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RESEARCH ARTICLE

Batch culture growth of *Chlorella zofingiensis* on effluent derived from two-stage anaerobic digestion of two-phase olive mill solid waste

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Keywords: batch culture, *Chlorella zofingiensis*, kinetic parameters, substrate, two-phase olive mill solid waste, two-stage anaerobic digestion.

Abbreviations: *Ca*: chlorophyll *a*
DO: dissolved oxygen
HRT: hydraulic retention time
LCFA: long chain fatty acids
OLR: organic loading rate
OMSW: olive mill solid waste
OPP: oxygen partial pressure
SCOD: soluble chemical oxygen demand
t: operation time
T: temperature
TC:total chlorophyll
TCOD: total chemical oxygen demand
VFA: volatile fatty acids
X: microorganisms concentration at time *t*
X_0: microorganisms concentrations at the beginning of the exponential growth phase
μ: microorganisms specific growth rate

This paper presents the use of an effluent derived from two-stage anaerobic digestion of two-phase olive mill solid waste (OMSW) as a substrate for the production of *Chlorella zofingiensis* in batch mode. *Chlorella zofingiensis* when grown autotrophycally can accumulate significant quantities of valuable carotenoids which are used as an additive in fish and poultry farming, as colorants in foods and in health care products. It was found that two-phase OMSW previously treated by two-stage anaerobic digestion and further sterilized may be used as a culture medium for the microalgae *Chlorella zofingiensis.* Typical growth curves were obtained using both the above-mentioned anaerobic effluent and a synthetic medium. Total chemical oxygen demand (TCOD) and soluble chemical oxygen demand (SCOD) removals of 37% and 45% respectively were achieved in batch experiments after 11 days' operation time. The specific growth rate was lower when the treated effluent was used as the feed substrate (0.02 h^{-1}) in comparison to the synthetic medium (0.03 h^{-1}). The specific growth rates of the exponential phases were determined by using a first-order kinetic model applied to chlorophyll *a* (C*a*) and total chlorophyll (*TC*) concentrations, as indirect measurements of the microalgae concentration. It was concluded that the effluent from two-stage anaerobic digestion of two-phase OMSW constituted an appropriate culture medium for the growth of *Chlorella zofingiensis*, providing a simple technology feasible for producing a very useful product for animal feeding.

Chlorella zofingiensis when grown autotrophycally can accumulate significant quantities of valuable carotenoids, astaxanthin and lutein to be specific, used as an additive in fish and poultry farming, as colorants in foods and in health care products (Del Campo et al. 2004). Under standard batch-culture conditions this microalgae strain exhibits high values of both growth rate (0.04 h^{-1}) and standing cell population (over 1011 cells/l). In addition, recent studies have shown that astaxanthin is a potent antioxidant, which is effective for the prevention of certain cancers. It has been suggested that a high carbon to nitrogen ratio (C/N) may be efficient for inducing astaxanthin biosynthesis. Nitrogen limitation in the presence of excess organic carbon substrates such as acetate and glucose has proved effective in astaxanthin production in mixothrophic cultures (Ip and Chen, 2005). However, the cultivation of this strain using a wastewater or treated effluent as a substrate has not been reported in the literature to date.

The new two-phase olive-oil extraction process produces a solid and very humid waste called two-phase olive mill waste (OMSW) or 'alperujo' (Borja et al. 2005a; Borja et al. 2005b), which contains large amounts of organic matter, including toxic and inhibitory compounds such as polyphenols, poly-alcohols, long chain fatty acids (LCFA), volatile fatty acids (VFA), etc. This manufacturing process generates large volumes of polluting wastes (approximately 4-5 million tonnes per year in Spain). Such a pollution charge represents a serious problem for the environment, taking into account the 2,000 Spanish olive oil factories currently existing, 90% of which use this new extraction process.

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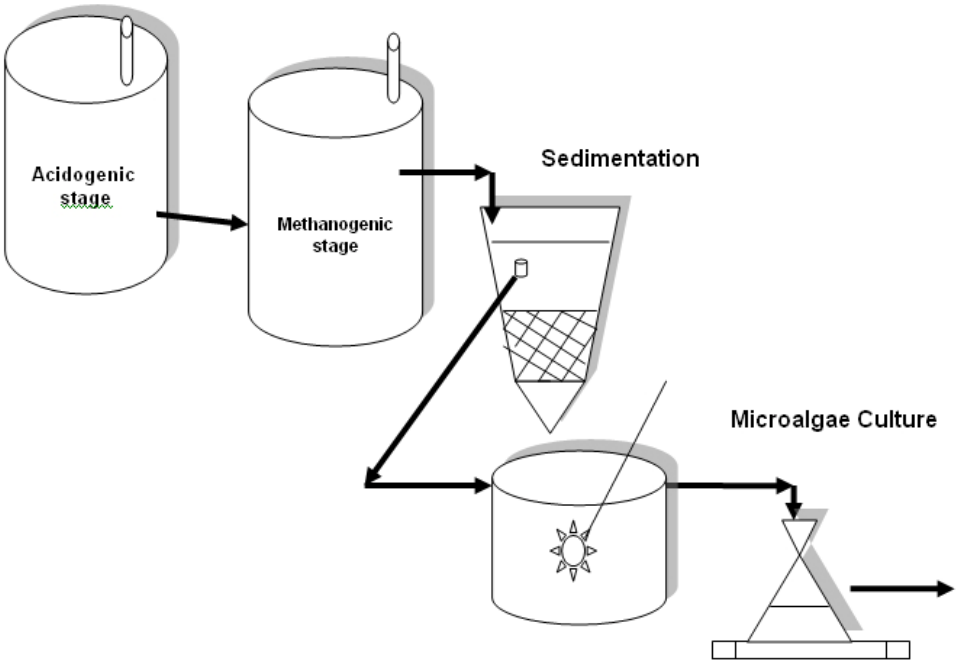

Figure 1. Schematic diagram of the different steps of the overall treatment process.

Anaerobic digestion of solid agricultural wastes such as sugar beet pulp, potato pulp, potato thick stillage, etc. is an economical solution for reducing environmental pollution in most cases. This technology makes excellent waste stabilization and energy recovery possible (Chynoweth et al. 2001). Previous studies have shown the anaerobic biodegradability of the two-phase OMSW at mesophilic temperature (35ºC) by using one and two-stage reactors (Borja et al. 2005a; Borja et al. 2005b). However, the high organic content of this solid residue and the presence of high concentrations of toxic and recalcitrant compounds make post-treatment of the effluent derived from the anaerobic digestion of this waste necessary prior to its disposal or re-use.

Due to the low cost of construction and operation, one of the most widely used and attractive treatment systems for the final treatment of different types of wastes is stabilization ponds with microalgae: no aeration equipment is needed and oxygen requirements are provided by natural surface aeration and photosynthesis by microalgae. The bacteria consume the oxygen released by the microalgae to decompose the organic matter producing carbon dioxide, ammonia and phosphates, which are assimilated by the microalgae (Quintero, 1981; Richmond, 1986; Craggs et al. 2004; Grönlund et al. 2004).

Light intensity, temperature, nutrients and pH are the most important factors for optimising the microalgae culture (Ogbonna and Tanaka, 1996; Sevrin-Reyssac, 1998; Zonneveld, 1998; Martinez et al. 1999; Zhang et al. 1999; John and Flynn, 2000; Carlozzi and Sacchi, 2001; Zhang and Lee, 2001; Babel et al. 2002; Kayombo et al. 2002; Yuan et al. 2002; Kayombo et al. 2003; Fiedler et al. 2003; Tukaj et al. 2003). At relatively constant values of light intensity and temperature, the culture depends mainly on the substrate concentration.

Table 1. Characteristics of the sterilized final anaerobic effluent used as culture medium of *Chlorella zofingiensis.*

Parameter	Unit	Anaerobic effluent [a]
Total Alkalinity	g/l $CaCO_3$	0.31 ± 0.01
Partial Alkalinity	g/l $CaCO_3$	0.21 ± 0.01
pH	-	7.0 ± 0.2
Total COD (TCOD)	g/l	1.93 ± 0.07
Soluble COD (SCOD)	g/l	0.70 ± 0.01
Volatile Solids	g/l	1.50 ± 0.06
Nitrogen content	g/l	0.014 ± 0.006
C/N ratio	-	29

[a]Values are averages of five determinations; ± standard deviations are also included.

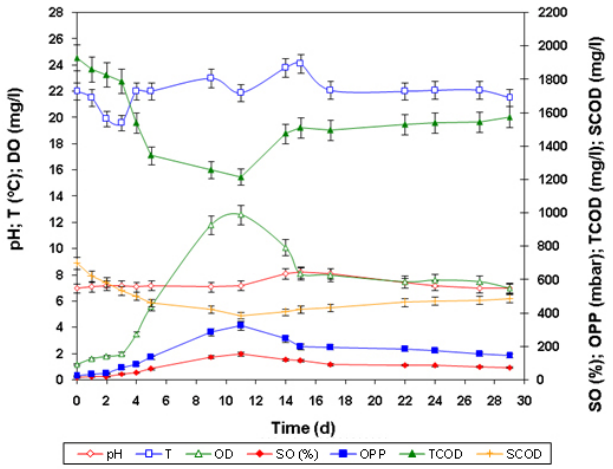

Figure 2. Variation of the parameters monitored with time in the culture of *Chlorella zofingiensis* carried out in the treated effluent.

Different kinetic models for batch culture growth have been developed. Martinez et al. (1999) studied the growth of *Scenedesmus obliquus* using the Monod model in a mineral medium with phosphorus concentrations of up to 372 µM and temperatures in the range of 20ºC-35ºC. The inhibition constants due to the presence of phosphorus increased when the temperature rose from 20ºC to 30ºC, and decreased at temperatures higher than 30ºC. The maximum specific growth rate was $0.047h^{-1}$ at 30ºC, while the highest yields were achieved at 20ºC. Zhang et al. (1999) used an inhibition model to describe the heterotrophic growth of microalgae *Chlamydomonas reinhardtii* in batch culture. Wachenheim et al. (2003) and Liu et al. (2003) applied logistic functions to describe the microbial growth pattern under batch conditions. Kayombo et al. (2003) used both a modified logistic model to describe the growth of *Chlorella vulgaris* and heterotrophic microorganisms and the Monod model to describe the effect of substrate concentration.

Thus, taking into account the knowledge drawn from the literature, the main objectives of this work were:

a) To carry out a preliminary study of *Chlorella zofingiensis* cultivation using sterilized effluent derived from the two-stage anaerobic digestion of two-phase OMSW.

b) To determine the growth curve of the strain and the specific growth rate comparing the above-mentioned effluent with a synthetic culture medium.

Previous works have demonstrated that *C. zofingiensis* showed excellent growth on glucose-supplemented media in batch culture. In the absence of light, formation of secondary carotenoids was mostly dependent on the initial carbon and nitrogen balance in the medium. Enhanced biosynthesis of these compounds was found in the medium with a high C/N ratio (*i.e.* C/N ratio = 180) (Ip and Chen, 2005).

MATERIALS AND METHODS

Equipment

Batch cultures were grown in six erlenmeyers of 500 ml with sealed with rubber caps with a hole for sample extraction. The culture was mixed at 300 rpm by means of magnetic stirrers (microMagMix). Three Erlenmeyers were used for the synthetic medium (blank) and the other three for the above-mentioned diluted effluent as substrate.

Waste used as culture medium of *Chlorella zofingiensis*

The experiments were carried out using the effluent from the two-stage anaerobic digestion of two-phase OMSW as substrate. The anaerobic effluent was sterilized at 121ºC with 2 bars of pressure for 20 min. The characteristics and features of the sterilized final effluent used as substrate are summarized in Table 1.

Synthetic culture media of *Chlorella zofingiensis*

The composition of the synthetic culture medium is shown in Table 2. The pH was adjusted to 6.5. The synthetic medium was sterilized at 121ºC for 20 min. The initial C/N ratio of the medium was adjusted to 30 by using a concentrated glucose solution (30 g/l).

Analytical techniques

The concentration of *Chlorella zofingiensis* was determinedby the measurement of Chlorophyll *a* (*Ca*) and Total Chlorophyll (*TC*), using the Spectrophotometric Standard Method (APHA, 1998). Dissolved oxygen (DO), oxygen partial pressure (OPP), saturation oxygen percentage (SO) and temperature (T) were measured by using an InoLab Oxy WTW oxymeter. The pH was measured using a CRISON Basic 20 pH meter. Total and soluble chemical oxygen demands (TCOD and SCOD) were performed according to Standard Methods for the Examination of Waters and Wastewaters (APHA, 1998).

Chlorella zofingiensis

A strain of bacteria-free *Chlorella zofingiensis* (supplied by the "Instituto de Bioquímica Vegetal y Fotosíntesis", "Consejo Superior de Investigaciones Científicas" (CSIC) of Seville, Spain) was used as inoculum. The culture of microalgae was previously carried out in a synthetic medium until the required amount for batch experiments was obtained.

Ambient conditions

The experiments were carried out at average temperatures of 28ºC during the day and 23ºC at night, with illumination

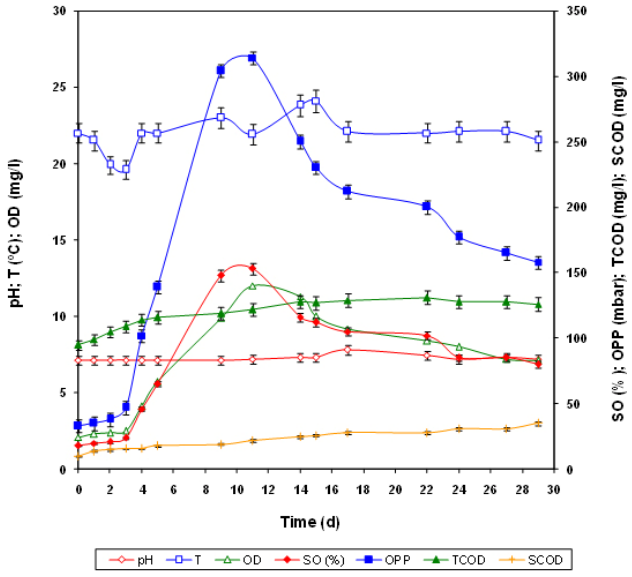

Figure 3. Variation of the parameters monitored with time in the culture of *Chlorella zofingiensis* carried out in the synthetic medium.

kept constant at 9 kw/m^2 per day by using fluorescent lamps. The cultures were placed in a chamber with controlled temperature and illumination.

Experimental procedure

The anaerobically pre-treated two-phase OMSW used in the batch experiments was previously settled for two hours and the supernatant obtained was sterilized as mentioned previously. The characteristics of the wastewater used as culture medium are shown in Table 1. All the experiments were carried out in triplicate; each experimental run lasted 29 days. During the experiments, triplicate samples were taken and analysed daily and the final results expressed as means. Figure 1 shows a schematic diagram of the different steps of the overall treatment process.

Experimental two-stage anaerobic reactor system

The waste used as the substrate in the batch experiments was the effluent coming from a two-stage anaerobic digestion of two-phase OMSW. The acidogenic step of this process was carried out at 35ºC ± 2ºC. The acidogenic reactor was operated at an organic loading rate (OLR) and hydraulic retention time (HRT) of 12.9 g TCOD/l day and 12.4 days, respectively (Borja et al. 2005b).

The effluent from the acidogenic reactor, operating at the above-mentioned optimum conditions, was processed in a methanogenic reactor. This was operated at mesophilic temperature (35ºC ± 2ºC) and at OLR of 20 g TCOD/l day and HRT of 5.0 days. These operational conditions were considered as optimum for the process after a long operation time (Borja et al. 2005b).

Finally, the effluent derived from the methanogenic reactor was used as a substrate in the batch experiments once it was sterilized as was previously described.

RESULTS AND DISCUSSION

Experimental results

Figure 2 and Figure 3 show the variation of the different parameters monitored during the experiment with the operation time for the microalgae culture in the anaerobic effluent and in the synthetic media, respectively. It can be seen in Figure 2, that pH, DO concentration, the saturation percentage and the partial pressure of oxygen increased during the first 11 days. The increase in these parameters may be attributed to the increase in the metabolic activity of the microalgae inoculated to the studied effluent. In addition, the increase in the microalgae concentration determined an increase in the oxygen production, causing a rise in DO, SO and OPP. As a consequence, the concentrations of TCOD and SCOD decreased by 37% and 45% respectively during the same period, showing that part of the organic matter was assimilated by the culture as a source of carbon and nutrients. Similar values of pH and DO concentration were observed in mixed batch cultures of heterotrophic bacteria and algae using settled domestic sewage as a sole source of carbon with influent substrate concentrations (TCOD) in the range of 200-800 mg/l (Kayombo et al. 2003). After the 11th day, the pH, DO, SO and OPP decreased with time and the TCOD and SCOD increased slightly, probably because the culture was in the endogenous phase which increases the rate of cell death.

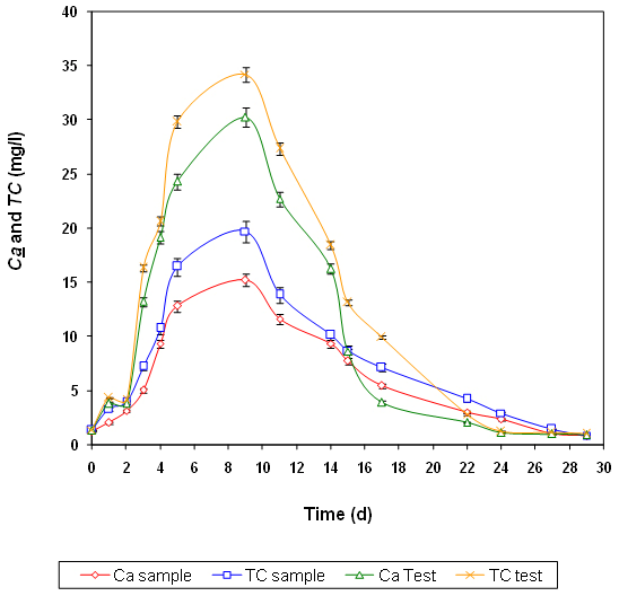

Figure 4. Variation of *Ca* and *TC* with time during the experiments carried out with the treated effluent (sample) and synthetic culture media (test).

Figure 3 shows the variation of the parameters monitored during the experiment in the case of the synthetic medium. As can be seen, a similar behaviour was observed when compared with the data of Figure 2. However, for the synthetic medium the increase of pH, DO, SO and OPP took place during the first 10 days. In addition, values of DO, SO and OPP were higher than those obtained when the anaerobic effluent was used as culture medium. Values of TCOD and SCOD increased with time during the whole experimental period because the source of organic matter was in this case the microalgal biomass itself. Therefore, an increase in the microalgae concentration brought about a rise in TCOD and SCOD values.

Kinetic model

Figure 4 shows the variation of chlorophyll *a* (*Ca*) and total chlorophyll (*TC*) concentrations with time for the studied effluent and synthetic medium during the 29 days of the operational period. These parameters are indirect indicators of the microalgae concentration, taking into account that *Ca* and *TC* are directly proportional to the microalgae concentration (Kayombo et al. 2003). Typical growth curves were obtained in both cases. The concentrations of microalgae were significantly higher for the synthetic medium when compared to the values obtained for the treated effluent. The lag phase may be located at the first 3 days for the treated effluent and at the first 2 days for the synthetic culture medium. The phases of exponential growth were located between the 2nd and 4th days of the experiment in the case of synthetic medium and between the 3rd and 5th days for the treated effluent. Therefore, the microalgae growth was lower in the case of the treated effluent medium than for the synthetic medium. After the 10th day, the dead phase appeared in both culture media and the concentration of *Ca* and *TC* decreased dramatically, the minimum value being observed at the end of the experiment. With the values of *Ca* and *TC* obtained during the exponential growth phase for both treated effluent and synthetic media, the values of the specific growth rates were determined by the following equation:

$$\mathrm{Ln}(X/X_0) = \mu t \qquad [1]$$

where X_0 is the concentration of microorganisms (concentration of *Ca* or *TC*) at the beginning of the exponential growth phase, X is the microorganisms concentration at time t, μ is the specific growth rate (d^{-1}) and t is the operation time (d). The plot of the naeperian logarithm of X/X_0 versus the time in the exponential growth phase must result in a straight line with intercept at the origin equal to zero and slope with a value equivalent to the specific growth rate. This plot was carried out for the experimental data obtained for both treated effluent and synthetic media. In both cases, *Ca* and *TC* were used as a measurement of the microalgae concentrations. In all cases, typical straight lines were obtained. For the treated effluent medium, the specific growth rate (μ) value was equal to 0.49 d^{-1} (0.02 h^{-1}), with a variance coefficient equal to 5%, the linear regression coefficient R^2 being equal to 0.97. In the case of the synthetic medium, the value of μ was found to be 0.72 d^{-1} (0.03 h^{-1}), with a variance coefficient equal to 5%, the linear regression coefficient R^2 being equal to 0.98. Therefore, the value of μ was 46% lower for the effluent treated medium, when compared with the synthetic medium, which may be considered as acceptable, and similar to other values reported in the literature for this microalgae (0.031 h^{-1}) by using glucose at a concentration of 20 g/l as culture medium (Ip and Chen, 2005) and other synthetic media (Del Campo et al. 2004). In addition, the value of μ obtained with the effluent from two-stage anaerobic digestion of two-phase OMSW was higher than that obtained in mixed cultures of *Chlorella vulgaris* by using settled and diluted piggery waste as substrate at similar initial TCOD concentrations (Travieso et al. 2006).

Table 2. Characteristics of Bristol's synthetic culture medium for *Chlorella zofingiensis.*

Reagent	Unit	Value
$NaNO_3$	g/l	0.75
K_2HPO_4	g/l	0.075
KH_2PO_4	g/l	0.175
$MgSO_4.7H_2O$	g/l	0.075
$CaCl_2.2\ H_2O$	g/l	0.025
NaCl	g/l	0.025
$FeCl_3.6\ H_2O$	mg/l	5
$ZnSO_4.7\ H_2O$	mg/l	0.287
$MnSO_4.\ H_2O$	mg/l	0.169
H_3BO_3	mg/l	0.061
$CuSO_4.5\ H_2O$	mg/l	0.0025
$(NH_4)_6Mo_7O_{24}.7\ H_2O$	mg/l	0.00124
C/N ratio	-	30

CONCLUDING REMARKS

It was found that two-phase OMSW previously treated by two-stage anaerobic digestion and further sterilized can be used as a culture medium for the microalgae *Chlorella zofingiensis.* TCOD and SCOD removals of 37% and 45% respectively were achieved after 11 days' operation time with initial TCOD and SCOD values of 1.93 and 0.70 mg/l respectively.

Typical growth curves were obtained in batch experiments using both treated effluent and a synthetic media. The specific growth rate (μ) obtained in the case of the treated effluent medium was lower (0.02 h^{-1}) than that obtained for the synthetic medium (0.03 h^{-1}). However, the μ value obtained with the anaerobically digested two-phase OMSW was higher than that obtained with other species of *Chlorella* (*i.e. C. vulgaris*) using different wastewaters as substrates such as diluted and settled piggery wastes with similar TCOD values.

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