

From a Pollutant Byproduct to a Feed Ingredient

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1. Introduction

Industrial activities have always been associated to the economic development of nations and their population. Nevertheless, they are also associated to the generation of industrial byproducts, generally considered undesirable due to the environmental damage they impose to society (Pipatti et al., 2009). Industrial byproducts have variable characteristics and compositions, since they are directly dependent on crude matter essence, kind of processing, facilities characteristics and volume of output, among so many other factors. Nowadays, the broad range of industries spread all over the world in an effort to supply the necessity of global population makes evident the need for the adoption of strategies capable of equilibrating economic development and environmental preservation as a way of reaching a sustainable industrial production (Parente & Silva, 2002).

In that way, transformation industries are currently searching for productive technologies of low environmental impact, which include practices like minimization of byproducts generation and/or recuperation and recycling of these residues, so aiming at the optimization of industrial processes (Juskaitė-Norbutienė et al., 2007; Leite & Pawlowsky, 2005; Souza & Silva, 2009). The adoption of such technologies is a differential for the establishment and maintenance of industries in the current social and economic world scenery (Leite & Pawlowsky, 2005).

The management of industrial byproducts generally combines techniques as recuperation, treatment and safe disposal. Regarding to liquid waste, also called wastewater or effluent, treatments performed in the food industry generally consist of physical, chemical and biological operations. Physical treatments provide the removal of suspended solids and the separation of oils and fats by means of filtration, grading, sedimentation or floating techniques, while chemical treatments provide the removal of dissolved matter and even of microorganisms by using different chemicals (Giordano, 2006). The biological treatments, in turn, count on the ability of bacteria, fungi, micro algae and protozoa in transforming organic matter into new cells, called biomass, and gases (Arvanitoyannis & Tserkezou, 2009; Giordano, 2006). This kind of treatment simulates the natural remediation processes that occur in nature and brings as an advantage the production of compounds with particular applications, which may be appropriately separated and used for distinct purposes (Liu, 2007). Microbial biomass, for instance, has been considered as an alternative source of

proteins for foods and feeds and may be produced in different substrates, including effluents from industries and farms (Nasseri et al., 2011).

Some organisms may be used for the removal of organic matter from agro industrial residues yielding a biomass with potential for use in animal feeding, such as the phototrophic bacteria (Azad et al., 2003; Izu et al., 2001; Ponsano et al., 2008). Purple Non Sulfur Bacteria (PNSB), for example, are phototrophic bacteria commonly found in rivers, ponds, lakes and wastewater treatment systems, that can grow both as photoautotroph and photoheterotroph under anaerobic-light or microaerobic-light conditions (Choorit et al., 2002; Kantachote et al., 2005). Some PNSB also can grow in the dark using fermentation when they are in anaerobic environments or respiration when in aerobiosis (Devi et al., 2008; Kantachote et al., 2005; Kim et al., 2004; Ponsano et al., 2002a). Due to the ability of phototrophic bacteria to utilize diverse metabolic activities in different substrates and growth conditions, they find a role in the depollution of wastewaters from food industries, still producing a biomass rich in proteins, vitamins and carotenoids that may be used in the supplementation of animal feed (Carlozzi & Sacchi, 2001; Izu et al., 2001; Kantachote et al., 2005; Ponsano et al., 2002a, 2003a, 2004a, b; Zheng et al., 2005 a, b).

Rubrivivax gelatinosus, formerly named *Rhodocyclus gelatinosus* is a PNSB commonly found in many wastewaters in which it grows as an autotrophic or a heterotrophic, depending on light and oxygen conditions (Ponsano et al., 2003a, 2008). As the bacterium produces oxycarotenoids as photosynthetic pigments, its biomass can find use as a pigmenting additive in animal production, as previously suggested and tested by Ponsano et al. (2002b, 2003b, 2004a, b) and Polonio et al. (2010).

The use of pigmenting additives in animal production is justified by the fact that animals are unable to synthesize their own carotenoids and therefore, rely on dietary supply to achieve their natural pigmentation (Gouveia et al., 2003). The effectiveness of oxycarotenoids or xanthophylls in providing pigmentation to animals is possible because these carotenoids have the ability to deposit on different parts in animal bodies, such as muscles, fat, skin, feather, legs, ovaries and eggs (Ponsano et al., 2002b, 2004b).

Primarily, pigmenting additives were added into food formulations in order to replace color lost during the industrialization processes but, when the remarkable acceptance of consumers for well colored products was identified, industries started coloring a broad range of food items, reaching consumers desire and so improving its sales (Calil & Aguiar, 1999). In case of poultry and fish production, for instance, either natural or synthetic additives are used when intensive rearing is adopted and/or when feed ingredients are poor in xanthophylls, so lacking in color in the final products. The most used synthetic additives for this purpose are apocarotenoic acid ethyl ester, canthaxanthin and astaxanthin, which show good stability and deposition rates on animal tissues. Nevertheless, more and more consumers around the world have been showing their preference for natural additives, what stimulates the search for natural sources of pigments, like those from biotechnological production. Among natural xanthophylls used in animal production, those from plants, algae, bacteria and yeasts have been previously described in literature (Akiba et al., 2000, 2001; Bosma et al., 2003; Gouveia et al., 1996; Liufa et al., 1997; Perez-Vendrell et al., 2001; Toyomizu et al. 2001).

The great acceptance that fish finds among consumers due to its nutritional and sensorial properties guarantees its market and yet claims for increases in production, which has been supplied by the aquaculture (Lem & Karunasagar, 2007). Nevertheless, fish is a perishable food and so requires the application of methods for its preservation, such as fermentation,

refrigeration, freezing, canning, smoking, drying and others, that may be performed separately or in combinations. As it happens in any other food industry, fish processing generates great amounts of wastewaters with variable Chemical Oxygen Demand which depends on fish species, fish products and methods of processing, since water is involved in several stages of manufacturing, like butchering, evisceration, filleting, salting, cooking, canning, freezing, sterilization and cleaning operations (Arvanitoyannis & Kassaveti, 2008; Liu, 2007). The utilization of these effluents for the biomass production is an alternative for minimizing costs with treatment and environmental impacts. Moreover, in case the composition of the biomass finds an appropriate purpose, it can represent extra profits for the industry.

So, the hypothesis to be tested in this chapter is that an industrial byproduct may undergo a biological treatment yielding a product with application. The objective of this chapter was to describe a study on the transformation of a fish processing wastewater into a product with potential of use in animal rearing.

2. Study conduction

2.1 Wastewater characterization and treatment

Tilapia fish processing wastewater used in the experiment was donated by Tilapia do Brasil Inc. (Buritama City, SP, Brazil) and was made up of effluents from killing, scaling, gutting, cleaning, skinning, filleting and freezing operations, and also from cleaning operations, which were gathered and roughly filtered (grating), averaging 10,000 L h⁻¹.

Crude wastewater was analyzed for turbidity, total solids (TS), pH, total nitrogen (TN) and oils and greases (OG), according to standard methods (American Public Health Association, American Water Works Association, Water Pollution Control Federation [APHA, AWWA and WPCF], 2005). Chemical Oxygen Demand (COD) was determined by chemical digestion (HR digestion solution for COD 0-1500 ppm; DRB200; DR2800; Hach), based on the protocol developed by Jirka & Carter (1975).

Before being used as a substrate for the bacterial growth, the wastewater was filtered in a 50 µm mesh fast filter (Gardena 1731; 3,000 L h⁻¹) for the withdrawal of gross particles and heat treated (Incomar LTLT tank) at 65°C/30 min to eliminate pathogenic agents and repress the level of competing microorganisms. After that, wastewater was cooled to room temperature and so it was ready to receive the bacterial inoculum.

Microbiological analyses of crude and heat treated wastewater comprised mesophilic aerobic bacteria, total and fecal coliforms, molds and yeasts, *Aeromonas* spp and *Salmonella* spp, and were performed according to standard methodology (APHA, AWWA and WPCF 2005).

2.2 Bacterial inoculum preparation

Rubrivivax gelatinosus previously isolated from poultry slaughterhouse wastewater and characterized by morphological and biochemical tests was used in this experiment. The cells were maintained in Pfennig medium containing (per liter): 0.5 g KH₂PO₄; 0.4 g MgSO₄·7 H₂O; 0.4 g NaCl; 0.4 g NH₄Cl; 0.05 g CaCl₂·2H₂O; 1.0 g sodium acetate, 0.2 g yeast extract; 0.005 g ferric citrate; 10.0 mL trace elements solution (FeSO₄·7H₂O 200 mg; ZnSO₄·7H₂O 10 mg; MnCl₂·4H₂O 3 mg; H₃BO₃ 30 mg; CoCl₂·6H₂O 20 mg; CuCl₂·2H₂O 1 mg; NiCl₂·6H₂O 2 mg; Na₂MoO₄·2H₂O 3 mg); 20.0 g bacteriological agar; 10.0 ml biotin sol. (0.0015%) and 10.0 ml thiamine-HCl sol. (0.005%). The pH was adjusted to 7.0 before autoclaving at 121°C for 15 min.

For the initial inoculum preparation, cells were grown in Pfennig liquid medium with the same pH and composition described above but bacteriological agar, under anaerobiosis (fully filled screw-crap tubes), $32 \pm 2^\circ\text{C}$ and $1,400 \pm 200$ lux for approximately 3 days, until a slight red color arose.

For the final inoculum, an aliquot from initial inoculum was transferred at 1% (v/v) to the same medium and incubation was carried out under the same conditions described before, until optical density at 600 nm reached 0.5 (Ponsano et al., 2003a).

2.3 Biomass preparation and recuperation

The bacterial inoculum was added, at 1% (v/v), to 100 L of treated wastewater. Cultivation was accomplished in anaerobiosis inside 100 L glass reactors at $32 \pm 2^\circ\text{C}$ and $2,000 \pm 500$ lux for seven days.

For the biomass recuperation, the culture was filtered at $0.2 \mu\text{m}$, $1.5 \text{ m}^3 \text{ h}^{-1}$ and 4.5 bar (Frings), giving origin to a concentrate containing the cells and a permeate. The concentrate was centrifuged at 3,400 g for 30 min at 5°C (Incibras Spin VI) and the resulting slime was frozen at -40°C and lyophilized (Liobras L 101) for 48 h. Hand grinding was performed to obtain the power biomass. Procedures were repeated six times.

2.4 Process analyses

Cell mass concentration was determined from 20 mL of concentrate, after successive centrifugation (900 g/15 min) and washing cycles followed by drying at 80°C until it gets constant weight.

For productivity determination, it was considered the mean production of dry biomass per liter per day.

TN, OG, COD and pH determinations in permeate were accomplished as previously described for crude wastewater (APHA, AWWA and WPCF, 2005).

2.5 Biomass analyses

For the microbiological characterization by biomass, total and fecal coliforms, molds and yeasts, coagulase-positive staphylococci, *Aeromonas* spp and *Salmonella* spp were investigated according to methodologies described by Vanderzant & Splittstoesser (1992).

For the proximate composition of biomass, the concentrations of moisture, lipids, proteins and ash were determined according to Association of Official Analytical Chemists (1995).

Amino acid determinations were carried out before and after acid hydrolysis (5 mg of extract) with a mixture containing 6 mol L^{-1} of HCl and 5% phenol/water (0.08 mL) for 72 h at 110°C . Samples were dried, diluted with citrate buffer pH 2.2 and filtered in a GV Millex Unity (Millipore). Amino acids analyses were performed by cation-exchange chromatography using a Shimadzu LC-10A/C-47A, sodium eluents and post-column derivatization with o-phthalaldehyde. Identification and quantification were accomplished by the comparison of retention time and area of each amino acid with a standard containing 16 amino acids (100 nmol mL^{-1}), respectively (Fountoulakis & Lahm, 1998).

The biomass color attributes *L* (lightness), *C* (chroma) and *h* (hue) were obtained from the average of three consecutive pulses launched from the optical chamber of the MiniScan XE Plus (Hunter Lab) using illuminant D65 and 2° observer, after calibration with black and white standards (Commission Internationale de l'Éclairage, 1986).

For the determination of oxycarotenoids, an adaptation of Valduga (2005) methodology was used. Pigments were extracted from biomass with dimetilsulfoxide at $55^\circ\text{C}/30$ min and

alternated cycles of ultrasound at 40 kHz (Unique/USC 1800A) and shaking (Phoenix/P-56). Next, a mixture containing acetone: methanol (7:3, v/v) was added, tubes were centrifuged at 3.400 g and 5°C/10 min and the supernatant was transferred to a 50 mL volumetric flask. Successive extractions were performed until no color remained in cells or solvent. Final dilutions were made up with methanol and the quantification of oxycarotenoids was accomplished at 448 nm (Hitachi U-1000/U-1100). Total carotenoids were estimated according to Davies (1976) using the absorption coefficient of carotenoids suggested by Liaaen-Jensen & Jensen (1971).

3. Main findings of the study

The microbiological investigation on crude and treated wastewaters showed a sharp decrease in indicator organisms after heat treatment (Table 1).

Aeromonas spp are spread in aquatic environments, what may explain the presence of such organism in the crude effluent. Nevertheless, some species such as *A. hydrophila* and *A. salmonicida* may be responsible for lethal infections in fish, bringing considerable economic losses to aquaculture (Maluping et al., 2005; Vieira, 2003) and some others have been described as emergent pathogens for humans (Vieira, 2003). So, the presence of this microorganism in the crude wastewater claims for periodic control in aquaculture, slaughter and processing of tilapia fish, as a way of avoiding financial injury to the fish industry and to consumers.

The presence of *Salmonella enterica* subsp. *enterica* serotype *Typhi* was detected in the wastewater, which represents a potential risk to public health and reveals deficient sanitary conditions during manipulation in the industry, since man is the natural reservoir of this serotype. This bacterium may be transmitted by water and foods contaminated with human feces, causing a serious infectious disease (Franco & Landgraf, 1996).

Microbiological analysis	Crude wastewater	Treated wastewater ²
Mesophilic aerobic bacteria (CFU* mL ⁻¹)	8.5 x 10 ⁵	7.0
Moulds and yeasts (CFU mL ⁻¹)	4.6 x 10 ³	6.0
Total coliforms (MPN** mL ⁻¹)	1.0 x 10 ⁵	<1.0
Fecal coliforms (MPN mL ⁻¹)	0.41	<1.0

¹Mean values. ²Filtration (50 µm)/heat treatment (65 °C/30 min). *Colony Forming Units. **Most Probable Number.

Table 1. Microbiological characteristics of tilapia fish industrial wastewater¹

Heat treatment was able to eliminate contaminants and pathogenic microorganisms detected in the crude wastewater, so reducing competition for substrate during *Rubrivivax gelatinosus* cultivation.

The knowledge on the wastewater physicochemical properties reveals its suitability for discharge. Total solids, for instance, represent dissolved or suspended substances, both of organic or inorganic structures and, if too high, may cause damages to water bodies and aquatic organisms. Turbidity units indicate the transparency of the wastewater and the presence of colloids that, when excessive, may alter the aspect of streams and rivers and so prevent photosynthetic organisms' metabolism. The acidic or alkaline characteristic of the wastewater is defined by pH and, together with temperature, find an important role on the control of biotechnological processes. Nitrogen in wastewaters may derive from synthetic

detergents used during cleaning operations or from protein degradation. Although this element may be essential to most living organisms, in high concentrations it may cause the proliferation of aquatic plants in water bodies and effluents. Oils and greases in wastewaters may originate from industrial kitchens, mechanic repairs garages, boilers and other equipments, as well as from raw material. They can easily be oxidized and so exhale bad odors in the environment. COD is an indirect measure of organic compounds concentration in wastewaters and so, reflects its pollutant load (Giordano, 2004; Liu, 2007).

The physicochemical data found for crude tilapia fish processing wastewater (Table 2) indicate the need for previous treatments for a safe discharge, according to Brazilian legislation. On the other hand, the presence of such organic matter in the wastewater was important to ensure the growth of *R. gelatinosus* with the resulting production of cells and oxycarotenoids.

Physicochemical parameter	Quantity
Effluent volume (l day ⁻¹)	120,000
Effluent flow (l h ⁻¹)	11,000 to 15,000
Temperature (°C)	20.3 ± 0.23
Total solids (g L ⁻¹)	1.5 ± 0.32
Turbidity (TU)	35.7 ± 2.25
pH	9.4 ± 0.09
Total nitrogen (mg L ⁻¹)	813.3 ± 54.65
Oils and greases (mg L ⁻¹)	1,166.3 ± 68.52
COD (mg L ⁻¹)	1,127.5 ± 33.84

¹Mean values and standard errors.

Table 2. Physicochemical characteristics of crude tilapia fish industrial wastewater¹

The physicochemical characteristics of wastewaters presented herein differ from others previously reported. This happens because the particular characteristics of each industrial effluent derive from crude matter composition, season of the year, water supply, reuse procedures, factory installations and industrial processing techniques, among others (Liu, 2007). For settled and unsettled wastewater from sardine processing industry, for example, pH values from 6.2 to 6.3; 63,000 mg L⁻¹ COD and 10.88 mg L⁻¹ TN were described (Azad et al., 2001; 2003). For white fish filleting plants, Arvanitoyannis & Kassaveti (2008) reported the generation of wastewater with 50 kg COD and Prasertsan et al. (1993) reported 5.3 to 8.3 pH; 5,950 to 157,080 mg L⁻¹ COD; 19.30 to 82.22 g L⁻¹ TS and 666 to 32,182 mg L⁻¹ OG for effluents from different seafood processing plants. Concentrations around 4,300 mg L⁻¹ COD, 800 mg L⁻¹ OG and 6.2 to 7.0 pH also were reported for wastewater from fish processing operations by Giordano (2004).

Changes in tilapia fish wastewater physicochemical parameters after biomass recuperation comprised removals of 82% in COD, 48% in OG and 22% in TN and a decrease in pH to 7.9, rendering it suitable for discharge in the environment, according to Brazilian laws. So, the biomass production process itself worked as a biological treatment for the reduction of pollution in tilapia fish industry wastewater.

Mean cell mass production and productivity achieved with the biological treatment were 0.18 g L⁻¹ and 0.0634 g L⁻¹ day⁻¹, respectively. Prasertsan et al. (1993) credit the low cell production to the anaerobiosis/light cultivation conditions, in which the synthesis of oxycarotenoids is intensified. Other authors found higher cell mass concentrations when growing phototrophic organisms in industry wastewaters but, in those cases, initial organic matter and inoculum levels were higher than the ones used in this study and/or nutritional supplementation was

adopted (Azad et al., 2001, 2003; Prasertsan et al., 1997). In this study, we opted to maintain the original wastewater composition and to use a low inoculum level in an attempt to minimize costs and render the biomass production process feasible for the industry.

The microbiological investigation on *Rubrivivax gelatinosus* biomass indicated low counts on total coliforms (20.27 NMP g⁻¹), fecal coliforms (< 1.0 NMP g⁻¹) and molds and yeasts (1.2 x 10³ UFC g⁻¹) and the absence of pathogenic organisms. This way, the product showed to be in agreement with Brazilian microbiological standards required for feed ingredients, which ensures its safe utilization.

Mean proximate composition of biomass and amino acid profile in the product are presented in Tables 3 and 4, respectively. As a typical feature of single cell proteins, the values indicate the high level of proteins in the biomass, which denotes its use in animal diets as a nutritional ingredient. Moreover, it also contained considerable amounts of all amino acids considered essential for animals, what reinforces the suggestion of its use in the supplementation of animal feeds in order to supply deficiencies that may cause, for instance, delay in protein utilization and reduction of growth, weight gain, feed conversion and immunity (Cyrino et al., 2004). In view of these findings, the bacterial biomass presents a potential for use as a nutritional ingredient for feeds.

Component	%
Moisture	4.55 ± 0.84
Ash	4.05 ± 0.66
Protein	57.39 ± 2.81
Lipids	11.08 ± 1.41

¹Mean values and standard errors.

Table 3. Proximate composition of *Rubrivivax gelatinosus* biomass produced in tilapia fish industrial wastewater¹

Amino acid	Quantity (g 100 g ⁻¹)
Aspartic acid	5.70 ± 2.35
Threonine	3.82 ± 1.50
Serine	2.81 ± 0.96
Glutamic acid	6.40 ± 2.29
Proline	2.93 ± 1.02
Glycine	3.46 ± 1.51
Alanine	5.32 ± 2.28
Valine	4.39 ± 1.84
Methionine	0.66 ± 0.29
Isoleucine	3.33 ± 1.43
Leucine	7.08 ± 2.41
Tyrosine	2.56 ± 0.96
Phenylalanine	3.43 ± 1.31
Histidine	1.92 ± 0.74
Lysine	4.52 ± 1.76
Arginine	3.85 ± 1.29

¹Mean values and standard errors

Table 4. Amino acid composition of *Rubrivivax gelatinosus* biomass produced in tilapia fish industrial wastewater¹

Oxycarotenoids content in the biomass was found to be 3.03 mg g⁻¹ dry biomass, which conferred a dark red color to the power product ($L = 22.42$; $C = 14.22$; $h = 25.48$). This is in agreement with Prasertsan et al. (1997), who found concentrations of 2.13 to 3.90 mg of carotenoids per gram of dry biomass of *Rhodocyclus gelatinosus* produced in tuna processing wastewater.

The main photosynthetic pigments produced by *Rubrivivax gelatinosus* are bacteriochlorophyll *a* and carotenoids from alternative spirilloxanthin series, which contains spheroidene, hydroxyspheroidene and spirilloxanthin as the major representants (Holt et al., 2000). The blend among these pigments gives the bacterial cultures a reddish color (Ponsano et al., 2002a, 2003a, 2008) that remains in the dry biomass, since sensorial and nutritional properties of lyophilized products remain intact after drying process (Pereda et al., 2005). Considering that these pigments are oxycarotenoids and so have the ability to deposit in animal tissues, this feature of the biomass suggests its application as a pigmenting ingredient for the rearing of different animals.

The use of natural or synthetic oxycarotenoids for the rearing of animals is reported by many authors. Salmonids, for instance, are noble fish natural from cold waters in North Hemisphere, but that are being commercially farmed in many parts of the world. According to Baker & Günther (2004), in wild salmon, the natural carotenoid astaxanthin provides a majority of the color expected from this flesh. Nevertheless, for farmed salmonids, the same effect may be achieved by the use of pigmenting additives in rations. They may also be used for the raising of ornamental fish to increase skin color and beauty. For the raising of red *Cyprinus carpio* (*Kawari*), for instance, Gouveia et al. (2003) relate the utilization of carotenoids produced by micro algae *Chlorella vulgaris*.

For poultry products, the pigmentation varies according to market demand. In Mexico, Belgium, Italy, Peru and some regions in Brazil, for instance, the use of pigmenting ingredients in poultry production is a common practice since people prefer strong colors for broilers carcasses and egg yolks (Gouveia et al., 1996; Toyomizu et al., 2001). People often associate strong colors of a food item to safety and health and so look for strongly pigmented products. Taking it into account, Ponsano et al. (2002b, 2004a, b) added *Rhodocyclus gelatinosus* biomass produced in poultry slaughterhouse wastewater in broilers rations and found an increase in the color of breast meat. Polonio et al. (2010) used different concentrations of the same product in hens rations and found an improvement in yolks color, with no deleterious effects on birds performance. In the sensorial test, these authors identified the concentration of the biomass that, when used together with corn xanthophylls, provides a desired golden orange color to the yolks. Yet, Garcia et al. (2002) found an increase in yolks color, with no influence in the performance and eggs characteristics, when canthaxantin was used in hens diets.

Besides the pigmenting feature of oxycarotenoids, they are also known to exert benefits on animal health and welfare due to antioxidant properties. According to Baker & Günther (2004), evidences suggest that the carry-over of these pigments into the human food chain could be beneficial to human health too. In humans, the consumption of oxycarotenoids is associated to aging prevention and to the decrease of the risk of diseases related to the accumulation of free radicals (Bhosale, 2004; Bhosale; Bernstein, 2005). So, for further studies on the properties of *Rubrivivax gelatinosus* biomass, the antioxidant ability of its carotenoids will be considered.

4. Conclusion

In this chapter we showed the feasibility of using an industrial byproduct for the production of a biomass with potential of use in animal rearing, not only for being a source of natural

pigments but also for having an elevated nutritional value. Moreover, we showed that the biomass production process worked as a biological treatment for the reduction of pollution in the industrial wastewater, requiring simple and feasible methods that can be operated in the industry, so minimizing byproducts and still rendering profits from the biomass commercialization.

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