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Microbial Astaxanthin Production from Agro-Industrial Wastes—Raw Materials, Processes, and Quality

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Abstract: The antioxidant and food pigment astaxanthin (AX) can be produced by several microorganisms, in auto- or heterotrophic conditions. Regardless of the organism, AX concentrations in culture media are low, typically about 10–40 mg/L. Therefore, large amounts of nutrients and water are necessary to prepare culture media. Using low-cost substrates such as agro-industrial solid and liquid wastes is desirable for cost reduction. This opens up the opportunity of coupling AX production to other existing processes, taking advantage of available residues or co-products in a biorefinery approach. Indeed, the scientific literature shows that many attempts are being made to produce AX from residues. However, this brings challenges regarding raw material variability, process conditions, product titers, and downstream processing. This text overviews nutritional requirements and suitable culture media for producing AX-rich biomass: production and productivity ranges, residue pretreatment, and how the selected microorganism and culture media combinations affect further biomass production and quality. State-of-the-art technology indicates that, while *H. pluvialis* will remain an important source of AX, *X. dendrorhous* may be used in novel processes using residues.

Keywords: residues; pretreatment; secondary wastewater; culture media; nutrients

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1. Introduction

Astaxanthin (AX) is a ketocarotenoid important for its role as a pigment. It is responsible for the pink to orange color in flamingoes, crustaceans, and salmonid fish. However, none of these animals synthesize their astaxanthin; they acquire it through the food chain. The primary producers of astaxanthin are some bacteria, fungi, and microalgae that accumulate the pigment intracellularly for its photoprotection role against excessive light. Astaxanthin has a very high antioxidant capacity, superior to beta-carotene, lycopene, and many other carotenoids [1–3], making it a growingly important nutraceutical.

The astaxanthin market is about US$ 647 million and projected to reach US$ 880–968 million in 2026 [4,5], at a market annual growth rate estimated from 8.3 to 16.8% [5,6]. Most of the astaxanthin produced commercially is synthetic [7]. However, natural astaxanthin seems to perform better than synthetic in aquaculture [8,9] (as *H. pluvialis* dry biomass) and in human nutrition [10,11]. The public perception that "natural" is better than "artificial" or "synthetic" stimulates the adoption of natural pigments [12,13] and has been an essential driver of the interest in microbial astaxanthin production [5,14].

1.1. Current Production

Natural astaxanthin is mainly produced using *Haematococcus pluvialis*, a flagellated Chlorophyte alga usually cultivated in closed photobioreactors to avoid contamination. Cultures reach modest cell concentrations ranging from 0.4 to 3 g L^{-1} [7,12], with the higher titers obtained with enriched media, small-scale culture, or mixotrophic settings [7,12]. The cultivation is done in two phases: first, a nutrient-rich, vegetative growth phase ("green phase") and then a carotenogenic ("reddening phase") that occurs after limiting nutrients such as nitrogen or phosphorus are depleted. Stress can also be established using high light and salinity (Figure 1). The stressed cells transition into a *palmella* form and, ultimately, a red cyst, with astaxanthin contents of up to 4.5% of the dry biomass [12,15]. *Haematococcus* sp. cysts are dense and easily separated but have a thick cell wall that must be disrupted to increase the astaxanthin availability. In order to obtain astaxanthin nutraceutical concentrates, the pigment can be extracted with traditional solvents or supercritical CO_2 [16].

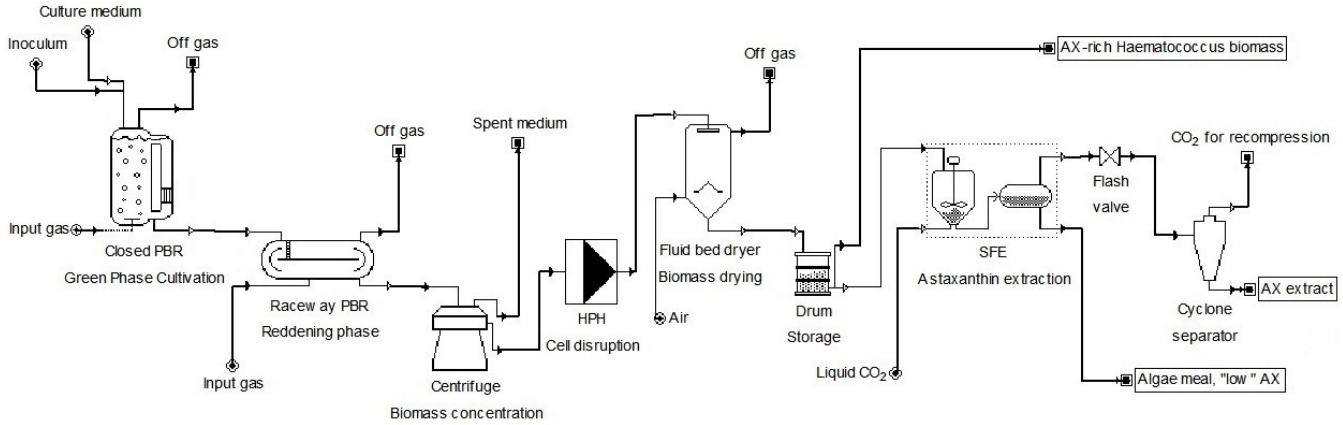

Figure 1. Overview of the astaxanthin production from *H. pluvialis*. The initial steps are large-scale bioreactors (about 25,000 L of culture medium for each kilogram of AX). The equipment following centrifugation is much more compact.

1.2. The Importance of Intracellular Concentration

Although many microorganisms can produce astaxanthin, processing aspects make just a handful of these really competitive for astaxanthin production. Historically, *Phaffia rhodozyma* (now *Xanthophyllomyces dendrorhous*) was investigated for production because traditional fermentation processes can be easily adapted to produce this yeast (In this text, *Xanthophyllomyces dendrorhous* is used *in lieu* of *Phaffia rhodozyma*, even when the original report used the name *Phaffia*. To avoid exhaustive repetition, sometimes only the genus of the most common organisms, *Xanthophyllomyces* and *Haematococcus*, is used). Although its concentration of astaxanthin (around 1 to 10 mg/g) is lower than that of *Haematococcus* (around 40 mg/g), *Xanthophyllomyces* has a higher growth rate [12] and, thus, good productivity. However, *Haematococcus* has competitive productivity because of the high titers, dominating the natural astaxanthin market nutraceuticals [17]. Several other microorganisms are investigated, but none has become important yet, except for *Paracoccus* sp., a bacterium producing a mix of carotenoids with 66% astaxanthin and granted FDA-GRAS status in 2017. A recently developed mutant of the yeast *R. toruloides* produced 0.6 mg astaxanthin/g biomass, a modest concentration. Still, the optimized culture medium required high concentrations of organic carbon sources (53 g/L of a mix of peptone, malt extract, and glucose), leading to only 3 mg/L of astaxanthin [18]. This is an improved but still relatively low-producing strain compared to the average titers in the best-producing species yeast *Xanthophyllomyces*.

The economic production of industrial biomolecules usually requires fast microorganism growth and high titers. Low product concentration in the culture medium is one of

the drawbacks of microbial astaxanthin production; another is the culture media cost. Low concentrations of AX in the biomass and, consequently, in the culture medium also mean that large culture volumes are required, and downstream processing must deal with large volumes of biomass in drying and extraction, increasing costs.

High titers impact the required production of biomass profoundly. As an example, an industry that should produce 1 kg/day worth of astaxanthin (oily extract, powdered concentrate, or raw biomass) would have to produce:

(a) 25 kg of *Haematococcus* with 40 mg/g of astaxanthin, which would translate into 25 m^3 of culture harvested each day, with a concentration of 1 g/L biomass.
(b) 1 ton of *Xanthophyllomyces* sp. with 1 mg/g of astaxanthin, which requires a volume of fermentation of 50 m^3 for a culture reaching 20 g/L of yeast biomass.

These large volumes can be easily reduced by filtration or centrifugation but at the expense of energy, impacting costs. Volume and power are inversely proportional to the intracellular concentration of astaxanthin and biomass concentration in the culture.

1.3. Culture Media Costs: Nutrients and Water

Even with the best astaxanthin-producing microalgae, large water volumes are required for culture media. This makes using classical laboratory media such as BBM, ES, or BG11 inadequate, because these media were developed for screening and maintenance, not for biomass production. The impact of the cost of media components in the biomass product can be high, estimated at US$ 6.20–40.36 by [19] using pure salts. The cost drops significantly with commercial, agriculture-grade fertilizers and can be estimated at US$ 540 per ton of *Haematococcus pluvialis* biomass, using a BBM-like medium to produce 0.64 g/L of biomass (Table 1). Although this seems a reasonably low price, one ton of this biomass will give about 25 kg worth of astaxanthin at a price that cannot be below US$ 13.55 per kilogram in order to cover media components cost, and water and processing prices must be added to that figure.

Table 1. Biomass cost using agricultural-grade components to mimic Bold's basal medium. Only the cost of the main ingredients is factored.

Component	Concentration, g/L or kg/m^3 of Medium	Component Price, US$/ton FOB, China *
$NaNO_3$	0.250	350
$CaCl_2 \bullet 2H_2O$	0.025	120
$MgSO_4 \bullet 7H_2O$	0.074	100
K_2HPO_4	0.075	1100
KH_2PO_4	0.175	950
NaCl	0.025	65

Medium cost per cubic meter	US$ 0.35
Biomass production **, kg biomass per cubic meter of medium	0.643 kg
Contribution of media components to biomass cost, US$/kg biomass	US$ 0.54
Astaxanthin production, kg per cubic meter of medium	0.0257 kg
Contribution of media components to astaxanthin cost, US$/kg astaxanthin	US$ 13.55

* Average prices from Alibaba.com (acessed on 30 March 2020); ** biomass with 40% protein and 4% astaxanthin.

The cost of water worldwide varies significantly but was estimated at an average of US$ 1.31/m^3 in 2013 [20]. With the yearly increase of 4.13% observed in the prior decade, an average cost of US$ 1.96/m^3 can be projected for the year 2024 for treated water. For microalgae culturing, even if only operating expenses, about 53% of the water cost, are

factored in culture media costs, that still represents US$ 1.04/m^3, higher even than the impact of salts. However, the water treatment for microalgal production does not have to comply with all urban water quality requirements. Still, the inbound water must be clear, devoid of xenobiotics, and adequately treated for microbiological contaminants, and that requires a minimal treatment. With the high costs involved in culture media, and the fact that microalgae *do* grow naturally in wastewaters, especially in stabilization lagoons, it is natural to think of wastewaters as a source of water for microalgae cultures, as will be discussed in the next section.

For heterotrophic microorganisms such as *Xanthophyllomyces* sp., culture media is even more expensive because of the need to include the carbon source—usually, a carbohydrate, whose price is about US$ 200–500/ton, depending on the source [21]. Additionally, although the biomass concentration is higher, the astaxanthin content is lower; therefore, the culture media costs end up being yet higher than that for microalgae. However, as shown in the next section, some agro-industry residues can be especially suitable for *Xanthophyllomyces* production.

2. Microbial AX Production from Agro-Industry Wastes

The use of residues in bioprocesses has two essential advantages: it cuts the culture media costs [19,22] and opens up the possibility of increasing the circularity and sustainability of processes [23,24], it introduces pretreatment steps and the potential variability of the raw materials and may require amendments with specific nutrients [25,26].

2.1. Production and Productivity in Residues

Residues used for astaxanthin-rich biomass production form a spectrum between two extremes: from highly concentrated, solid-derived hydrolysates to highly diluted, low-carbon, nutrient-rich liquid residues (the "low-carbon" concept here really depends on whether heterotrophic, mixotrophic, or autotrophic growth is expected. For autotrophs such as microalgae, the source of carbon is inorganic, derived from dissolved CO_2 species. Further organic carbon present in the residue may be used for mixotrophic growth, but that usually requires concentrations lower than 10 g/L to guarantee light penetration. Conversely, heterotrophs such as yeasts will thrive with carbohydrate concentrations above 50 g/L.)

Concentrated residues are suitable for heterotrophic growth, while the low-carbon residues are adequate for auto- or mixotrophic growth. After the consolidation of the first (*Xanthophyllomyces*) and second (*Haematococcus*) generations of microbial astaxanthin production, it was only natural that *residues*—often evaluated for application in several other bioprocesses—were investigated for astaxanthin production. Figure 2 shows the final biomass concentrations, astaxanthin titers, and productivity of astaxanthin production in residues, in proof-of-concept processes. Yet, to date, there have been no reports of the commercial production of AX-rich biomass from residues.

Similar to the production with synthetic media, *Haematococcus* cultures in residues reach low final biomass concentrations around 1 g/L but a high astaxanthin content up to 4–6%. The reverse is true for *Xanthophyllomyces dendrorhous,* with the biomass exceeding 30 g/L but typical astaxanthin concentrations of 0.03–0.1%. Other organisms, such as *Chlorella* sp., were also tested in residue-based media and have low astaxanthin contents but high biomass concentrations, possibly translating into a future alternative for astaxanthin production.

The key to comparing such different trends is to evaluate the overall astaxanthin content and productivity. The overall content is the astaxanthin "concentration" in the culture volume, calculated multiplying the biomass concentration in the medium by the astaxanthin content in the biomass. *Haematococcus* has an intracellular AX content far superior than other microorganisms; therefore, it is the winner in the overall concentration, even with low biomass production. However, factoring in how long it takes to make that product—the productivity—is crucial in the economic evaluation of the process. When the astaxanthin

volumetric content is divided by the time it takes to be produced in liquid residues, *Xanthophyllomyces* (6.4–6.7 mgAX L^{-1} d^{-1}) and *Chlorella* (2–5.3 mgAX L^{-1} d^{-1}) species can be considered fair producers, maybe superior to *Haematococcus* (4.7–6.5 mgAX L^{-1} d^{-1}) (Table 2).

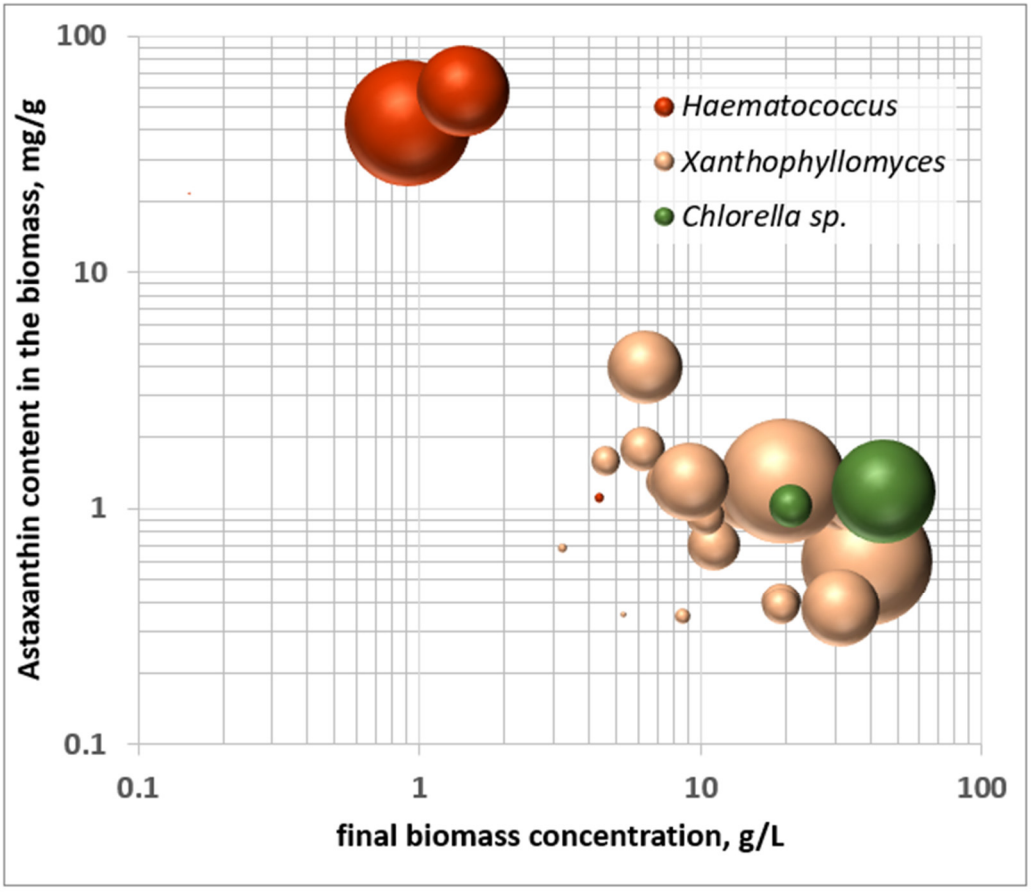

Figure 2. Astaxanthin content in the biomass, biomass concentration in the culture medium, and productivity (bubble sizes, from 0.02 to above 12 mg astaxanthin·L^{-1}·day^{-1}), for microorganisms produced in culture media prepared from agro-industry residues. Data extracted from Table 1.

The decision on which microorganism to use depends on the agro-industry residue available, as well as the final usage of the biomass. Residues can have high or low carbon contents. Primary residues such as whey or solid residues have high organic carbon, favoring the cultivation of obligate heterotrophs; the fast-growing *Xanthophyllomyces dendrorhous* is an adequate organism in this case. Secondary or diluted residues such as biodigester effluents have lower carbon concentration, but frequently high N and P contents and may therefore be adequate for microalgal production. Table 2 illustrates that trend: the production of *Haematococcus* is done in mineral-rich, carbon-depleted media such as sugarcane vinasse [27,28] or secondary waste from piggery wastes [29] that already passed by a preliminary fermentation or biodigestion step, where easily digestible carbon is converted into methane and CO_2, leaving much of the mineral nutrients and also reducing the turbidity of the medium. As for *Xanthophyllomyces dendrorhous*, the production is done in carbohydrate rich-residues from molasses or sugarcane juice [30–32] to biomass hydrolysates [33,34].

Table 2. Microorganisms cultivated in agro-industry residues for astaxanthin (AX) production.

Ref.	Microorganism	AX Content, $mg \cdot g^{-1}$	Time, Days	Biomass, $g \cdot L^{-1}$	Volumetric AX Productivity, $mg \cdot L^{-1} \cdot d^{-1}$	Waste/Residue
[35]	*Chlorella protothecoides*	0.096	7	3.6	0.049	Ricotta production waste, *scotta*
[36]	*Chlorella zofingiensis*	1.03	10	21	2.163	Pretreated cane molasses
		1.19	10	44.6	5.307	
[37]	*Haematococcus pluvialis*	43	6	0.906	6.493	Minkery wastewater
[28]		1.11	11	4.37	0.441	Bioethanol plant wastewater
[27]		0.4	9	0.7	0.031	
[38]		0.011	13	0.97	0.001	Swine slurry
[39]		21.59	35	0.151	0.093	Domestic wastewater
[29]		58.7	18	1.43	4.663	Treated piggery wastewater
[40]		98	8			Synthetic medium (for reference)
[41]	*Paracoccus* NBRC 101723	0.963	5			Synthetic medium (for reference)
[42]	*Thraustochytrium striatum*	0.6	8	4.2	0.315	Corn stover hydrolysate
[43]	*Xanthophyllomyces dendrorhous*	1.448	7	9.21	1.905	Low-cost agro products
[44]		0.35	4	8.6	0.753	Cassava residues substrate
[45]		0.355	7	5.3	0.269	Fruit and vegetable waste
[46]		3.962	2	6.35	12.579	Mussel processing wastewater
[30]		1.6	5	4.6	1.472	Peat hydrolysate
		0.4	4	19.3	1.930	Sugar cane juice and urea
		1.3	5	7.5	1.950	Grape juice
		1.8	5	6.2	2.232	Coconut milk
		1.1	5	14.1	3.102	Molasses
		0.6	3.5	39	6.686	Date juice
		1.3	4	19.6	6.370	Mustard waste isolates
		1	14	30.6	2.186	*Eucalyptus* hydrolysate
		1.1	12	36	3.300	Sugar beet molasses
[47]		1.141	6	18.43	3.505	Citrus waste isolates
[48]		1.31	4	19.6	6.419	Mustard waste isolates
[34]		0.03	7	7.5	0.032	Enzymatic hydrolysates of pre-hydrolyzed wood
[49]		0.68	5	3.23	0.439	Thai traditional rice vermicelli plant
		0.93	5	10.3	1.916	
[33]			4		5.750	Barley straw
[50]		0.018	5	5.5	0.020	Residual-brewery yeast extract
[31]		0.383	4	19.35	1.853	Sugarcane juice
[32]		0.38	3	31.4	3.977	
[51]		1.3	3	9.2	3.987	Vinasse supplementation of sugarcane juice
[52]		0.702	3	11.16	2.611	Synthetic medium (for reference)

Hybrid production, i.e., using autotrophs such as microalgae to grow in mixotrophic conditions, is less common but possible, especially when the microorganism has a high growth rate and can be later stimulated to induce carotenogenesis, as seems to be the case with some species of *Chlorella* sp. [35,36].

Culture adaptation is also paramount. It is necessary to work with adapted cultures cultivated in growing concentrations of the production media; the inoculation of an agro-industry residue with a culture adapted for growth in synthetic culture media can lead to a culture crash. With a slow adaptation, a culture will have time to express enzymes and contamination antagonists and adapt intracellular concentrations of osmoprotective compounds. It is even possible that adaptation selects for subpopulations of adapted microorganisms, although this is best done by the isolation and selection of suitable strains through classical microbiological methods.

However, high titers and productivity are not the only thing to look at in producing biomass for food and feed. Considering that the residues are obtained at a low cost or for free, the next thing to worry about is the process efficiency: the biomass recovery, which is not different from traditional bioprocesses and will not be discussed in this paper, and the particularities of handling and pretreatment of the residues, discussed in the next section.

2.2. Transport, Handling, and Pretreatment of Agro-Industry Wastes

It is common to evaluate processes in a small scale looking at medium composition and the ability of a microorganism to grow in a residue. However, residue availability, transportation, and handling are essential when it comes to industrial production.

The actual availability of a residue depends on how it is produced, where, and in which condition. Liquid residues such as secondary wastewaters from the preliminary digestion of piggery wastes can be collected at the output of a biodigester and pumped into tanks. Solid residues such as lignocellulosic biomass from sugarcane are produced in concentrated units and can be collected, balled, or transported in bulk for further processing. Residues such as domestic food waste are produced in substantial amounts. They can be used for bioprocessing [53,54], but they are not commonly available or reliable due to their scattered production, heterogeneous quality, and possible contamination.

Residues of low water activity a_w (Figure 3), such as lignocellulosic wastes, are generally resistant to degradation and can be transported in bulk from generation to production without fear of decomposition. However, microorganisms can still grow slowly in such residues and become a nuisance, requiring adequate pretreatment.

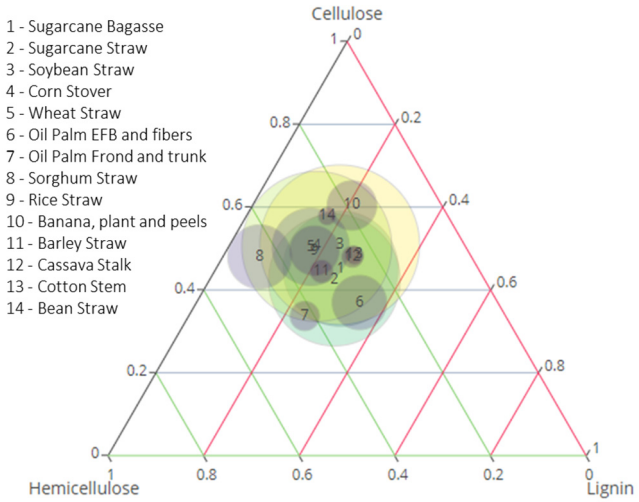

Figure 3. Lignocellulosic residues composition after [24]. Bubble size is proportional to the generation volumes projected for 2025 in South America, amounting to 900 million tons.

Residues with a high water activity (Table 3), such as cassava wastewater, whey, or vinasse, are more susceptible to the growth of bacteria during storage and transportation and, ideally, should be processed in a short time or in situ. Transport adds costs for the process, and the more diluted the residue, the higher is its contribution to the final bioproduct. The cost of transportation involves a fixed price per ton and a variable cost

dependent on the distance; for example in Brazil, for transportation by trucks, the charge is about US$ 7.5 per ton, plus US$ 0.06 per km per ton [55]. Molasses has a very high solids concentration of 50–80% in weight, and although somewhat "liquid", has a low aw of 0.6–0.8 [56], can withstand storage and transportation, and requires dilution to be used.

Table 3. Average composition of the selected liquid agro-industrial wastes.

Components	Cassava Wastewater (Average) [23]	Secondary Effluent (Treated Piggery Wastewater) [29]	Digested Cassava Wastewater [57]	Cane Molasses, Diluted to 10% *w/v* Sugars [36,37]	Anaerobically Digested Vinasse [58]	Mixed *Scotta* (Cheese Whey) [35]
Total carbohydrates (g/L)	32.9			100		40.7
Total lipids (g/L)				0.12		1.8
Protein (g/L)	1.53			9.1		
Total nitrogen (g/L)	0.84		2.38		0.02	1.1
Phosphorus (mg/L)	485.6	98.7	87		14	930
Potassium (mg/L)	1324.9		1680		38	1190
Calcium (mg/L)	162.63		190			480
Magnesium (mg/L)	242.96		420			300
Sulfur (mg/L)	50.33	660	40			
Iron (mg/L)	7.57		0.4			164
Zinc (mg/L)	3.83		9.2			73
Manganese (mg/L)	1.21		0.4			3.13
Copper (mg/L)	0.48		0.8			
Sodium (mg/L)	51.7		9.6			2000
pH	5.46	7.85	8.05		6.7	6.02
COD (g O_2/L)	20.21	800	17.1		0.3	
Total Solids	15.49		44	157.9		77.5
Ammonium, mg/L	41.42	12.9			20	
Nitrate, mg/L	24.27	469			2	
C:N ratio	15.8		24.5			

Adapted from [25] and compiled from six other sources as listed in the columns. Empty cells are values not reported by the authors but not necessarily zero; in particular, molasses was reported to have ~18.7 g/L of mixed metal ions (calcium, potassium, sodium, iron, magnesium, and copper) after dilution.

The production of value-added molecules from agro-industry wastes requires pretreatment of the media for a few reasons: (i) guarantee suitability (digestibility) of the medium for the culture, e.g., by an hydrolytic pretreatment step; (ii) improve transparency and light penetration (for microalgae growth), usually by filtration of dilution; (iii) guarantee biomass production (destroying predator, parasite and competitor species), also through pretreatment; and (iv) guarantee product quality (destroying potential animal and human pathogens and contaminants).

The **pretreatment** methods for substrates depends obviously on their nature. Solid carbon sources must be converted into digestible carbon. As shown in Figure 3, the main components of lignocellulosic residues are: 10–30% lignin that typical carotenogenic microorganisms cannot use; 30–60% cellulose, which can be extracted as valuable fibers or converted into easily assimilable glucose; and 20–40% hemicellulose, which is the easiest to hydrolyze, generating xylose and other sugars. For example, Nghiem et al. [33] processed barley straw by soaking in aqueous ammonia and treating it with Accelerase XY® (a hemicellulase complex from Genencor). After 46–72-h hydrolysis and filtration, the xylose-rich solution was used for astaxanthin production by *X. dendrorhous* UBV-AX2. The harsh conditions in the pretreatment or subsequent hydrolysis are enough to kill contaminant microorganisms. If properly handled, hydrolysates can proceed directly to fermentation: high concentrations of acids [34] or solvents or modifiers [33] in the

pretreatment, plus extended periods of enzymatic hydrolysis at 48–50 °C [33,34]. Although many novel pretreatment options exist for lignocellulosic residues, the most economical processes are still based on acid or thermal pretreatments, followed by mild acid, alkaline, or enzymatic hydrolysis. These steps are thoroughly described in specialized texts such as [24,59]; for the present discussion, it is important to stress that *Xanthophyllomyces* sp. can grow xylose [33], reaching high biomass concentrations, which makes it a candidate for inclusion in lignocellulose-based biorefineries.

Anaerobic digestion (AD) is an adequate pretreatment process for high BOD residues, such as manure slurries and vinasse. Although AD is a valuable process in itself (both for the reduction of polluting potential and for the recovery of energy as biogas), the secondary effluent of AD conserves much of the mineral nutrients of wastewaters while having a lower carbon content. This makes secondary effluents more competitive for microalgal growth, and in fact, these effluents are much more common for microalgal production [23,60–63] than liquified agro-industry wastes.

Residue amendment must be done to produce high titers of astaxanthin and the optimal growth of microorganisms. Residues such as piggery wastewaters have high concentrations of N, P, and other nutrients. Conversely, the lignocellulosic hydrolysates have low concentrations of these essential nutrients. They must be supplemented, as done by Fontana et al. [51] by adding ammonia and phosphate to vinasse-supplemented sugarcane juice or by Wu et al. [47] by adding phosphate, nitrate, magnesium, and yeast extract (both for *Xanthophyllomyces* production). Yeast and malt extract are common additives in laboratory media but can be successfully substituted by industrial, feed-grade biomass, or extracts [64].

Some components in residues may actually *inhibit* the development of a culture. For example, molasses is known to have a mineral concentration that is so high that it can hinder the proper culture development. Molasses can be treated by classical methods such as ferrocyanate precipitation or more sophisticated options such as ion exchange, as suggested by J. Liu et al. [36], which cultivated *C. zofingiensis* with excellent results. While the ammonia present in secondary effluents may be a nuisance for several microalgae, selected or adapted microalgae can resist higher concentrations of the cation [65]. Yeast tends to tolerate better ammonium. The same care must be taken with cassava wastewater, which can contain cyanide anions but sustains the growth of selected isolates [23].

Transparency is necessary for light penetration, which is essential in autotrophic microalgae production and in the induction of carotenogenesis through irradiation cues. Both solid removal and color reduction can aid in increasing light penetration in a culture medium. A simple dilution may suffice if the concentration of nutrients is high enough or even excessive, as in media such as molasses. Haque et al. [27] cultivated *H. pluvialis* using a medium based on corn ethanol-thin stillage and found that a dilution of 60× reduced the high turbidity while maintaining adequate levels of N and P, reaching 0.8 g/L of biomass after 10 days of culture. Supplementing this culture media with 5% CO_2—another coproduct or residue from bioethanol production—led to higher biomass concentrations of 4.37 g/L in 11 days but with a low astaxanthin content [28].

Besides biodigesters, biological filtration units (porous beds with high hydraulic retention times) can be advantageous in reducing the organic load of residues but maintaining an adequate concentration of nutrients. Ledda et al. 2016 [38] used a set of biofilters with a 30-day HRT to treat piggery wastes and filtered the effluent through a 0.45-µm pore membrane before cultivating *H. pluvialis*.

Biological contaminants elimination—Wastes can contain microbiological contaminants: all sorts of bacteria, fungi, and plant and animal pathogens, etc. For heterotrophic production with highly concentrated substrates, that is not an issue, because the substrate pretreatment (e.g., hydrolysis) usually kills contaminant microorganisms. Even if the substrate does not require a harsh pretreatment (e.g., whey for *Xanthophyllomyces* cultivation), a classical thermal sterilization is economical if done with a concentrated residue.

However, a diluted residue such as secondary wastewaters may require sterilization, as with the BG-11+ whey used by Ribeiro et al. [35] for *C. protothecoides* growth; however, the sterilization of whey can actually cause the precipitation of proteins and require either solid separation or cultivation with adequate agitation. Also, the large volumes of liquid residues make sterilization costly. Instead, the direct filtration of pretreated residues with membranes can guarantee sterility, as done by Kang et al. [29] with piggery secondary wastewater, filtered through membranes with 0.2-µm pores. Rodriguez Amado et al. [46], working with mussel processing wastewater, used a 100-kDa filtration step after protein precipitation by acidification to guarantee sterility. In industrial processes, a prefiltration could ensure solid removal, while a second filtration step could guarantee sterility.

The main problem with using nutrient-rich municipal wastewaters (MWW) and municipal solid wastes (MSW) is precisely the quality control. Theoretically, it is possible to use both residues, with harsh treatment conditions that will destroy or block contaminants such as human pathogens while enhancing the access to substrates by the microorganism. In practice, the use of municipal residues brings the danger of creating a loop for pathogens and xenobiotics recycling into food chains, and that must be avoided. The use of agro-industrial residues, even if animal production ones, is much safer because of inherent orthogonality, i.e., land plant pathogens will hardly propagate in microalgal or yeast cultures. Legislation specific to microalgae is still rare, and therefore, provisions such as those in place for agricultural waters must be used, such as USA CFR 112.44.

2.3. Products and Quality Control

Inoculation and culture as microbiological control tools—Working with axenic cultures of fast-growing eukaryotes is relatively widespread and straightforward in fermentation processes. Since yeast multiplies quickly in rich media and resists several antimicrobial agents, it is easy to establish a pure culture and, from that, a pure inoculum.

Consequently, producing *Xanthophyllomyces* in pure fermentations, even in large volumes, is relatively easy. That is harder with *Haematococcus*, because the stock cultures of microalgae propagate slowly, frequently with the presence of small populations of bacteria that can persist through scale-up steps. Still, closed photobioreactors can have good microbiological control. The same cannot be said about open systems such as raceways, where airborne pathogens can contaminate the culture and become a nuisance. Besides following the culture state carefully throughout the process and using control agents [66], the best strategy for microalgal culture is to use strong inocula, i.e., cultures with high cell concentrations of at least 10% but preferably 20–30% of the target (final) concentration expected in each step. In the typical two-step process used with *Haematococcus*, cultures usually develop for 5–15 days in controlled conditions, with CO_2 and the temperature favoring the microalgal development. In the second phase, cultures are subjected to osmotic and irradiation stress for another 3–5 days in open systems. Similar reasonings can be used for other organisms, such as the fast-growing prokaryote *Paracoccus* sp. Or the slower-growing, photosynthetic eukaryote *C. zofingiensis*.

***Haematococcus* as a human supplement**—*Haematococcus* biomass and astaxanthin are permitted as feed colors for salmonid fish in the USA and Europe [67]. Both can be used as nutrient ingredients in foods. *Haematococcus* biomass and its supercritical CO_2 extracts were recognized as a GRAS ingredient in 2010 after analyzing the data submitted in 2009 by Fuji Chemical Industries, Toyama, Japan (AstaREAL® brand). Additionally, in 2010, Algatechnologies, Kibbutz Ketura, Israel (AstaPure® brand) withdrew their submission from 1998 for similar products. In 2015, Innobio Ltd., Dalian, China (Innobio® brand), requested and received, in the same year, a GRAS notice for *Haematococcus* solvent extracts [68], while the GRAS response letters stated that the *Haematococcus* products could not be considered a food color, they could be used as a nutrient in food ingredients, which opened up the possibility of using them as de facto coloring agents. Legislation for supplements includes herbs and extracts, provided that they do not make claims of intended effects on diseases [69] Therefore, dozens of astaxanthin producers market *Haematococcus* products as biomass,

oleoresin gel caps, or capsule supplements. Other natural sources of astaxanthin are still uncommon for human use.

Product specifications and the challenge for variable residues—*Haematococcus* supplement products usually guarantee heavy metals to be below 10 ppm; bacterial counts below 10,000 CFU/g; yeasts or molds below 100 CFU/g; and the absence of *Salmonella* sp., *S. aureus*, and pesticides [68]. Maintaining the same quality for astaxanthin from agroindustry residues may prove challenging, because biomagnification effects may exist. Depending on the residue, residual antibiotics, pesticides, or transition metals may exist. Processing the algal biomass is expected to destroy most microorganisms during the pretreatment and drying steps. Still, control of the media ingredients and the process must be carefully developed to guarantee the quality. For astaxanthin extracts, the low polarity nature of the solvent and product ensures that it will be devoid of microbial contamination (inactivated through membrane dissolution) and heavy metals (insoluble in nonpolar solvents), but not from xenobiotics (possibly soluble); again, quality control is a bottleneck that must be addressed early in process development.

3. Trends and Perspectives

Astaxanthin production in residues is still in development, and plenty of challenges must be solved before large-scale production can be done. Astaxanthin for human use has a relatively high price, and the impact of the culture media in this process is less critical than in astaxanthin-rich biomass used for feed production. Therefore, astaxanthin for nutraceutical uses will keep being produced by *Haematococcus* in synthetic media. In contrast, in the next years, astaxanthin-rich meals will probably be produced using *Xanthophyllomyces*, *Chlorella*, and *Paracoccus*. Even so, competition (and the need to reduce costs) and sustainability might onde day drive the producers of *Haematococcus* biomass towards using residues.

The need to develop circular, zero-waste processes using residues comes with opportunities and many challenges, from economical pretreatment to improved downstream processes. These challenges are shared with traditional bioprocesses; however, in using residues, three areas are especially important: integrating existing processes, developing novel strains, and integrating downstream processing.

The Integration of production with existing processes is essential to reduce transportation costs, reduce contamination, and facilitate pretreatment. Several processes generate essentially sterile residues (vinasses from distillation and biomass hydrolysates) or have a microbiota that cannot survive aerobic condition growth (digestates from anaerobic reactors with long residence times). Other residues, such as sugarcane bagasse, are generated in huge facilities with surplus waste heat and cheap energy. Whenever possible, processes must be developed to effectively profit from direct integration, ensuring better quality control and feedstock security.

Novel strains—As with other fermentation bioprocesses, the strain is critical in astaxanthin production. Moreover, with the widespread use of molecular biology tools, both the bioprospection of novel microorganisms and transformation of others will keep creating better strains of both classical genera and the expression of carotenogenic pathways in modified organisms, such as the fast-growing *E. coli*. However, while mutants, constructs, and new strains such as *R. toruloides* or *C. zofingiensis* seem good producers [18,36], the astaxanthin content as a fraction of the total carotenoids is still much higher in *H. pluvialis*.

Several carotenoids (lutein, zeaxanthin, etc.) can also be used to color animal products such as eggs and fish. Therefore, it is likely that the market keeps divided into two classes of products: astaxanthin produced using any suitable microorganism for production application of the biomass for feeds and *Haematococcus* still dominating the products directed toward human use.

Downstream processing in astaxanthin production using classical microorganisms is somewhat mature: enhancement of bioavailability by cell disruption, followed by drying for integral biomass; and extraction with solvents or CO_2 supercritical fluid extraction,

followed by standardization in edible oil for astaxanthin concentrates, or encapsulation with a dispersing agent for color additives. There is space for improvement in the downstream process, especially in cell disruption and the use of green solvents. However, an exciting possibility in microbial carotenoid production is merging it with second-generation processes that use xylose to produce other biomolecules—bioethanol by *Xanthophyllomyces* sp., proteins by *Chlorella* sp., and lipids by *Yarrowia* sp. With this approach, the carbon sources in the residues are better used, generating a bulk, low cost product such as biofuels, and the low-concentration, high-value mixed carotenoids from microbial sources.

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