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A simplified process of swine slurry treatment by primary filtration and *Haematococcus pluvialis* culture to produce low cost astaxanthin

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ABSTRACT

A simplified process for treating swine slurry through primary filtration and subsequent depuration of the filtrate with the astaxanthin-rich microalga *Haematococcus pluvialis* is proposed. The first step comprises a low-cost filtration system capable of reducing 66% of ammonia, 7% of phosphorus and 19% of chemical oxygen demand, and increasing the concentration of nitrate, being this useful for subsequent growth of the algae. The second step comprises the discontinuous cultivation of *H. pluvialis* in diluted filtered slurry. The optimal dilution was researched by testing undiluted and 2, 4 and 8-fold diluted filtrate. This step led to a drastic reduction in macro and micronutrients concentration (up to 99% for NO_3-N and NH_4-N, 98% for TP and 26% for chemical oxygen demand). After *H. pluvialis* growth the accumulation of astaxanthin took place for 14 d in nutrient-deprived conditions: an astaxanthin accumulation of 1.27% on a dry weight basis was measured. These results indicate the possibility to couple low-cost filtration and microalgae production to recover nutrients from swine wastewaters and to add value by producing valuable astaxanthin for the feed market or for an on-farm utilization as feed addictive.

1. Introduction

Managing the input of organic and mineral nutrients derived from livestock rearing activities into the atmosphere and water-bodies poses both technical and economic challenges to the agricultural sector. Storage and land application of animal slurries cause the loss of large amounts of N into the air due to volatilization of ammonia (Clarisse et al., 2009), and also to nitrate leaching and the accumulation of P and K compounds in agricultural soils (Ledda et al., 2013). Manure management is thus crucial to minimize losses of valuable nutrients and to prevent contamination and/or eutrophication of the surrounding environment (Kebede-Westhead et al., 2006).

One opportunity for the treatment of these effluents is the utilization of microalgae- based processes. Compared to physical and chemical treatment processes, algae-based treatment can potentially achieve nutrient removal from wastewater in a less expensive and ecologically safer way, with the added benefits of producing added-value biomass (Oswald, 2003). Common nitrogen removal methods such as bacterial nitrification/denitrification remove the majority of the nitrogen as N_2 gas, whereas algal treatment retains useful nitrogen compounds in the biomass. Usually in conventional biological wastewater treatment processes the biomass produced must be treated and disposed of safely and in an economically feasible way, increasing operating costs. In microalgae cultivation, nitrogen can be converted into a valuable biomass to produce bioactive substances, bioenergy, or valuable chemicals that can enhance the economic viability of the process (Kang et al., 2006). Notwithstanding these benefits, microalgae treatment presents some difficulties as an integrative process for common nutrient removal, mainly due to the low rates of growth and nutrient uptake by microalgae. Many efforts have been made over several decades to study and optimize microalgae cultivation in wastewaters, both in stand-alone and integrated systems, mainly to achieve nutrient reduction and/or abatement in the final effluent. In particular, swine manure is characterized by high contents of nitrogen (up to 872 mg L^{-1}, Borin et al., 2013), organic matter (Roche, 1984) and suspended solids (Buelna et al., 2008), so that a pre-treatment system reducing these components is often necessary before final depuration by algae.

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As a primary step, filtration could contribute to an economic solution; traditional methods of wastewater filtration consist of trickling filters, rotation biological contactors, intermittent sand filters and infiltration percolation systems (Loupasaki and Diamadopoulos, 2013). Although sand layer filters are quite common (Roseth, 2000; Liu et al., 2003; Nakhla and Farooq, 2003; Tao and Wang, 2009; Zheng et al., 2012), the aim of the treatment can be different, depending on the substrate used. Biological filters filled with semi-soft plastic media can give good results in chemical oxygen demand (COD) removing (Wie et al., 2010).Yasuda et al. (2009) demonstrated that rock wool could be a possible substrate for ammonia removal from manure composts. Laterite material has also been shown to be a suitable medium to reduce COD, biological oxygen demand (BOD_5), ammonia, nitrite and turbidity (Kadam et al., 2009). Saliling et al. (2007) suggested wood chips and wheat straw as alternative biofilter media to treat high nitrate content wastewaters.

Regarding the microorganisms, different microalgal strains of the *Chlorella*, *Scenedesmus*, *Arthrospira* and *Chlamydomonas* genus have commonly been used for the phytoremediation of industrial, urban and agricultural wastewaters (Ji et al., 2013; Franchino et al., 2014), producing widely chemically diversified biomasses which have claimed to have potential applications, above all in the biofuels and feed sectors. However, more valuable microorganisms such as the species *Haematococcus pluvialis* are not usually tested for this application because of its sensitivity to adverse culture conditions. Moreover, many studies have been made on the development of an optimal synthetic growth medium for *H. pluvialis* (Gong and Chen, 1997; Fábregas et al., 2000) but, as far as we are aware, few studies focused on the possibility of using agricultural wastewaters for *H. pluvialis* culture and subsequent astaxanthin production (Kang et al., 2006). This is relevant because *H. pluvialis* produces a valuable ketocarotenoid named astaxanthin with applications in nutraceuticals, cosmetics, food and feed industries. *H. pluvialis* represents the richest source of natural astaxanthin, which is a high-value product for human applications (US$ 2500 per kg with an annual worldwide market estimated to be over US$ 200 million) whereas for feed, the synthetically-derived astaxanthin is mainly used due to its lower cost (Del Río et al., 2008). Consumers' demand for natural products provides an opportunity for the natural molecule to be used as feed if it can be produced at low cost using wastes as raw material. Commercial production of *Haematococcus*-derived astaxanthin has been commonly reported by using a two-step culture where the first stage is done photoautotrophically under highly controlled culture conditions in either tubular, bubble column or airlift photobioreactors and the reddening stage, which is less prone to contamination, is done in open cultivation ponds (Hata et al., 2001). The conditions triggering the accumulation of astaxanthin in *H. pluvialis* cells have been widely studied: nutrient starvation is known to be effective. In this work a simplified process for the treatment of pig slurry through a low-cost filtration system and the subsequent effect of *H. pluvialis* in removing nutrients and producing astaxanthin was studied. The results of this work are useful as a first insight into a proposed integrated farm facility where a low cost pre-treatment and microalgae cultivation would deal with nutrient overload in animal slurries and allow on-farm high added-value production of astaxanthin.

2. Materials and methods

2.1. Swine slurry filtration

The primary treatment plant consisted of an upstream filtering system that served a downstream phytodepuration system, located at the Experimental Farm "Lucio Toniolo" at Legnaro in Padova, Italy. The plant treated a wastewater portion coming from a piggery made up of feces, urine, and fresh water used daily for cleaning. The filtering system was made up of six filters each consisting of an iron structure of 0.5 m^3 of capacity (0.8 m × 0.8 m × height 0.8 m); each filter was filled with a different substrate (Table 1). The filtering system worked in parallel, so that each filter was fed simultaneously with wastewater moving vertically on to the filling substrate. A daily load volume of 30 L of swine wastewater coming from the storage tank of the piggery (5 L per filter) with 5 re-circulations, was applied to the system. All effluents coming from the filters were finally mixed before being introduced into the phytodepuration system. The filtering system was run for 35 d. Inlet and outlet streams were collected during this period to monitor both chemical and physical parameters. During the last day of filter monitoring, filtered wastewater was sampled and preserved for algae cultivation trials.

2.2. H. pluvialis cultivation

The green microalga *H. pluvialis* (strain number 34-1d) was obtained from the SAG Culture Collection of the University of Goettingen (Germany). Pre-cultures were maintained in 200 mL Erlenmeyer flasks in Bold Basal Medium for freshwater microalgae, under constant illumination provided by fluorescent cool white lamps (Osram L13W/840) at an irradiance of 50 $\mu E\ m^{-2}\ s^{-1}$. Growth experiments were performed in batch mode.

The filtered slurry was further filtered with 0.45 Whatman GF/C filters and diluted with distilled water to obtain a final growth medium containing 100%, 50%, 25% and 12.5% of filtered slurry. A preliminary test with 100% filtered slurry did not result in any microalgal growth (data not shown). Cultures were inoculated at an initial biomass concentration of 0.05 $g\ L^{-1}$: this step was performed in discontinuous mode in 500 mL Erlenmeyer flasks containing 300 mL of cell suspension. The flasks were continuously illuminated using two 30W OSRAM-Sylvania GRO-LUX 6400 °K lamps, with an irradiance on the flasks' surface of 150 $\mu E\ m^{-2}\ s^{-1}$. The temperature of the culture was maintained constant at 25 °C. Mixing was provided by bubbling air at 0.2 v/v/min, no additional CO_2 being supplied. Experiments were performed in duplicate.

2.3. Biomass concentration and astaxanthin content

Growth of the cultures was monitored by optical density (OD) measurement at 750 nm wavelength. A linear relationship was previously found between absorbance at 750 nm and dry weight of the culture ($R^2 = 0.99$; $p < 0.01$; $n = 7$). Additionally, dry weight of biomass in the cultures was periodically measured by drying filtered cells at 85 °C for 24 h after filtration of 20 mL of culture through a pre-weighed GF/C filter (Whatman). Astaxanthin accumulation took place in the experimental conditions of the growth trials. Its content was spectrophotometrically determined according to Boussiba et al. (1992) after the beginning of the stationary phase and the depletion of nitrogen compounds, for a total of 14 d in starvation conditions. All the analyses on cultures were done in triplicate.

2.4. Wastewater physical and chemical analyses

The pH of the wastewater was measured on-site using a Hach Lange HQD 40d multi-parameter with interchangeable probes according to standard methods (APHA, 1998). Turbidity (NTU) was measured using a portable HI83414 (HANNA Instruments) turbidimeter. Chemical concentrations in the wastewater were analysed in a laboratory immediately after the sample collection. Chemical Oxygen Demand (COD), total nitrogen (TN), ammonia nitrogen (NH_4^+-N) and nitric nitrogen (NO_3-N) were determined using Mackerey-Nagel NANOCOLOR® kit for COD (COD 1500), Total