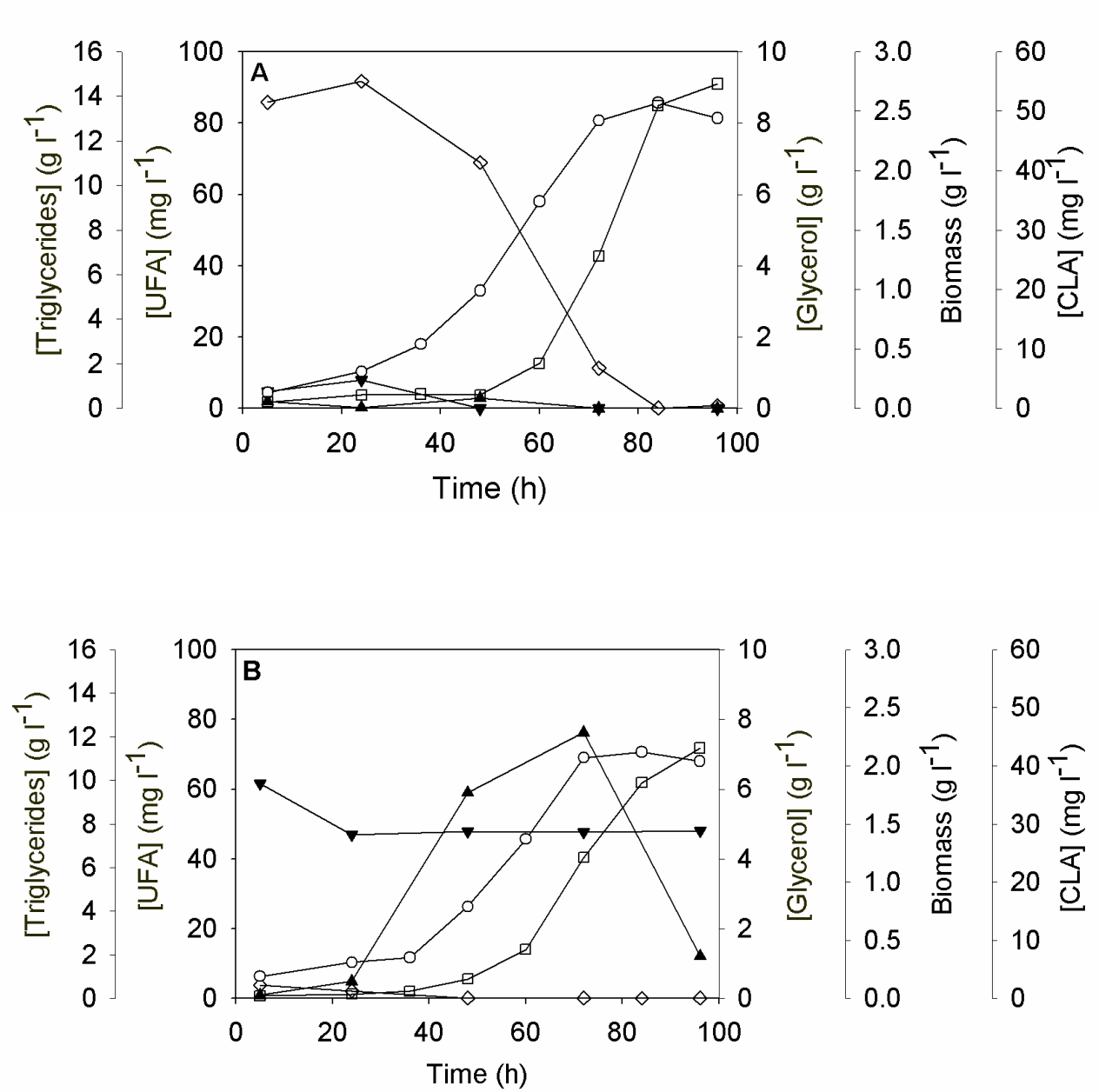

Figure 2. Changes of triglycerides (▼), unesterified fatty acids (UFA, ▲), glycerol (◊), biomass (○) and clavulanic acid (CLA, □) levels in (**A**) glycerol- and (**B**) olive oil-containing batch cultures of *S. clavuligerus*. The results are shown as the average of five biological replicates for biomass and clavulanic acid (n=5) and of three biological replicates for triglycerides, unesterified fatty acids and glycerol (n=3).