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Evaluation of the Cultivation of *Aspergillus oryzae* on Organic Waste-Derived VFA Effluents and Its Potential Application as Alternative Sustainable Nutrient Source for Animal Feed

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Abstract: Considering the projected demand for protein supplementation in animal feed, as well as prioritizing plant-based protein provision for the growing human population, great stress is imposed on conventional protein sources, calling for new sustainable alternatives. In this regard, the production and application of single-cell proteins (SCPs) has proven to be a promising alternative. Therefore, in this study, volatile fatty acids (VFAs) effluents recovered from anaerobically digested FW, CKM, CM, and their combinations were applied for the cultivation of edible filamentous fungi *Aspergillus oryzae*. The biomass was further evaluated considering its protein, fat and alkali insoluble material contents. The maximum fungal biomass yielded of 0.47 ± 0.00 and 0.37 ± 0.00 g dry biomass/g $tVFAs_{CODeq.consumed}$, with up to 47% protein and 5% fat content successfully cultivated in shake flasks and bench scale reactors, respectively. In addition to the production of protein-rich biomass, significant reductions in medium COD (25–58%) and ammonium (33–48%) were achieved. The results presented in this research work imply that using waste-derived VFAs for the production of animal feed grade SCP is an innovative approach that can contribute to the economy and sustainability of animal feed production process.

Keywords: animal feed; organic-rich residues; volatile fatty acids; *Aspergillus oryzae*; single-cell protein

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1. Introduction

As a consequence of world population growth, food demand is outpacing sustainable supply. The ever-increasing human population is predicted to reach more than 9 billion people by 2050 and is to bring an over 70% increase in food demand [1]. This will impose great stress on the animal production sector as the animal-derived protein global demand is estimated to nearly double in this period [2]. In addition, about 40–70% of animal diets' protein fraction comes from food-grade protein sources such as soybean and corn, which has created human food versus animal feed competition [3]. The scarcity of protein sources primarily arouses competition for arable land, water and fertilizers and subsequently rises prices. This has motivated the introduction of new protein sources such as single-cell proteins (SCPs) to cover for animal and human food protein deficiency as alternative supplements.

Considering their production independence from climate conditions and the availability of arable land, SCPs have gained significant attention in the past several decades by addressing protein scarcity concerns through the potential replacement of conventional protein sources such as soybean [4]. SCPs or microbial protein source refers to protein production from the biomass of edible microorganisms such as microalgae, yeast, bacteria, and filamentous fungi. Among all, edible filamentous fungi have received significant industrial and research attention in the production of organic acids, enzymes and other valuable metabolites, along with SCPs. In addition, the amino acids, vitamins and fatty acids profiles of fungal biomass makes fungal SCPs a promising candidate for food and

feed applications [5]. Some species of filamentous fungi such as *Fusarium venenatum*, *Aspergillus oryzae*, *Neurospora intermedia*, *Rhizopus oligosporus*, *Rhizopus oryzae* and *Monascus purpureus* are generally regarded as safe microorganisms as they have been applied in food and beverage fermentation (such as sake, shoyu (soy sauce) and miso (soybean paste)) for centuries [6–8]. Edible filamentous fungus, *F. venenatum*, is a well-known mycoprotein source for human food [9]. The *Aspergillus oryzae (A.oryzae)* strain has been reported as the source of mycoprotein [5,10].

The biggest advantage of the edible filamentous fungi is their versatility and the ability of growing on a wide range of substrates, including industrial by-products and residues such vinasse, pea-processing industrial byproduct, thin stillage, fat-rich dairy by-product, olive oil mill wastewater, brewer's spent grain, etc. [11]. However, clean synthetic media containing sugar-based substrates (glucose, maltose, starch, etc.) supplemented with nutrients such as ammonium, urea, etc., are generally used for the cultivation of fungal biomass. The use of pure nutrient streams to provide results in typically high-quality products; however, the high cost of biomass production hinders the economic feasibility of SCP provision when it comes to large-scale animal feed production [4]. This issue can be alleviated by the application of low-value organic-rich waste (underutilized or discarded by-products or residues) streams. If such an approach is realized, not only are the issues associated feed provision remediated but also a solution for the negative environmental impacts of waste generation is proposed.

The main issue with the application of organic wastes as substrates for fungal cultivation is their heterogeneity and complexity. The main fraction of nutrients in many organic waste streams such as food waste and animal manure may not be directly available for fungal utilization as they are either a part of a recalcitrant structure resistant to fungal degradation (e.g., lignocellulosic materials) or are in form of complex macromolecules hard to degrade using fungal natural enzyme machinery (e.g., proteins and lipids) [12]. In addition, the waste stream may also contain different level of inhibitory compounds (acids, furans, phenols, etc.) that hinder or prevent fungal growth [13,14]. A potential indirect path to homogenize the mixed organic waste into value added compounds such as volatile fatty acids (VFAs) that can be consumable by filamentous fungi is anaerobic digestion (AD). VFAs such as acetic, propionic, and butyric acids are intermediate metabolites produced in the acidogenesis and acetogenesis stages of AD as a result of microbial degradation and conversion of the complex organics. According to our previous findings, VFAs [10,15] have the potential to be used as the carbon source for fungal cultivation. In addition to VFAs, the AD effluent contains different levels of essential nutrients such as nitrogen in the form of ammonium resulting from protein degradation, solubilized saccharides and minerals that support fungal growth and reduce the demand on the ex-situ supplementation of costly chemicals.

Among the filamentous fungi strains studied, *A. oryzae* has proven to be able to grow on different waste-derived effluents yielding a fungal biomass with 20% to 62% protein content [11,16,17]. In addition to the high protein content, *A. oryzae* has potential application in animal feed supplementation, especially in ruminant feeding [18,19]. Furthermore, the fungal biomass fat and cell wall content are reported to affect immunostimulant activities, potentially boosting animal health (antioxidants level, anti-inflammatory, anti-obesity, etc.) [20]. Therefore, the production of protein-rich fungal biomass on waste-derived VFAs post AD presents an attractive strategy within the circular bioeconomy concept, reducing the competition over the animal feed protein source while playing a significant role in waste reduction, nutrient recovery, waste valorization, and resource efficiency.

This study aims to bring a novel perspective in animal feed sustainability by evaluating the potential of *A. oryzae* fungal biomass produced from waste-derived VFAs-rich effluent. In this regard, in order to prove the flexibility organic waste sources that comply with the proposed concept, effluents from the AD of cow manure (CM), chicken manure (CKM) and food waste (FW) were taken into consideration. Moreover, a particular focus was given to applying VFAs post-AD effluents to investigate the sole potential of the nutrient source

applied for *A. oryzae* growth. The nutritional value of the harvested biomass including protein, alkali insoluble materials (AIM), minerals and fat contents, as well as changes in the ammonium and chemical oxygen demand (COD), were analyzed.

2. Materials and Methods

2.1. Filamentous Fungal Strains

In this study, *Aspergillus oryzae* var. *oryzae* CBS 819.72 (Centraalbureau Voor Schimmel cultures, Utrecht, The Netherlands) was used as an edible fungus and grown on Potato Dextrose Agar (PDA) plates containing 15 g/L agar, 4 g/L potato infusion and 20 g/L glucose. Then, the prepared agar plates were incubated at 30 °C for three days. After the incubation, the plates were stored at 4 °C until use.

2.2. Substrates

In this study, VFAs produced from acidogenic anaerobic fermentation of various wastes (CM, CKM and FW) conducted in previous studies [15,21] were used as fungal cultivation substrates. In brief, CM, CKM and FW were collected from Hushållningssällskapet Sjuhäras (Länhem, Sweden), Renova AB (Gothenburg, Sweden) and an egg-laying farm Sjömarkens Hönsgård AB (Borås, Sweden), respectively, and were anaerobically digested in a continuous stirred tank reactor (CSTR) equipped with a back-washable, flat sheet immersed membrane panel. The particle and microorganism-free VFAs-rich permeate was recovered from the AD process running in a semi-continuous mode. The recovered VFA effluents were kept in the freezer at −20 °C until use. The characteristics of the VFA effluents derived from anaerobic digestion of cow manure, food waste and chicken manure are presented in Table 1. The concentrations of the total VFAs converted based on the chemical oxygen demand (COD) equivalent ranged as follows: 21.04–57.02% acetic acid, 6.49–16.14% propionic acid, 5.57–27.01% butyric acid and 0–28% caproic acid, plus traces amount of valeric, isovaleric and isobutyric acids. The VFA effluents were autoclaved at 121 °C for 20 min for the sterilization, then used for fungal cultivations.

Table 1. General composition of VFAs solution derived from anaerobic digestion of chicken manure (CKM), cow manure (CM), food waste (FW) and their combinations.

Parameters	CKM	CM	FW	FW + CKM	FW + CM	CM + CKM	FW + CM + CKM
tCOD (g/L)	13.8 ± 0.57	13.40 ± 0.57	11.60 ± 1.70	12.8 ± 1.13	12 ± 0.00	13.6 ± 0.00	12.8 ± 0.00
NH_4^+-N (mg/L)	1820 ± 28.28	420 ± 0.00	160 ± 0.00	1320 ± 56.57	300 ± 28.28	1220 ± 28.28	740 ± 28.28
C/N ratio	3.92 ± 0.03	12.03 ± 0.74	30.35 ± 1.12	5.36 ± 0.36	14.55 ± 1.43	4.72 ± 0.03	7.49 ± 0.18
tVFAs (g/L)	9.09 ± 0.05	6.16 ± 0.09	7.90 ± 0.37	9.20 ± 0.09	6.06 ± 0.01	7.23 ± 0.08	7.48 ± 0.05
Ac (%) [1]	57.02	45.12	21.04	54.38	30.10	48.30	40.37
Pr (%) [1]	16.14	9.61	4.78	15.28	6.49	12.20	10.15
Bu (%) [1]	16.71	5.75	27.01	19.39	14.20	10.81	16.75
Ca* (%) [1]	0.00	0.00	28.27	2.13	10.00	0.00	7.30
pH	6.63	8.07	5.62	6.26	6.14	6.78	6.20
Ca (ppm) [1]	33.00 ± 0.00	119.00 ± 2.05	66.45 ± 2.05	49.72 ± 0.07	37.00 ± 0.00	29.5 ± 0.71	43.5 ± 3.54
Mg (ppm) [1]	61.5 ± 2.12	112.5 ± 0.07	13.40 ± 0.14	34.00 ± 1.41	340.00 ± 2.83	44.00. ± 0.00	47.5 ± 2.12
Fe (ppm) [1]	2.5 ± 0.71	1.50 ± 0.71	0.20 ± 0.00	1.00 ± 0.00	3.00 ± 0.41	2.00 ± 0.00	1.5 ± 071
K (ppm) [1]	1554 ± 18.4	2691.19 ± 19.8	173.75 ± 5.57	1019.5 ± 13.44	852.00 ± 9.90	1703.00 ± 63.64	1204.00 ± 15.56
Na (ppm) [1]	1016.00 ± 29.7	224.20 ± 0.04	702.00 ± 0.07	829.5 ± 16.26	774.5 ± 2.12	568.5 ± 16.26	578.5 ± 21.92

[1] Ac: Acetic acid, Pr: Propionic acid, Bu: Butyric acid, Ca*: Caproic acid, Ca: Calcium, Mg: Magnesium, Fe: Iron, K: Potassium, Na: Sodium.

2.3. The Cultivation of A. oryzae in Shake Flasks

The *A. oryzae* cultivations were performed in 250-mL Erlenmeyer flasks filled with 50 mL of sterile VFA effluents (CKM, FW, CM and their combinations as CKM + FW (1:1), CM + FW (1:1), CKM + CM (1:1) and CKM + CM + FW (1:1:1)). In each flask, 1 mL of spore suspension was inoculated. The spore suspension of 1.86×10^7 spores/mL was measured with a Neubauer hemocytometer followed by several dilutions. After the inoculation, all flasks were incubated in a water bath (Grant OLS-Aqua pro, Cambridge,

UK) operated at 35 °C and 125 rpm for 72 h. After the cultivation, the grown biomass in medium was harvested, sieved (1 mm^2 pore size) and washed with distilled water to remove medium components. The collected fungal biomass was then oven dried overnight at 70 °C. The biomass yield was reported as grams of dry biomass/g $VFAs_{CODeq\ consumed}$. All cultivations were performed in triplicates.

2.4. The Cultivation of A. oryzae in Bubble Column Bioreactors

A. oryzae fungal cultivations were scaled up to 4.5-L capacity glass bubble column bioreactors (56 cm height and 11 cm width, Belach Bioteknik AB, Skogås, Sweden). Initially, the empty bioreactors and VFA effluents were autoclaved at 121 °C for 20 min, separately. After the sterilization, 2.7 L of VFA effluents were added into the bioreactor, and each reactor was inoculated with 20 mL/L of spore suspension (1.86×10^7 spores/mL). During cultivation, the bioreactors were continuously aerated (0.5 vvm) at 35 °C for 54 h without pH adjustment. At the end of the cultivation, the biomass was harvested, sieved, washed and oven dried as mentioned in Section 2.3.

2.5. Analytical Methods

The VFAs profiles were analyzed and quantified according to Uwineza et al. [10]. The gas chromatography (GC) equipped with a capillary column and a flame ionized detector (FID) (GC: Clarus 550; Column: Elite-Wax ETR, 30 m$\times$ 0.32 mm $\times$ 1.00 µm, Perkin-Elmer, Shelton, CT, USA) was used to analyze the VFAs.

The levels of total soluble chemical oxygen demand (sCOD) and ammonium ($NH4^+$-N) of the substrates were analyzed with a COD kit (Nanocolor® COD 15000, Düren, Germany) and ammonium kit (Nanocolor® Ammonium, Düren, Germany), respectively. The Nanocolor 500D photometer (MACHEREY-NAGEL GmbH & Co. KG, Germany) was used to determine the concentration of COD and ammonium. The COD equivalent of the volatile fatty acids were calculated according to Wainaina et al. [15] using the following conversion factors: for acetic acid, 1.07, for propionic acid, 1.51, for butyric acid, 1.82, and for caproic acid, 2.21.

The crude protein content biomass was analyzed and determined according to the Kjeldahl method and by using a factor of 6.25, respectively [10]. The total fat content analysis was based on petroleum ether extraction using an extraction analyzer (ST243 Soxtec™, FOSS Analytical Co., Ltd., Suzhou, China). After the extraction, the amount of fat was determined by weight of the evaporated organic materials. Alkali-insoluble material (AIM) and mineral (ash) contents of fungal biomass were determined according to a previous work [16].

The mineral compositions (calcium (Ca), magnesium (Mg), iron (Fe), potassium (K) and sodium (Na)) of effluents were analyzed using a microwave plasma atomic emission spectroscopy (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA).

The neutral detergent fiber (NDF) of fungal biomass was extracted and determined using $ANKOM^{200}$ Fiber Analyzer. This method was accomplished using a filter bag (F57 and F58, ANKOM Technology) sealed with a heat sealer (HS or His, ANKOM Technology, Macedon, NY, USA) and a digestion instrument for extraction ($ANKOM^{2000}$ with 65 rpm agitation, ANKOM Technology, Macedon, NY, USA).

2.6. Statistical Analysis

All cultivations were performed in triplicates. The data were statically analyzed using MINITAB 17 statistical software (Version 17.1.0, Minitab Inc., State college, PA, USA). The analysis of variance (ANOVA) using general model linear models was performed on results and a confidence interval of 95% plus the pairwise comparisons between the data using Turkey's test were considered. All presented data are the means of the resulted values, and all errors bars represent their standards deviations.

3. Results and Discussion

In this work, a novel bioprocess was used to convert wastes (cow and chicken manure and food wastes) to single-cell protein (SCP) using a two-step process of anaerobic digestion of the wastes to VFAs by a membrane bioreactor followed by the cultivation of the VFAs solution to the fungal biomass of *Aspergillus oryzae*. While the first step of this process was previously published [15,21] with compositions presented in Table 1, this work was focused on step 2 of the fungal biomass production, and the results are summarized in Tables 2 and 3 and Figures 1–4.

Table 2. Summary of biomass yields from *A. oryzae* cultivation in VFAs from shake flasks and bubble column reactors.

	Shake Flask			Bubble Column Reactors		
	g dry biomass/ $tVFA_{COD\ eq.fed}$	g dry biomass/ $tVFAs_{COD\ eq.\ Consumed}$	Consumption Rate (g VFAs/L.h)	g dry biomass/ $tVFA_{COD\ eq.fed}$	g dry biomass/ $tVFAs_{COD\ eq.\ Consumed}$	Consumption Rate (g VFAs/L.h)
CKM	0.24 ± 0.01	0.36 ± 0.02	0.11 ± 0.00	0.24 ± 0.00	0.30 ± 0.00	0.12 ± 0.00
FW + CKM	0.21 ± 0.00	0.44 ± 0.03	0.07 ± 0.00	0.22 ± 0.00	0.26 ± 0.00	0.12 ± 0.00
FW + CM	0.26 ± 0.00	0.47 ± 0.00	0.06 ± 0.00	0.28 ± 0.00	0.37 ± 0.00	0.07 ± 0.00
FW + CM + CKM	0.25 ± 0.01	0.42 ± 0.00	0.07 ± 0.00	0.25 ± 0.01	0.32 ± 0.00	0.09 ± 0.00
CM + CKM	0.27 ± 0.02	0.39 ± 0.00	0.09 ± 0.00	0.25 ± 0.00	0.31 ± 0.00	0.10 ± 0.00
CM	0.13 ± 0.02	0.21 ± 0.00	0.07 ± 0.00	0.10 ± 0.02	0.18 ± 0.01	0.05 ± 0.00
FW	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00

CKM: chicken manure, FW: Food waste, CM: cow manure, COD: chemical oxygen demand.

Table 3. Mineral content of VFAs solution derived from anaerobic digestion of CKM, CM and FW after fungal cultivation in bubble column reactors.

Parameters	CKM	CM	FW + CKM	FW + CM	CM + CKM	FW + CM + CKM
Ca (ppm)	3.00 ± 0.00	86.00 ± 4.24	4.50 ± 0.71	0.00 ± 0.00	2.50 ± 0.71	4.00 ± 0.00
Mg (ppm)	0.26 ± 0.01	83.00 ± 2.83	0.23 ± 0.01	20.50 ± 0.71	0.38 ± 0.01	0.65 ± 0.07
Fe (ppm)	1.52 ± 0.01	0.95 ± 0.21	2.00 ± 0.00	1.71 ± 0.09	1.00 ± 0.00	1.00 ± 0.00
K (ppm)	924.00 ± 43.84	1854.50 ± 101.12	936.50 ± 48.79	765.00 ± 8.49	1682.50 ± 43.13	1120.00 ± 55.15
Na (ppm)	709.50 ± 41.72	147.50 ± 14.85	263.50 ± 0.71	293.50 ± 4.95	378.00 ± 4.24	513.50 ± 20.51

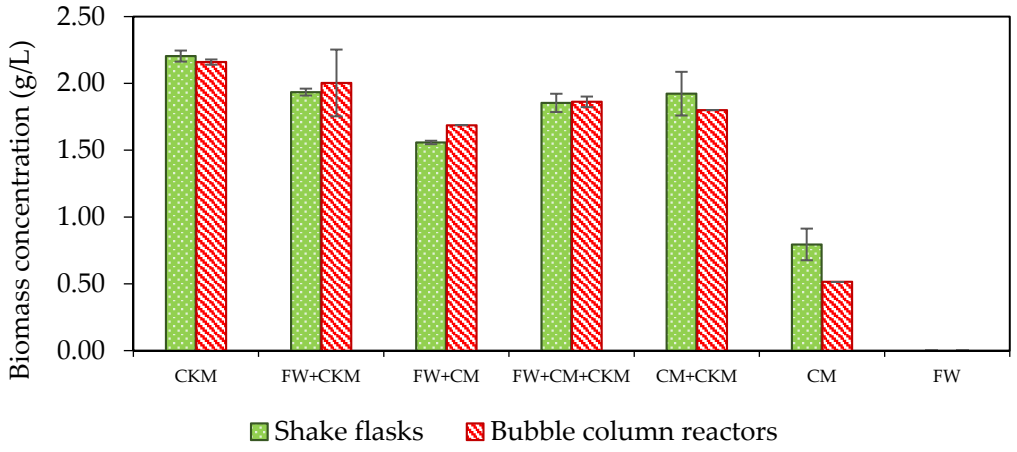

Figure 1. *A. oryzae* fungal biomass produced during cultivation in shake flask and in bubble column reactors.

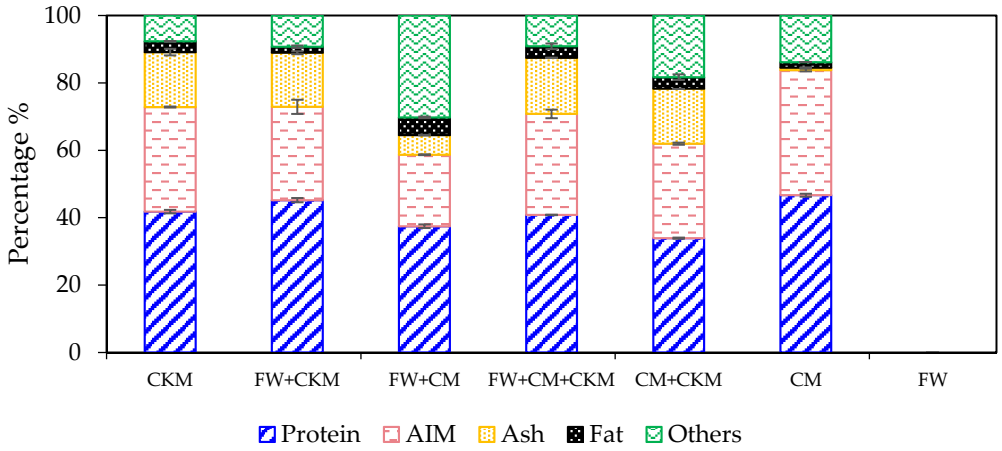

Figure 2. Composition of *A. oryzae* fungal biomass obtained during cultivation in bubble column reactor.

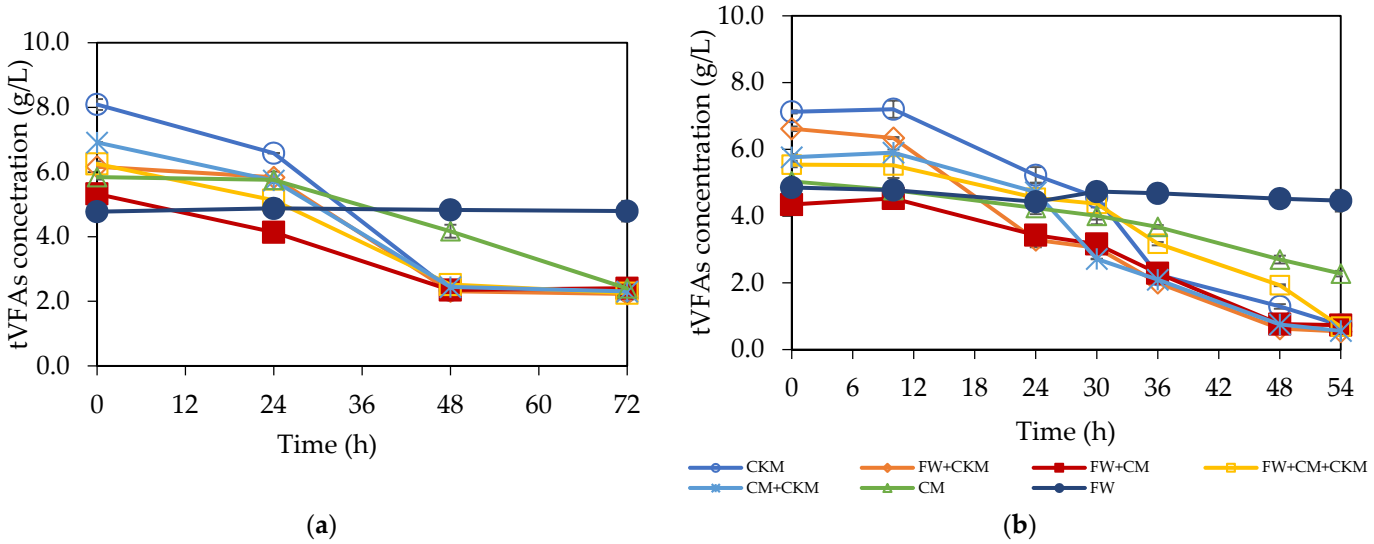

Figure 3. Total VFAs consumption profile by *A. oryzae* in VFAs effluent derived from cow manure, chicken manure and food waste (**a**) in shake flasks and (**b**) in bubble column reactor.

Initially, the production of protein-rich biomass in shake flasks was attempted, and then the cultivation was scaled up to bubble column reactors (4.5-L capacity) to help overcome the challenges of using waste organic materials. Then, the compositional analyses (protein, fat, alkali insoluble material and minerals contents) were evaluated to determine the nutritional values of the fungal biomass. Concomitantly, the contents of the substrates after the cultivation were reanalyzed regarding their ammonium, chemical oxygen demand (COD) and VFAs levels.

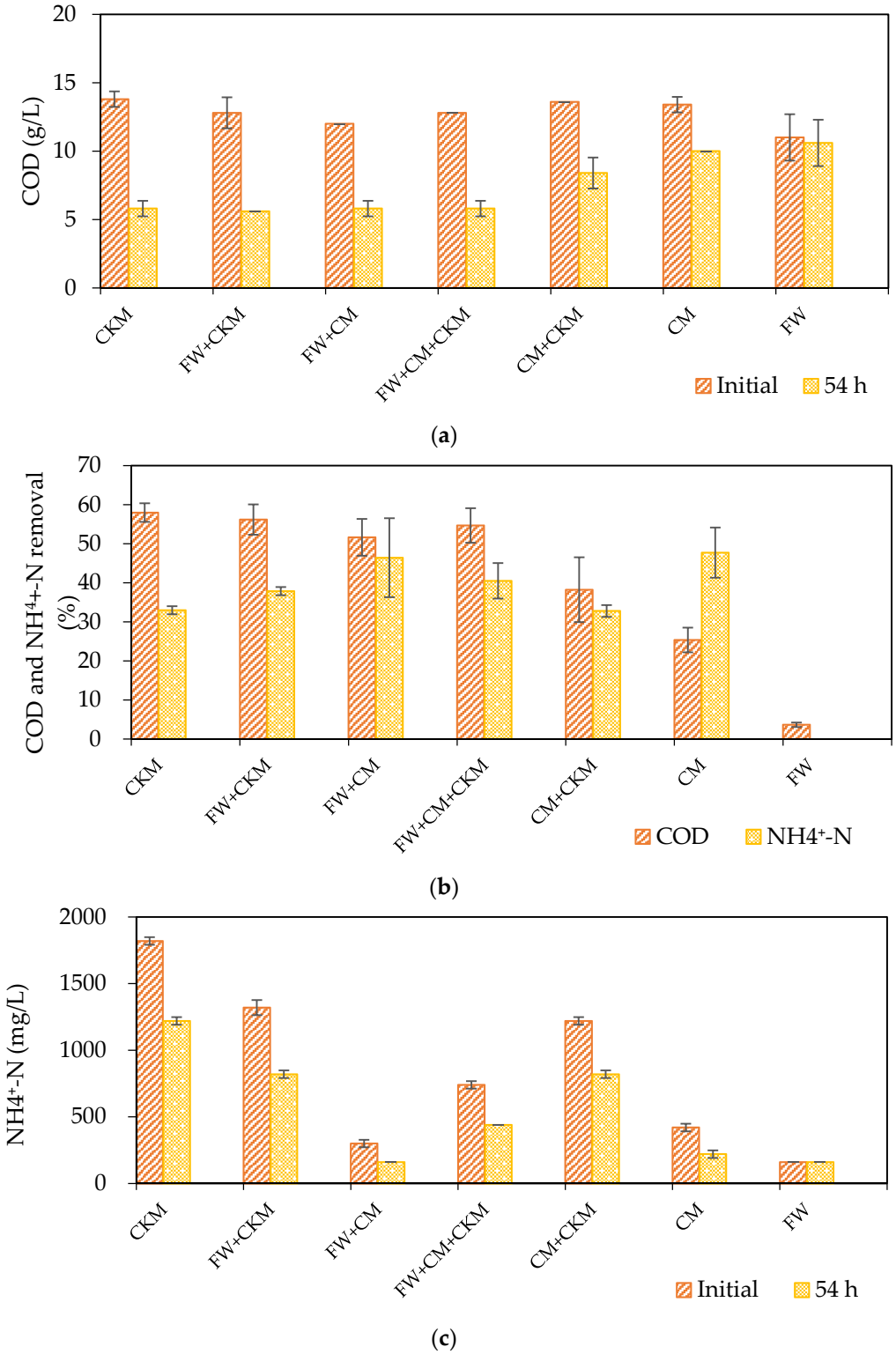

Figure 4. COD and ammonium content after cultivation of *A. oryzae* in VFA effluents derived in bubble column reactors: (**a**) COD content, (**b**) Ammonium content, (**c**) COD and Ammonium removal ratios (%).

3.1. Characterization of VFA Effluents before Fungal Cultivation

The chemical composition of the VFA effluents produced from AD of CKM, CM, FW and their combinations are presented in Table 1. The initial pH of the VFAs ranged from 5.62 to 8.07. All effluents contained similar levels of COD (11.60–13.80 g/L), while their

ammonium concentrations varied between 160 and 1820 mg/L. These differences make it possible to investigate the initial amount of ammonium in fungal cultivation.

The total VFAs of the effluents converted based on the COD equivalent, mainly composed of acetic acid, were also varied (6.06–9.20 g/L; Table 1). Depending on their concentrations, some of the VFAs, such as acetic and propionic acids, can be considered as inhibitors due to their toxic effects on the growth of some microorganisms such as *Saccharomyces cerevisiae, Candida shehatae* and *Listeria monocytogenes* [22,23]. On the other hand, these acids can be evaluated as an alternative carbon source in some microbial fermentations [24]. The important thing for the use of the inhibitory substances in the production of biometabolites is to be determined for their appropriate concentrations. For this, Uwineza et al. [10] reported that the biomass of *A. oryzae* can be successfully obtained in the presence of acetic acid at up to 9 g/L concentrations. Similarly, the VFA effluents containing acetic acid and propionic acid obtained from anaerobic digestion were used for the cultivation of fungal strains (*Rhizopus* and *Aspergillus*) [10,15]. Considering the highest total amount of VFAs used in this study (9.20 g/L; Table 1), it was thought that the VFAs could be used directly without any dilution.

Some minerals, mainly Ca, Mg, K, Na and Fe, are also known as essential nutrients in microbial production, and they are usually supplied in the form of cations of inorganic salts. [25,26]. The elemental analysis of the VFA effluents was carried out and presented in the Table 1. The highest concentrations for K (up to 2691 ppm) and Na (up to 1016 ppm) were found in CM and CKM, respectively. Other metal elements (Ca, Mg and Fe) were found in low concentrations. The fact that the VFA effluents contain the nutrients in terms of metal elements indicates that it can be used without any nutritional supplementation regarding the fungal growth.

3.2. Biomass Cultivation in Shake Flask vs. Bubble Column Reactor

Fungal biomass productions by *A. oryzae* were comparatively evaluated in seven different VFA effluents obtained from the anaerobic digestion of FW, CKM and CM as carbon and nutrient sources in shake flasks and bubble column reactors. Except for the VFA effluents from FW, CM and their mixtures, the fungal biomass of *A. oryzae* in the shake flasks were similar in the VFA effluents obtained from CKM and its varied combinations (1.85–2.20 g/L, *p*-Value = 0.234, Figure 1). Although there was no fungal growth in the FW-derived VFA effluents due to the pH inhibition, the biomass production yields were slightly higher in the mixture of the VFA effluents obtained from CKM and FW (Table 2). Similar to that seen in shake flasks, the fungal biomass levels in the bubble column reactors were high in the CKM and its combinations. The highest amount of biomass (2.16 g/L, Figure 1) was obtained in the medium of VFA effluents from the CKM containing the highest total VFAs (Table 1). High carbon content might have a positive effect on biomass production, and the initial pH and nitrogen content of the medium also have a critical role in fungal growth and sporulation and cultivation yield [17,27–29].

In this study, the initial pH value and nitrogen amounts were varied when the VFA effluents were combined. When VFAs from chicken manure with the highest nitrogen content (NH_4^+ -N; 1820 mg/L) was mixed with other substrates (CKM + FW, CKM + CM, CKM + CM + FW), the final biomass concentration was decreased to 1.80 to 2.00 g/L (Figure 1). Despite these decreases, the biomass production yields increased from 0.26 to 0.44 g biomass/g$VFAs_{CODeq.consumed}$ (*p*-Value = 0.01; Table 2), probably due to the C/N balance ranging from 3.93 to 30.35 with CKM as the lowest and CM the highest (Table 1). An appropriate C/N ratio is required in microbial fermentations to prevent catabolic repression and to obtain an efficient product [30,31]. The fungal biomass was relatively higher in VFAs medium from the mixture of CM with FW, (Figure 1). In addition, it was found that the VFAs obtained from the mixture of CM with FW, whose C/N ratio was much higher than the others (except for FW), was preferable for the growth of *A. oryzae*, achieving a higher biomass production yield of 0.37–0.47 g dry biomass/g$COD_{eq.consumed}$ (Table 2), whereas the amount of fungal biomass produced in this mixture medium was relatively

lower than in the other media. Similarly, the biomass production efficiency was increased by adding nitrogen such as ammonia, ammonium sulfate or sodium nitrate to balance the C/N ratio [17,32,33].

There was no fungal growth in the VFAs from food waste, which had the lowest nitrogen content (160 mg/L) in both growth conditions. Herein, the absence of fungal growth in this substrate may be related to its initial pH level of 5.62. While *A. oryzae* are naturally able to grow in substrates between pH 3 and 8 [34], it was recommended that the initial pH value of the VFAs medium could be between 6 and 7 to reduce the inhibitory effects of organic acids such as acetic acid [10]. Except for the VFAs medium obtained from food waste, the initial pH values of all other media were higher than 6. In particular, the initial pH value of the VFAs medium obtained from cow manure was 8.07, and the fungal biomass production was also low in this medium. The high pH value of the medium may also cause a long lag phase, which may adversely affect the growth of *A. oryzae* [10,35].

3.3. Fungal Biomass Characterization in Bench Scale Bubble Column Reactors

In addition to the fungal biomass production in VFAs-containing media, the characteristics and compositions of the produced fungal biomass were also analyzed. The results of the protein, fat, AIM and minerals (ash) are presented in Figure 2. The crude protein content of the produced dry fungal biomass ranged between 34% and 47%. Those results support those of other studies, for instance, the protein content of fungal biomass from fish processing wastewater streams through *A. oryzae* [16]. Souza Filha et al. [36] reported that the produced *A. oryzae* biomass in 2% pea-processing byproduct yielded between 0.11 and 0.14 (g/g substrate) protein content. In another research study by Sar et al. [17], *A. oryzae* fungal biomass produced in olive oil mill wastewater was generally characterized by varied protein contents (between 15 and 45% of dry weight). Interestingly, *A. oryzae* was found to grow in the mixture of VFA effluents performed in shake flasks and resulted in a fungal biomass with 37–41% protein content in a recent study by Uwineza et al. [10]. In this regard, a 6% increase in the protein content of the fungal biomass was observed in the bubble column reactors in this study compared to the shake flasks. In addition, the protein content of the produced *A. oryzae* fungal biomass was comparatively related to soybean meal (40–50% crude protein) frequently used as the source of protein in animal feed [37–39]. In addition, the obtained protein content of *A. oryzae* biomass was in range compared to other SCPs such as microalgae and yeast (ranging between 40 and 60% and 44 and 55% of dry weight, respectively [40,41]). Added to the high protein content of *A. oryzae* biomass, the supplementation of *A. oryzae* in ruminants has been reported to promote protein degradation, stimulate fiber digestibility, increase the rate of rumen fermentation, increase the number of cellulolytic bacteria, improve feed intake and result in increased in total VFAs and hence increased milk yields in dairy cows [42–44]. Considering the protein content of the biomass produced from VFA-containing substrates, this concludes the use of *A. oryzae* biomass as an alternative protein supplement in animal feed other than soybean meal. Considering the content of animal feed, the protein content and amino acid profile are of great biological and economic aspects [5]. Nevertheless, the amino acid profile of the biomass needs to be determined.

A more comprehensive compositional analysis including fats, AIM, minerals (ash) (Figure 2) and the neutral detergent fiber (NDF) of the fungal biomass were performed. The fats content of the biomass ranged between 2 and 5%. The fats content of the fungal biomass may vary according to the content of the substrate used, and for instance, the fats content of the produced *A. oryzae* biomass from vinasse and olive oil mill water ranged between 3 and 7% [5,17]. In general, the inclusion of fat in animal diets, especially ruminants, has the primary function of increasing the energy density of the animal's diet [39,45]. Usually, the fat requirement in dairy cattle is about 3–5% of the feed [39]. In the last decades, dietary fat inclusion in ruminants comes from cereal grains, vegetable oils, soybean oils, and oilseeds, and usually, when they are mixed with forages, they can provide up to 3% of feed fat. Since the produced fungal biomass meets ruminants' fat and protein requirements, the

A. oryzae fungal biomass can provide protein and fat needs in animal feeding. The fungal biomass contained approximately 21–37% AIM of the dried material of the biomass (Figure 2). The AIM are mainly the constituents of the fungal cell wall, which consists of large amounts (15–30%) of glucosamine derivatives [46], similar to the results determined in this study (21–37%), except the biomass obtained from the VFAs from cow manure. The cell wall is reported for its role as an immunostimulant, antioxidant and antimicrobial that makes it more attractive in various applications, including pharmaceuticals, feed and food and biomedicine industries [47]. Its immune modeling effects in animal feed can result in the reduction of bacterial infection, the activation of innate immune response as well as improving animal productivity [48].

The NDF content in produced fungal biomass ranged between 44 and 50% on a dry biomass basis. NDFs are the fiber residues which are predominantly hemicellulose, cellulose and lignin [39]. Based on the National Research Council's (NRC) recommendation, the amount of NDF in the diet of dairy cattle would be varied from 25–28% concerning the production stage. The NRC suggests 25% NDF in the ratio for cows, which are primary of milk production, where at least 75% of the NDF is mainly supplied by forage [39]. Based on the animal's NDF requirement and the production stage, the produced fungal biomass would provide the percentage of NDF in the animal diet. The ash (mineral) ratio of biomass from the VFAs derived from the chicken manure and its other mixtures was determined as 16 to 17%. Because of its high mineral content, an elemental analysis of biomass, which are important for nutrition, can be determined.

The study investigated the technical possibility to produce *A. oryzae* fungal biomass from different VFA effluents post anaerobic digestion of organic waste. *A.oryzae* is originally edible fungi and is already used for food production such as in miso (a Japanese fermented soya) and shoyu (soy sauce). In addition, *A. oryzae* extract known as Amaferm has been used as feed supplement in animal diet since 1990s [49]. In this regard, the Amaferm has shown the potential to increase the performance of ruminal microorganisms, mainly fibrolytic bacteria population and fiber degradation [50]. It has more probiotic effects on different parts of ruminant digestive tracts [51].

However, the *A.oryzae* fungal biomass produced from different organic waste, still need further investigation for its safety. Different aspect such as ethical approval, safety aspect from the medium used until the produced biomass need to be taken into account to ensure that it meets the set regulations (e.g., the European Commission's Food Safety policy or the Food and Agriculture Organization of the United Nations) for animal feed. It should be tested for the quality and degradability for different type of animals (dairy cattle, sheep, fish, etc.).

3.4. Characterization of VFA Effluents after Fungal Cultivation

The VFA effluents, containing various organic acids, can provide significant reductions in the amount of organic load concomitantly the production of protein-rich biomass by the cultivation of filamentous fungi. Therefore, the consumption levels of COD, ammonium, VFAs and metal elements of these substrates were determined after the cultivation of *A. oryzae*.

The levels of acetic acid and total VFAs were consumed up to 95% and 71% in shake flasks, respectively, in all effluents except FW (Figure 3a). In further experiments carried out in bubble column reactors, acetic acid's consumption rates were slightly increased (about 2–4%),whereas the total VFAs consumptions were increased by about 23 to 28% (Figure 3b). Aeration, agitation and the reactor type are among the cultivation factors that stimulate the growth of filamentous fungi [11]. Therefore, the effect of cultivation factors in the bubble column, regarding the ease of aeration, agitation, mass transfer and the reactor type, indicates the increase in VFAs consumption comparing to the cultivation in shake flask.

The concentrations of total COD and ammonium were reduced by ca. 25–58% and 33–48% after the cultivation, respectively (Figure 4a–c). Similarly, many researchers sug-

gested that *A. oryzae* can play an active role in total COD and nitrogen removal when the fungal strains were cultured in some wastewaters (potato, fish, vinasse, olive oil mill water, etc.) [5,16,17]. In general, it can be considered that COD and nitrogen removal from various wastewaters through filamentous fungi can improve the easier further treatment. However, the removal of COD and nitrogen in these VFA-containing substrates indicates that VFAs can also be evaluated as a carbon/nitrogen source for the production of various valuable by-products.

In addition to the consumption of VFAs, COD and ammonium, it was determined that 100% of Ca, 99.6% of Mg, 50% of Fe, 40% of K and 74% of Na were eliminated by fungal cultivation (Table 3). Generally, the microorganism necessitates mineral elements, especially K, Mg, Ca and Fe, for their energy and growth materials from their growth medium [25]. However, the analysis of mineral elements in biomass needs to be determined. Moreover, the conversion of heterogeneous and complex organic waste substrates into VFAs as a carbon source (VFAs), ammonium as a nitrogen source and other minerals salts through AD is confirmed as the sustainable substrates for the production of SCP.

4. Conclusions

The present novel approach reports an original perspective in animal feed sustainability by evaluating the potential of *A. oryzae* fungal biomass produced from VFA effluents post anaerobic digestion of chicken manure, cow manure and food waste. The VFA effluents from CKM and the mixture of CKM and other effluents favored the growth of *A. oryzae* fungal biomass. The nutritional value of fungal biomass, including protein, AIM, minerals, fat and fiber contents, as well as the removal levels of ammonium, COD and VFAs, were investigated. Considering the protein (34–47%) and fat (2–5%) contents of the harvested biomass from VFA-containing substrates, the produced fungal biomass can be considered as a sustainable protein source and other nutrients for animal feed. Therefore, the production of protein-rich fungal biomass on VFAs post AD could offer an attractive strategy within the circular economy concept. It is furthermore recommended that a systematic study should be planned to investigate all aspects of feeding the green constructed SCP to ensure that this product is safe for animals and meets the set regulations (e.g., the European Commission's Food Safety policy or Food and Agriculture Organization of the United Nations) for animal feed. More research on a pilot scale and techno-economic feasibility study is needed in order to further optimize operational parameters and maximize productivity.

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