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Cultivation of edible filamentous fungus *Aspergillus oryzae* on volatile fatty acids derived from anaerobic digestion of food waste and cow manure

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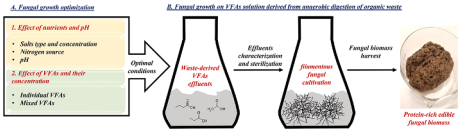
HIGHLIGHTS

- Effect of salts supplementation, pH and nitrogen source was investigated on fungal growth on VFAs.
- The growth of *A. oryzae* on VFAs was affected by type, concentration and distribution of VFAs.
- *A. oryzae* prioritized acetic acid consumption in synthetic and waste-derived VFAs effluents.
- VFAs derived from AD of cow manure and food waste are promising fungal growth media.
- Fungal biomass with 37–41% protein content was obtained by growing *A. oryzae* on waste-derived VFAs.

GRAPHICAL ABSTRACT



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ABSTRACT

In a circular economy approach, edible filamentous fungi (single cell protein) can be cultivated on volatile fatty acids (VFAs) derived from anaerobic digestion (AD) of organic-rich waste streams. In this study, the effect of pH, concentration/distribution of VFAs, nutrient supplementation, and type of waste on *Aspergillus oryzae* cultivation on synthetic VFAs, and actual VFAs derived from AD of food waste and cow manure were investigated. The optimal pH for *A. oryzae* growth on VFAs were 6 and 7 with maximum acetic acid consumption rates of 0.09 g/L. h. The fungus could thrive on high concentrations of acetic (up to 9 g/L) yielding 0.29 g dry biomass/gVFAs$_{fed}$. In mixed VFAs cultures, *A. oryzae* primarily consumed caproic and acetic acids reaching a biomass yield of 0.26 g dry biomass/gVFAs$_{fed}$ (containing up to 41% protein). For waste-derived VFAs at pH 6, the fungus successfully consumed 81–100% of caproic, acetic, and butyric acids.

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1. Introduction

Due to the world population growth, demands for human food and animal feed have significantly increased. Moreover, the generation of excessive amounts of organic-rich wastes is a fact of human societies. In a circular society, such waste should be treated and recovered using an environmentally friendly approach (Taherzadeh, 2019). Anaerobic digestion (AD) is a well-established and sustainable method of treating organic waste for biogas production. A complete AD cycle is comprised of four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, through which microbial assisted bioconversion takes place (Kiran et al., 2016). Because animal manure and food waste are produced in extensive amounts, they are the common feedstock of AD (Meyer et al., 2018; Pramanik et al., 2019; Recebli et al., 2015). However, due to recent market and economic challenges with biogas, there has been growing interest in the carboxylate platform in which short-chain volatile fatty acids (VFAs) are produced as intermediate products of AD from a wide variety of organic waste (Kleerebezem et al., 2015).

VFAs are short-chain fatty acids containing two to six carbon atoms (acetic, propionic, butyric, valeric, and caproic acids) in which acetic acid is the main volatile fatty acid produced through AD of organic wastes (up to 95%) (Jomnonkhaow et al., 2020; Lim et al., 2008; Owusu-Agyeman et al., 2020; Wainaina et al., 2020a). VFAs are attractive compounds with potential applications in food, cosmetics, pharmaceutical, and biofuel production industries and as the carbon source for nitrogen removal in wastewater treatment plants (Strazzera et al., 2018; Wainaina et al., 2019). The VFAs can also be used as building blocks to produce a wide range of valuable compounds like bioplastics and single-cell protein (Merrylin et al., 2020).

Among single-cell protein, *Aspergillus oryzae* (*A.oryzae*) from the Ascomycetes group is one of the most studied filamentous fungi at the industrial scale (Ferreira et al., 2016). There are several published articles on production of fungal biomass as a protein source from clean substrates such as sugars, starch, or even byproducts such as thin stillage (Ferreira et al., 2014), starch plant wastewater (Souza Filho et al., 2018), spent liquors from pulping processes (Asadollahzadeh et al., 2018), and vinasse (Karimi et al., 2019). Furthermore, fungus has also been considered for human consumption and as animal feed (Ferreira et al., 2016). *A. oryzae* is widely used in Asian fermented products, such as sake, shoyu, soy sauces in Japan; or Korean and Chinese rice wines (Nout & Aidoo, 2002). Most of these materials come from well-controlled industrial environments, and are considered as defined substrates whose organic compounds are easily converted into valuable products.

However, the use of the mixed VFAs produced from anaerobic digestion of complex mixed waste such as agricultural waste and food waste (including animal manure and household waste) for protein-rich filamentous fungi production remains unexploited. In a recent study, *Rhizopus oligosporus (R. oligosporus)* was found to have the ability to grow in a mixture of VFAs recovered from food waste as sole carbon source with promising crude protein content about 39% (Wainaina et al., 2020a). To the authors' knowledge, the application of VFAs solution derived from anaerobic digestion of organic waste as exclusive carbon sources for the production of edible *A. oryzae* fungal biomass has not yet been studied. This would therefore be the first study conducting an experimental analysis on A. oryzae cultivation using synthetic VFAs solution and VFAs derived from anaerobic digestion of actual organic waste streams such as cow manure and food waste. Media constituents and pH are the essential factors influencing the A. oryzae growth.

In this study, the effect of various factors such as salts supplementation, nitrogen source, pH and VFAs concentrations and distribution were firstly investigated on A. oryzae growth using acetic acid as the most dominant produced VFA. Then, the VFAs solution derived from anaerobic digestion of cow manure and food wastes were used as the sole nutrient source to produce edible A. oryzae fungal biomass.

2. Material and methods

2.1. Microorganisms

Aspergillus oryzae var. *oryzae* CBS 819.72 obtained from Centraalbureau voor Schimmel cultures, Utrecht, The Netherlands, was grown on petri dishes containing solid PDA (Potato Dextrose Agar) medium with the composition of (in g/L) glucose 20, potato infusion 4 and agar 15, for 3–5 days at 30 °C. After the sporulation, the plates were maintained at 4 °C until use. For the inoculation, 20 mL of sterile milli-Q water was poured on each plate and then the disposable spreaders were used to bring the spores into suspension. The spore suspension obtained was used to inoculate the liquid medium in order to reach a concentration between 8.0×10^5 and 15.9×10^6 spores/mL in the final medium.

2.2. Materials

The synthetic VFAs (acetic acid, propionic acid, butyric acid and caproic acid) and other chemicals of analytical grade $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, Urea (CH_4N_2O), $NaNO_3$, NaOH and HCl were obtained from Sigma-Aldrich and Merck. VFAs solutions were micro-filtered and recovered from membrane assisted anaerobic digestion according to the procedure and conditions by Wainaina et al. (2019) for food waste and by Jomnonkhaow et al. (2020) for cow manure. The above researchers carried out a long-term semi-continuous AD of either Food Waste (FW) or Cow Manure (CM) in which submerged microfiltration membranes assisted *in-situ* recovery of the metabolized VFAs solution from the mixture of undigested materials (including the bacteria consortium retained inside the reactor). VFAs solution recovered from anaerobic digestion of FW and CM were stored in a freezer at −20 °C until use. Before use for fungal cultivation, the effluents were kept in a cold room at 5 °C for 24 h. The characteristics of waste-derived VFAs effluents are presented in Table 1.

2.3. Synthetic medium

Two main synthetic solutions were prepared: a synthetic VFAs solution containing acetic acid with concentrations ranging from 1 to 18 g/L and synthetic mixed VFAs solutions with total concentration ranging between 3 and 18 g/L containing mixture of acetic, propionic, butyric and caproic acid at the ratio (in g/L) of 8:2:1:1, respectively. VFAs concentrations in the synthetic solutions were chosen to approximate VFAs levels obtained from fermentative VFAs production, using a variety of substrates mainly cow manure and food waste (Table 1). The synthetic medium used as control, contained acetic acid (6 g/L), KH_2PO_4 (3.50 g/L), $MgSO_4{\cdot}7H_2O$ (0.75 g/L), $CaCl_2{\cdot}_2H_2O$ (1.00 g/L) and $(NH_4)_2SO_4$ (7.50 g/L). The supplemented nutrients were chosen

Table 1
Characteristics of waste-derived VFAs effluents used for *A. oryzae* cultivation.

Parameters	Cow manure (CM)	Food waste (FW)	Mixture of CM and FW (CF)
Total VFAs (g/L)	6.49 ± 0.12	8.06 ± 0.38	7.93 ± 0.28
Acetic acid (g/L)	4.64 ± 0.16	2.84 ± 0.15	3.94 ± 0.11
Propionic acid (g/L)	0.76 ± 0.21	0.68 ± 0.08	0.80 ± 0.01
Butyric acid (g/L)	0.30 ± 0.05	2.35 ± 0.18	1.53 ± 0.10
Caproic acid (g/L)	0.00 ± 0.00	1.09 ± 0.08	0.68 ± 0.02
Total COD (g/L)	15.5 ± 0.7	13.5 ± 0.7	14.0 ± 0.00
NH_4^+-N (mg/L)	380 ± 0.00	358 ± 28.3	360 ± 14.1
pH	8.09 ± 0.00	5.49 ± 0.01	6.18 ± 0.02
Mg (mg/L)	90.93 ± 1.07	18.87 ± 0.11	60.05 ± 0.36
Ca (mg/L)	140.5 ± 0.06	115.3 ± 0.12	119.9 ± 0.13
Fe (mg/L)	1.51 ± 0.01	0.38 ± 0.01	1.03 ± 0.01
K (mg/L)	2300.19 ± 0.09	186.65 ± 0.03	1279.5 ± 0.12
Na (mg/L)	224.2 ± 0.04	702.0 ± 0.07	574.00 ± 0.04

following Ferreira et al. (2014). Urea (CH_4N_2O), $(NH_4)_2SO_4$, $NaNO_3$ were used as nitrogen source.

2.4. Optimization of synthetic VFAs medium for A. Oryzae cultivation

Acetic acid with concentration of 6 g/L was used as the main carbon source to determine the effect of salts, carbon to nitrogen ratio, nitrogen source and pH on growth of *A. oryzae* and VFAs consumption. The investigated pH ranged from 5 to 8. The ranges of supplemented salts varied with regards to the control as described in section (2.3) ranging from 1/3 to 9-times the control values. The control amounts supplemented to the fungi was adjusted based on the amount reported in the literature for another species of filamentous fungi (Ferreira et al., 2014; Sues et al., 2005). As changes in the initial salt content affected fungal growth in this research work, different levels of individual and the mixture of salts were used referring to the composition of waste-derived VFAs effluents used in this study (Table 1), to provide a clearer image of the effect of salt supplementation on A. oryzae growth and metabolic activity. The C:N ratios applied were between 0.75 and 6.00, where carbon source concentration was maintained constant. The effect of salts and nitrogen sources were studied at pH 6. The outcomes from the optimization experiments on acetic acid was used on the mixture of synthetic VFAs to investigate their synergic and/or antagonistic effect as a mixture for fungal growth.

2.5. A. oryzae cultivation on waste-derived VFAs effluents

The growth of *A. oryzae* on cow manure and food waste-derived VFAs was studied with and without pH adjustment to 6 every 24 h. Three different media combination of cow manure (CM), food waste (FW) and mixture of cow manure and food waste (CM + FW) (ratio of 1:1), were prepared (without dilution or any nutrient supplementation). A working volume of 100 mL was prepared for all media in 250 mL Erlenmeyer flasks, covered with cotton plugs and sterilized in autoclave (Systec, Germany) at 121 °C for 20 min. The pH was adjusted using 2 M NaOH and 2 M HCl prior to inoculation. All fermentation were carried out in shaking water bath (Grant OLS 200, Grant instrument ltd, UK) at 35 °C, 125 rpm for 72 h. At the end of each cultivation, the biomass was harvested, sieved, washed with distilled water and dried in oven at 70 °C for 24 h.

2.6. Analytical method

The concentration of VFAs in synthetic media was analyzed using a high performance liquid chromatography (HPLC) (Waters, Corporation, Milford, CT, USA) equipped with a hydrogen based column (Animex, HPX87-H; Bio-RAD, Hercules, USA) and Ultraviolet (UV–Vis) and RI detectors at 240 nm (Waters Corporation, Milford, CT, USA) working at 60 °C with 0.6 mL/min flowrate of 0.5 mM H_2SO_4 solution as eluent. The VFAs profile in cow manure and food waste-derived VFAs were analyzed and quantified by the help of a gas chromatography (GC) (Clarus 550; Perkin-Elmer, Shelton, CT, USA). The GC was equipped with a capillary column (Elite-Wax ETR, 30 m × 0.32 mm × 1.00 μm, Perkin-Elmer, Shelton, CT, USA), together with a flame ionized detector (FID). The GC-FID analysis was conducted with injection and detection temperature of 250 °C and 300 °C, respectively, using nitrogen as carrier gas at a flow rate of 2 mL/min and pressure of 20 psi. Prior to analysis, liquid samples were mixed with acid mix containing (25% (v/v) formic acid and 25% (v/v) *ortho*-phosphoric acid at a ratio of 1:3), the mixture was vortexed and then centrifuged at 10000 × g for 5 min. Then, the supernatant was filtered through a 0.2 μm syringe filter to remove undissolved particles. 250 μL of sample was mixed with 250 μL of butanol 1 g/L used as internal standards, and the volume was raised to 1 mL with milli-Q water.

The tCOD and $NH4^+$-N were analyzed using CSB 15.000 test kit and ammonium 100 test kit (Nanocolor, MACHEREY-NAGEL GmbH & Co. KG, Germany) respectively, while their concentration were determined using Nanocolor 500D photometer (MACHEREY-NAGEL GmbH & Co. KG, Germany). Biomass concentration and total kjeldahl nitrogen (TKN) was examined according to the standards method (APHA-AWWA-WEF, 2005), and to determine the protein content, a factor of 6.25 was used (Wainaina et al., 2020a). The harvested wet fungal biomass was dried at 70 °C for 24 h and the biomass concentrations were determined considering the final volume.

2.7. Statistical analysis

All cultivations were performed in triplicates, in which the presented values are the means of the resulted values and their standards deviations. The data were statistically analyzed using MINITAB 17 statistical software (Version 17.1.0, Minitab Inc., State college, PA, USA). The analysis of variance (ANOVA) was performed on the results using general linear models with 95% confidence interval followed by pairwise comparisons using turkey's test.

3. Results and discussion

The VFAs produced via anaerobic digestion of various organic waste are attractive compounds that can be used as building block chemicals for the production of a wide range materials, including filamentous fungi such as *A. oryzae*. The fungus can be used in food processing and as an alternative to plant- and animal-based protein sources. The fungus is a versatile microorganism that can grow on a wide array of different substrates, including carboxylate-containing waste streams such as thin stillage and vinasse. Accordingly, this is the first study investigating the use of VFAs solution derived from anaerobic digestion of organic waste as the sole nutrient source for the cultivation of *A. oryzae*. In this study, the fungal growth on VFAs were evaluated using synthetic VFAs medium, the effect of different factors including pH, salts, nitrogen source and mixed VFAs concentration were investigated. VFAs solution derived from anaerobic digestion of cow manure and food waste were utilized as carbon source (without any dilution or addition of nutrients) for the cultivation of *A. oryzae*. The analysis of fungal protein production and VFAs consumption is also included and reported for the first time.

3.1. The effect of salt concentration and composition on substrate consumption and biomass yield

Supplementation of essential elements (i.e., phosphorus, sulfur, calcium, potassium, magnesium, and potassium) is required to support fungal growth (Deacon, 2006; Kavanagh, 2005; Moore, 2011). The effect of salts on acetic acid consumption was investigated by cultivating A. oryzae in acetic acid 6 g/L with different salt concentrations and compositions, all compared to control culture containing KH_2PO_4 (3.50 g/L), $MgSO_4{\cdot}7H_2O$ (0.75 g/L), and $CaCl_2{\cdot}2H_2O$ (1.00 g/L). The results are summarized in Fig. 1 and Table 2. Fig. 1a shows that the medium with salt concentration of 3-, 6- and 9-times control provided a similar trend of the acid consumption profile and a total acid consumption to that of control (P-value of 1). In contrast, for one-third of the control's salts, the fungi consumed about 91% acid in 72 h. The biomass concentration and yield improved proportionately with the increase in salts concentrations, resulting in the treatments with 6- and 9-times salts as the control to reach the highest biomass concentration of 5.85 ± 0.22 and 4.07 ± 0.74 g/L, respectively (approximately two-fold increase compared to that of the control) (Table 2). Haenseler (1921) also studied the effect of increasing total salt concentration containing KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$ on the growth of *Aspergillus niger*. He reported that the fungal dry weight increased as the total salt concentration increased. He pointed out that sugar was thought to become a limiting factor for growth, thus preventing the fungal growth from being affected by the total salt concentration. In the medium containing 9-times control salt content, the positive effect became less pronounced, possibly due to

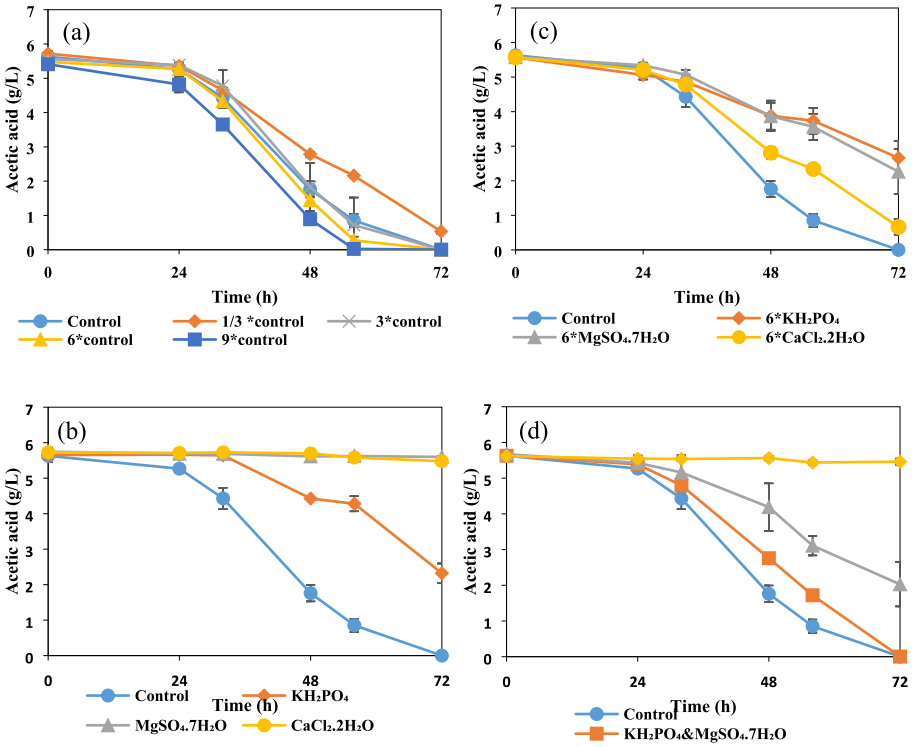

Fig. 1. The effect of salts concentration and composition on acetic acid consumption profile: (a) The effect of salts concentration, (b) the effect of single salts supplementation, (c) the effect of the concentration of single salts and (d) the effect of double salt supplementation.

Table 2
Biomass concentration, yield and VFAs consumption rate resulted during *A. oryzae* growth in media supplemented with different salts and acetic acid (carbon source).

Parameters	Biomass (g/L)	Yield (g dry biomass/ $gVFAs_{fed}$)	VFAs consumption rate (g/L.h)
Control (KH_2PO_4 3.50 $MgSO_4.7H_2O$ 0.75 & $CaCl_2.2H_2O$ 1.00)	2.63 ± 0.11	0.47 ± 0.02	0.08 ± 0.00
1/3 × Control	1.27 ± 0.06	0.22 ± 0.01	0.07 ± 0.00
3 × Control	3.88 ± 0.48	0.70 ± 0.08	0.08 ± 0.00
6 × Control	5.85 ± 0.22	1.07 ± 0.02	0.07 ± 0.00
9 × Control	4.07 ± 0.74	0.75 ± 0.14	0.07 ± 0.00
KH_2PO_4	0.60 ± 0.03	0.11 ± 0.01	0.05 ± 0.00
$MgSO_4.7H_2O$	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00
$CaCl_2.2H_2O$	0.16 ± 0.01	0.03 ± 0.00	0.00 ± 0.00
6 × KH_2PO_4	1.84 ± 0.13	0.33 ± 0.02	0.04 ± 0.01
6 × $MgSO_4.7H_2O$	3.36 ± 0.82	0.60 ± 0.15	0.05 ± 0.01
6 × $CaCl_2.2H_2O$	2.82 ± 0.38	0.51 ± 0.07	0.07 ± 0.00
KH_2PO_4&$MgSO_4.7H_2O$	1.73 ± 0.04	0.31 ± 0.01	0.08 ± 0.00
KH_2PO_4&$CaCl_2.2H_2O$	1.28 ± 0.10	0.23 ± 0.02	0.05 ± 0.01
$MgSO_4.7H_2O$ & $CaCl_2.2H_2O$	0.05 ± 0.02	0.01 ± 0.00	0.00 ± 0.00

carbon and nitrogen source exhaustion before harvest.

Furtheremore, to study the effect of single salt on fungal growth and acetic acid consumption, medium containing only a single or combination of two salts were used. As illustrated in Fig. 1b, the presence of KH_2PO_4 supported fungal growth on acetic acid with up to 60% acid consumption and a biomass concentration of 0.60 ± 0.03 g/L. while no fungal activity was detected in the presence of either $MgSO_4{\cdot}7H_2O$ or $CaCl_2{\cdot}2H_2O$ in 72 h. The hindrance in metabolic activity in the presence of $CaCl_2{\cdot}H_2O$ and $MgSO_4{\cdot}7H_2O$ could be attributed to absence of the key salt, KH_2PO_4, whereas in its present Ca and Mg salts encourage fungal metabolic activity (Fig. 1d). Totani et al. (2002) studied the effect of mineral salts on the filamentous fungus *Mortierella alpina* growth, and they found that 0.5 g/L of MgSO4·7H2O was critical to the growth while 75 mg/L of CaCl2·2H2O resulted in higher fungal biomass weight. Although it has been previously argued that magnesium is an indispensable requirement of fungal growth (Mann, 1932), the acquired result offers a rather different conclusion. The positive effect of KH_2PO_4 on fungal growth has also been reported by Safaei et al. (2016) when cultivating the fungal strain *Mucor indicus*. They showed an increase in the phosphate concentration from 2.50 to 5 g/L was translated to increase in biomass yield from 3.32 ± 0.08 to 4.11 ± 0.12 g/L, respectively. However, comparing to control, it is clear that the fungal growth were favored in medium containing a mixture of all nutrients.

Furthermore, when the three supplemented salts' concentrations were increased independently, the biomass concentration increased relatively (Fig. 1c). On the other hand, the effect of $CaCl_2{\cdot}2H_2O$ on *A. oryzae* growth becomes comparative to the control as its concentration was leveled up to 6-times that of control (P-value = 0.288). Although Mann (1932) has considered a high concentration of calcium salts as detrimental to fungal growth, our results agreed with the result from Taherzadeh et al. (2003) who showed that increasing $CaCl_2{\cdot}2H_2O$ amounts as high as 7.0 g/L could increase fungal biomass yield. Moreover, when the concentration of $MgSO_4{\cdot}7H_2O$ was increased to 6–times the control, the acid consumption rate and biomass yield increased from 3% to 60% and from 0.00 ± 0.01 to 0.60 ± 0.15 g dry biomass/$gVFAs_{fed}$,

respectively. A similar result was also observed in the work of Abu-Mejdad (2013), where the presence of magnesium boosted fungal growth and led to the highest Aspergillus niger mycelium content.

When it comes to duals (Fig. 1d), the combination of KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ enhanced the total acetic acid consumption significantly with biomass production of 1.73 ± 0.04 g/L compared to KH_2PO_4 together with $CaCl_2{\cdot}2H_2O$, and $MgSO_4{\cdot}7H_2O$ with $CaCl_2{\cdot}2H_2O$ (P-value = 0.001). In a study by Walker and Ian Maynard (1996), magnesium was shown to improve the growth of *Saccharomyces* cerevisiae. Mann (1932) also reported that the highest dry weight of Aspergillus niger mycelium was achieved when $MgSO_4$ dominated the salts proportions. Although the biomass concentration and yield increased proportionately with higher salt concentrations, the effect decreased when the concentration increased to 9-times Control.

In summary, KH_2PO_4 was the salt that significantly affected fungal growth in acetic acid. The importance of KH_2PO_4 can be accounted for phosphate's role in all organism's primary functions, i.e., energy transduction, nucleic acid, and membrane structure (Kavanagh, 2005). Another interesting finding is that $MgSO_4{\cdot}7H_2O$ is not necessarily needed in acetic acid metabolism, but it supports fungal growth in combination with other salts. Magnesium is necessary for macromolecular synthesis and formation of the energy-rich compound ATP (Moore, 2011). Besides, surprisingly, high concentrations of $CaCl_2{\cdot}2H_2O$ had significant effect on acetic acid consumption. In this regard, Moore (2011) claimed that calcium concentration in microbial cells is maintained at deficient levels by peculiar transport processes.

3.2. The effect of nitrogen source on cultivation of A. oryzae in acetic acid

The *A. oryzae* can assimilate both nitrate/nitrite and ammonium, and produce all the other essential amino acids through transamination reactions (Deacon, 2006), while urea can also be utilized following its conversion to ammonium by urea aminohydrolase (Kavanagh, 2005). Considering the fact that availability and concentration of nitrogen, influence the activity and growth efficiency of fungi (Di Lonardo et al., 2020), different nitrogen sources and C/N ratios were used to investigate their effect on cultivation of *A. oryzae* in acetic acid (6 g/L) medium.

The difference in acetic acid consumption (up to 98%) was not significant when different nitrogen sources, comprised of $(NH_4)_2SO_4$, urea, and $NaNO_3$, were applied to *A. oryzae* cultures. However, there was a slightly lower consumption rate observed in acetic acid consumption when $NaNO_3$ was incorporated, in which the acetic acid was only consumed as much as approximately 5 g/L (83%) during 72 h of cultivation. A statistical analysis showed no significant difference (P-value > 0.05) between $(NH_4)_2SO_4$ and urea in all applied C/N ratio regarding the total consumption of acetic acid in which around 99–100% of acetic acid was consumed. There was also no significant difference in the biomass production between $(NH_4)_2SO_4$ (0.39–0.47 g dry biomass/$gVFAs_{fed}$) and urea (0.39–0.40 g dry biomass/$gVFAs_{fed}$) (P-value > 0.05), while the biomass reduced about 10% when $NaNO_3$ was the sole nitrogen source. This results are in agreement with Pedersen and Nielsen (2000), who reported that the effect of ammonium sulfate was much higher than sodium nitrate on *A. oryzae* biomass yield. Zambare (2010) have also reported the positive effect of urea and ammonium sulfate on *A. oryzae* growth compared to other inorganic nitrogen sources including sodium nitrate.

Although the concentration of carbon source was similar in all treatments when comparing the effect of different C/N ratios ranging from 0.75, 1.50, 3.00, and 6.00, it was observed that C/N ratios in the mentioned range did not cause any significant difference (P-value > 0.05) in substrate consumption and biomass production.

3.3. The effect of pH on A. oryzae growth and acetic acid consumption

Acidity or alkalinity of the cultivation media is one of the major factors affecting fungal growth (Walker & White, 2017). According to Ali et al. (2017) the optimum pH for the growth of different fungal strains ranges between 3 and 8. For instance, *Aspergillus* spp. is reported to perform best at alkaline pH, while the opposite applies for *Penicillium* species.

However, as the effect of pH on the growth of *A. oryzae* on a VFAs supplemented media has not been fully investigated, it is of great interest to investigate the effect of pH on its growth in acetic acid as the most preferable in VFAs. Fig. 2 shows the effect of pH on acetic acid consumption by *A. oryzae* in a time span of 72 h. The results show a noticeable consumption of acetic acid in cultures starting with pH of around 6 and 7. The fungus assimilated 100% of the acetic acid within 72 h at pH 6, but just consumed 65% of the acid at pH 7, resulting in a biomass yield of 0.48 ± 0.03 and 0.33 ± 0.01 g dry biomass/$gVFAs_{fed}$, respectively. On the other hand, at pH 8, a marginal consumption of acetic acid was detected after 48 h of cultivation, while at pH 5, the no acid consumption was observed. In a study by Gomi (2014), the optimum pH range for *A. oryzae* growth was reported as between 5 and 6. As the cultivation proceeds, the fungus consumes the acid and the pH increases. Therefore, pH adjustment was carried out to adjust the pH every 24 h.

Control medium experiment without pH adjustment and no initial pH adjustment was also carried out to see the effect of pH adjustment on the fungal growth. *A. oryzae* was able to utilize all of the acetic acid in cultivation with initial pH adjusted to 6, whereas only 70% of acetic acid was consumed in an uncontrolled pH treatment (data not presented). For the uncontrolled pH cultivation, the pH increased from 6.0 to 8.3 in only 48 h. As reported in different literatures (Bergeim, 1940; Cole & Keenan, 1987; Hassan et al., 2012; Levine & Fellers, 1940; Taherzadeh et al., 1997), undissociated form of acetic acid plays the role of an inhibitor in fungal growth at low pH. According to Taherzadeh et al. (1997), the diffusion of undissociated acids and its equilibrium inside the cells depend on medium acid concentration and pH. Finally, pH higher than 8 might inhibit the growth of *A. oryzae* and cause long lag phases and cell death in long time intervals. Moreover, the rate of acid consumption was slower when there were no pH adjustment applied.

3.4. The effect of acid concentration on A. oryzae growth

Utilizing acid as sole carbon source in fungal cultivation is relatively new. As organic acids can be toxic for fungal growth at certain concentrations, cultivation of *A. oryzae* in media containing 1–18 g/L acetic acid were carried out. *A. oryzae* showed growth in the whole range of concentrations of acetic acid applied. However, the higher the initial

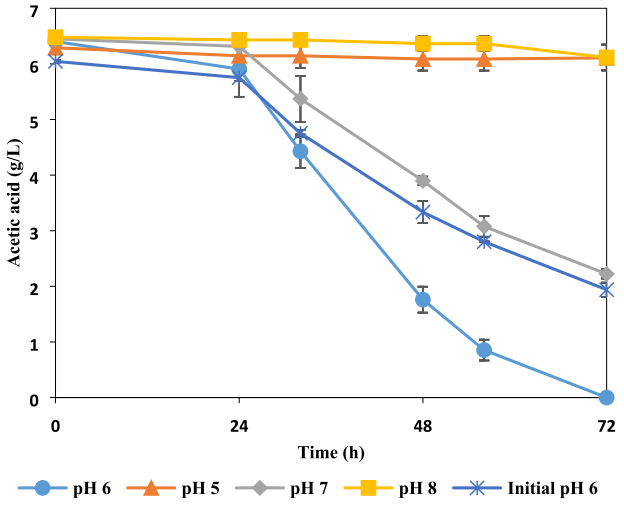

Fig. 2. The changes in acetic acid consumption trend at different pH.

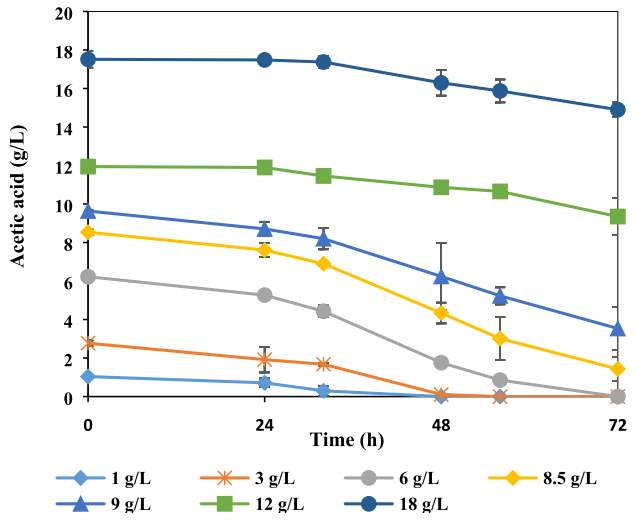

Fig. 3. The effect of the initial acetic acid concentration on acid consumption by *A. oryzae*.

acid concentration, the longer the lag phase (Fig. 3). Acetic acid was totally consumed in cultures with starting concentrations of 1 g/L, 3 g/L, and 6 g/L and the highest biomass yield was obtained for 3 g/L and 6 g/L initial acetic acid with values around 0.47 ± 0.02 to 0.50 ± 0.11 g dry biomass/gVFAs$_{fed}$, respectively (Table 3). This indicated a high tolerance of *A. oryzae* for acetic acid concentration. The consumption rate was found at best in 6, 8.5 and 9 g/L. Although 9 g/L was a high concentration of acetic acid and the fungi was not capable of fully consuming the acid in the time span of 72 h due to long lag phase, the fungi showed the highest acid uptake rate among all treatments. Ghasemian et al. (2018), reported similar result that the increase in acetic acid concentration of the medium caused long lag phase which affected fungal growth rate. This long lag phase and cell growth inhibition which is more pronounced when the concentration of acetic acid is increased, is coupled to the lower capacity of ATP regeneration during acetic acid metabolism. In normal conditions, acetic acid is directly converted into acetyl-CoA that afterwards enters the Krebs cycle to produce adenosine triphosphate (ATP), providing energy for cell growth (Pampulha & Loureiro-Dias, 2000). In low pH or near pka value, acetic acid is in an undissociated form that is liposoluble, entering the cell membrane through simple diffusion. In this regard, the acid dissociates into the anion ($CH3COO^-$) and protons (H^+) inside the near-neutral cytosol, causing the intracellular pH to drop. The plasma membrane ATPase can remediate this by pumping out the released excessive proton content in the cytoplasm at the expense of ATP. Consequently, increase in acetic acid concentration may induce the cells to restore the pH, exhausting the cell energy (ATP) to pump the released excessive proton in the cytoplasmic environment resulting in the depletion of ATP responsible for biosynthesis and cell growth. Moreover, the inhibitory effect of high acetic acid concentration might also be caused by the intracellular accumulation of high levels of acetate anions (Geng et al., 2017; León Peláez et al., 2012; Liu et al., 2015; Pampulha & Loureiro-Dias, 2000; Piper et al., 2001; Tang et al., 1989). This phenomena could be explained by the acetic inhibition model expressed by Tang et al. (1989) in the following equation:

Table 3
Summary of VFAs consumption profile, biomass concentration and yield of *A. oryzae* cultivated on VFAs.

Conditions	Acid composition	Biomass (g/L)	Yields (g dry biomass/ gVFAs$_{fed}$)	Consumption rate (g VFAs/L.h)
Acetic acid	1 g/L	0.28 ± 0.05	0.27 ± 0.04	0.01 ± 0.00
	3 g/L	1.13 ± 0.28	0.50 ± 0.12	0.04 ± 0.00
	6 g/L	2.63 ± 0.11	0.47 ± 0.02	0.08 ± 0.00
	8.5 g/L	3.06 ± 0.31	0.32 ± 0.03	0.09 ± 0.01
	9 g/L	2.79 ± 0.11	0.29 ± 0.01	0.08 ± 0.02
	12 g/L	1.75 ± 0.12	0.15 ± 0.01	0.04 ± 0.01
	18 g/L	1.81 ± 0.19	0.10 ± 0.01	0.04 ± 0.01
Mixed VFAs[a]	3 g/L tVFAs	2.02 ± 0.07	0.64 ± 0.01	0.04 ± 0.00
	6 g/L tVFAs	1.94 ± 0.08	0.34 ± 0.01	0.06 ± 0.00
	9 g/L tVFAs	2.33 ± 0.43	0.26 ± 0.04	0.07 ± 0.02
	12 g/L tVFAs	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	18 g/L tVFAs	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ac: Pr (8.5:2) g/L	1.34 ± 0.08	0.13 ± 0.01	0.04 ± 0.01
	Ac: Pr (12:3) g/L	0.87 ± 0.09	0.06 ± 0.00	0.07 ± 0.01
	Ac: Bu (8.5:1) g/L	1.34 ± 0.18	0.14 ± 0.01	0.05 ± 0.02
	Ac: Bu (12:1.5) g/L	1.42 ± 0.36	0.10 ± 0.03	0.06 ± 0.02
	Ac: Ca (8.5:1) g/L	1.47 ± 0.26	0.15 ± 0.03	0.05 ± 0.01
	Ac: Ca (12:1.5) g/L	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

[a] AC= Acetic acid, Pr= Propionic acid, Bu= Butyric acid, Ca= Caproic acid

$$\mu = \frac{\mu_0^{'} K_{TA}}{K_{TA} + [TA]}$$

where μ is the specific growth rate, μ_0' is the maximum specific growth rate in the absence of acetic acid, K_{TA} is the inhibition constant of the total acid, TA is the total acid concentration. They suggested a stronger acetic acid inhibition as a result of the decrease of K_{TA} with decreasing pH when $\mu_0'$and K_{TA} are dependent on pH. In addition, the increase in total acetic concentration indicates undissociated acetic acid as the main acid inhibitor, which has been explained by León Peláez et al. (2012) in the following expression:

$$[HA] = \frac{([C_a] \times [H^+])}{([H^+] + K_a)}$$

With [HA] as the undissociated acid concentration, K_a the equilibrium constant and C_a as the total concentration. Taherzadeh et al. (1997), reported that for fungal cultivation using acetic acid, the final concentration of undissociated acid should not exceed 5 g/L in order to prevent excessive energy loss by cell. However, the limited knowledge about this acid's inhibitory mechanisms in filamentous fungi, requires further research work.

3.5. The effect of mixed VFAs on A. oryzae growth

The previous experiments carried out with acetic acid, confirmed that VFAs could be used as the carbon source for the growth of A. oryzae. The optimum conditions from the above experiments has been applied to the mixture of VFAs, to examine their effect on fungal growth. The A. oryzae growth and acid consumption were studied using mixed synthetic VFAs (mainly acetic, propionic, butyric, and caproic acids with the corresponding ratio of 8:2:1:1) selected based on their percentage in waste-derived VFAs effluents (Jomnonkhaow et al., 2020; Parchami et al., 2020). A comparison was carried out considering different total mixed VFAs concentrations from 3 to 18 g/L. As observed in Fig. 4a, b and c, the fungus consumed up to 92%, 78% and 59% VFAs under total

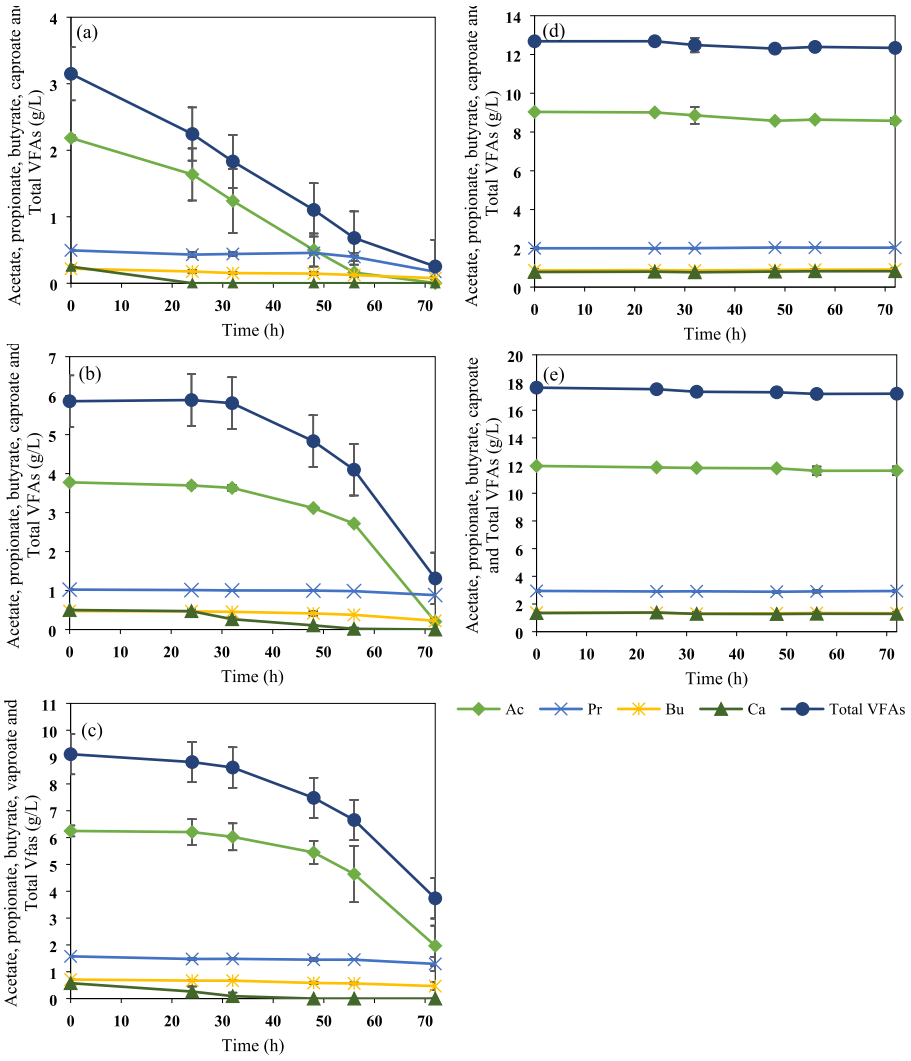

Fig. 4. VFAs consumption profile by *A. oryzae* in VFAs mixture of total concentrations of 3 g/L (a), 6 g/L (b), 9 g/L (c), 12 g/L (d); and18 g/L (e).

concentrations of 3, 6 and 9 g/L respectively. Although there was no difference in produced biomass (Table 3), the biomass yield of 0.64 ± 0.01, 0.34 ± 0.01, and 0.26 ± 0.04 g dry biomass/g$VFAs_{fed}$ decreased by increasing the total VFAs concentration of 3, 6, and 9 g/L, respectively. The biomass yield was inversely proportional to the VFAs concentration. This behavior indicates that fungal growth was affected by the concentration of VFAs, which led to the longer lag phase in high VFAs concentration.

However, at the highest concentrations of VFAs mixture, i.e. 12 and 18 g/L, neither VFAs consumption nor fungal growth were observed (Fig. 4 d and e) in 72 h. One of the reasons for this behavior at high acid concentrations, is that the fraction of undissociated acid that diffuse through the plasma membrane and dissociate intracellularly increases, which decreases intracellular pH (Sanders et al., 1981). It was also observed that *A. oryzae* showed an ability to grow in mixed VFAs with consumption preference of acetic and caproic acids, followed by, butyric acid as the least preferred carbon source. A similar result was observed in a work by Wainaina et al. (2020a), in which a filamentous fungus, *R. oligosporus*, also showed a preference of the three different even-chained acids with the simultaneous utilization of caproic and acetic acids.

On the other hand, because acetic acid was the preferable VFA, its consumption rate decreased by increasing concentration, as discussed in the previous section and it plunged noticeably in 12 and 18 g/L total VFAs. Narendranath et al. (2001) reported on the effect of acetic acid and lactic acid on growth of fungi *Saccharomyces cerevisiae*. It was clear that the increase in acetic and lactic acid concentration decreased yeast growth rate while increasing lag times. León Peláez et al. (2012) pointed out that the combination of acids might cause high inhibitory effect due to synergy on the specific growth than individual acid. However, in this case with *A. oryzae,* it was either total VFAs concentration inhibiting or single VFA inhibiting fungal growth and acetic acid consumption.

3.6. The effect of single VFAs on acetic acid consumption

To further understand the combined effect of acetic acid with other VFAs on *A. oryzae* growth, the effect of co-supplementation of acetic acid and other acids such as propionic, butyric, and caproic was investigated. The aim was to evaluate the interactive effect of acetic acid and individual propionic, butyric, or caproic acids based on their concentrations in medium comparing to acetic acid medium.

Firstly 8.5 g/L acetic acid was mixed with individual 2 g/L, 1 g/L, and 1 g/L propionic, butyric, and caproic acids, respectively; and then, the concentration of each acid was increased to; 12 g/L, 3 g/L, 1.5 g/L and 1.5 g/L for acetic, propionic, butyric and carproic acids, respectively. As shown in Fig. 5a, the activity of the fungus showed different behavior on acetic acid assimilation in only acetic acid medium (P-value < 0.05) compared to a mixture of acetic acid and other single acids.

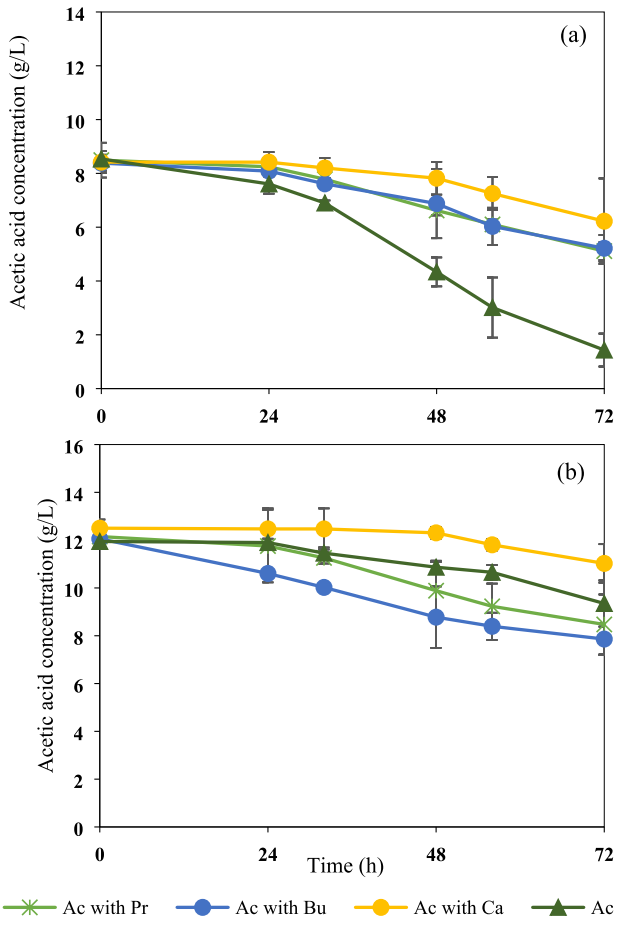

Fig. 5. The effect of dual acids supplementation and their concentration on acid consumption: (a) Single acetic acid 8.5 g/L compared with acetic acid 8.5 g/L mixed with either 2 g/L propionic, or 1 g/L butyric acid or 1 g/L carproic acid; (b) Single acetic acid 12 g/L compared with acetic acid 12 g/L mixed with either 3 g/L propionic, 1.5 g/L butyric and 1.5 g/L caproic acid.

After 72 h, the fungus consumed up to 58% of acetic acid at the rate of 0.1 g/L.h from a medium containing only acetic acid, 40% acetic acid at the rate of 0.05 g/L.h in a mixture of acetic and either propionic or butyric acids, and only 26% acetic acid at the rate of 0.03 g/L.h from the mixture with caproic acid. Similar inhibition was detected when supplied acetic acid for A. oryzae combined with propionic acid and butyric acid in which acetic acid content of the medium in the mixture reduced to about one half of that of only acetic acid medium. However, in the presence of acetic acid, propionic and butyric acid content was left relatively intact. On the other hand, a slower consumption of acetic acid was experienced in a mixture of acetic and caproic acids with slight caproic acid consumption.

When the concentration of acids was increased (Fig. 5b), the fungi consumed acetic acid similarly in either only acetic acid medium or mixed with propionic or butyric acids up to 22%, 30% and 35%. However, a considerable difference was observed in the mixture of acetic acid with caproic acid, where only 12% of acetic acid was consumed after 72 h. This result was further confirmed, as there was no biomass growth detected at the end of the cultivation. Moreover, it was noticed that the increase in acid concentrations negatively affected fungal growth. Fungal biomass reduced about 53% in the only acetic acid medium, while in acetic acid mixed with propionic, butyric, and caproic acids, biomass concentration decreased about 54%, 29%, and 100%, respectively (Table 3). These results agreed with previous studies which demonstrated that higher concentration of weak acids, mostly acetic, butyric, propionic, and caproic acids could result in a slower fungal or yeast growth rate (Fukushima et al., 1991; Maeda et al., 2005; Moon, 1983; Narendranath et al., 2001). According to Maeda et al. (2005) acetic and propionic acids played mixed inhibition, while butyric and caproic or hexanoic acid played a competitive inhibition on A. oryzae cutinase enzyme. They pointed out that carboxylic acids with carbon chain longer than four might result in higher fungal growth inhibition than shorter carbon chain carboxylic acids. Therefore, high caproic acid content in a VFAs mixture can be referred to as a growth-inhibiting factor for A. oryzae.

However, the weak acid inhibition effect on filamentous fungi has not received as much attention as the effect on yeast. The interesting finding, backed by Wainaina et al. (2020a) findings, is that although butyric acid is a smaller acid (4-carbon chain) compared to caproic acid (6-carbon chain), the latter can be simultaneously utilized along with acetic acid by the filamentous fungi while the consumption of the former only initiates after a considerable amount of acetic is already consumed. This could happen due to different metabolic pathways for metabolization of different VFAs. Acetic acid is converted directly into acetyl-CoA, whereas butyric acid requires conversion to acyl-coA through β-oxidation, and then continued to further oxidation through glyoxylate or tricarboxylic acid pathway (Perez-Garcia et al., 2011). In the case of filamentous fungi such as *A. oryzae*, caproic acid is mostly converted into 2-pentanone through peroxisomal β-oxidation that afterward can be converted to acetyl-CoA (Lewis, 1970; Walker & Mills, 2014).

In both concentration levels, combination of acetic/propionic, acetic/butyric and acetic/caproic delayed acetic acid consumption or reduced the acetic acid consumption rate, thus inhibiting fungal growth compared to the media containing only acetic acid. The inhibitory effect could explain this mechanism in the presence of either propionic, butyric or caproic acid. Propionic and butyric acid showed a similar impact when mixed with acetic acid, while the inhibitory effect of both acetic and caproic acids mixture was more pronounced when the concentration increased to 12 and 1.5 g/L, respectively. Fungal cultivation in medium containing individual propionic, butyric or caproic acid as a sole carbon source at different concentrations from 1 to 6 g/L led to complete fungal growth inhibition (data not shown). However, the specific inhibitory effect depends on environmental factors such as pH of the medium, type and strain of microorganism and organic acid concentration (Ullah et al., 2012). The inhibitory effect or antifungal activity of weak organic acids such as acetic, butyric and propionic acids as well as sorbic and lactic acid on yeast and bacteria have been extensively researched (Liewen & Marth, 1985; Moon, 1983; Narendranath et al., 2001; Piper et al., 2001; Restaino et al., 1982; Ullah et al., 2012). However, due to the limited information on the inhibitory effect of VFAs on filamentous fungi, further studies are needed to support the acquired data in this study.

3.7. Cultivation of A. oryzae on VFAs derived from anaerobic digestion of cow manure and food waste

It is clear that the high demand for animal feed imposes stress on natural resources for example by acquisition of arable land for animal feed production (Steinfeld et al., 2006). In order to alleviate issues related with animal feed provision, production of fungal single cell protein from organic waste or residual streams can be promising.

Two of the globally abundant and available organic waste streams are food waste and animal manure (Cavinato et al., 2017; Jomnonkhaow et al., 2020; Wainaina et al., 2020b; Yin et al., 2016). Considering the nutrient content of these waste streams, an effective bioconversion practice can convert these streams to essential biochemical and biomaterials.

Conversion of waste-derived VFAs for production of fungal biomass for animal feed protein supplementation can open new horizons in animal feed sustainability. Therefore, in this study, it was a great interest to investigate the use of VFAs derived from anaerobic digestion of cow manure (CM) and food waste (FW) as sole carbon source for the

cultivation of A. oryzae. CM and FW-derived VFAs effluents are characterized as a source of VFAs, ammonium and other trace minerals, which could play an essential role in the growth and metabolism of microorganism. The characteristics of both VFAs solution derived from anaerobic digestion of FW and CM are presented in Table 1. The CM and FW-derived VFAs solutions and the mixture of them with the as-received pH and pH adjusted to 6 were used as the cultivation medium for A. oryzae without any dilution or addition of nutrients in 72 h cultivation cycles.

The changes in the concentration of CM-derived VFAs during fungal cultivation is presented in Fig. 6 a and b. *A. oryzae* was capable of growing in both pH conditions leading to a total VFAs (mainly acetic acid) utilization of 74% and 68% (biomass yield of 0.23 ± 0.00 and 0.13 ± 0.01 g dry biomass/ $gVFAs_{fed}$) containing total protein of up to 37 and 39% in CM effluent with pH 6 and as-received, respectively (Table 4). Although in both conditions fungal growth and acid consumption was observed, the drop in total VFAs concentration (P-value = 0.018) and increase in fungal biomass content (P-value = 0.002) was significantly higher for pH 6.0. Moreover, as expected and proved through screening experiments with synthetic medium; among the VFAs, the fungi preferably consumed acetic acid (4.64 g/L) up to 91% and 89% in pH 6 and as-received pH after 72 h of cultivation, respectively. According to the results, in the CM effluent, acetic acid is the main targeted acid to be removed by *A. oryzae*. In addition, it was observed that the fungi was capable of assimilating nearly half and one-fourth of the initial butyric acid during cultivation at pH 6 and as-received pH, respectively. This indicates that pH 6 could significantly affect the selective consumption of VFAs other than acetic acid (P-value < 0.05) compared to that of as received pH of 8.0.

In the case of FW-derived VFAs effluent, the cultivation conditions were similar to that of CM with FW as-received pH being 5.5. Here in case of FW, the effect of pH became very pronounced (Fig. 6 c and d) as *A. oryzae* did not show any signs of growth in the effluent of as-received pH (Fig. 6 d). This is in confirmation with the results observed with *A. oryzae* grown in acetic acid at pH below 6 (section 3.3). As discussed at low pH, the concentration of undissociated acids are high, organic acids penetrate into cells by simple diffusion through the plasma membrane into cytoplasm and this causes substantial acidification, which could inhibit cellular metabolism (Hassan et al., 2015; Stratford et al., 2009; Taherzadeh et al., 1997; Thomas et al., 2002). Stratford et al. (2009) reported that Aspergillus niger showed intracellular pH drop during spore germination by weak acid preservatives (acetic acid) and concluded that the intracellular acidification resulted in growth inhibition. When pH of FW was adjusted to 6, the fungi consumed up to 75% of total VFAs, yielded 0.26 ± 0.03 g dry biomass/$gVFAs_{fed}$, resulting in a fungal biomass with 41% protein content (Table 4).

However comparing to CM, FW at pH 6 showed no significant difference in either total VFAs consumption, biomass yields or biomass protein content. Considering the fact that fungal growth was observed at pH 6 regardless the VFAs concentration, it can be concluded that the

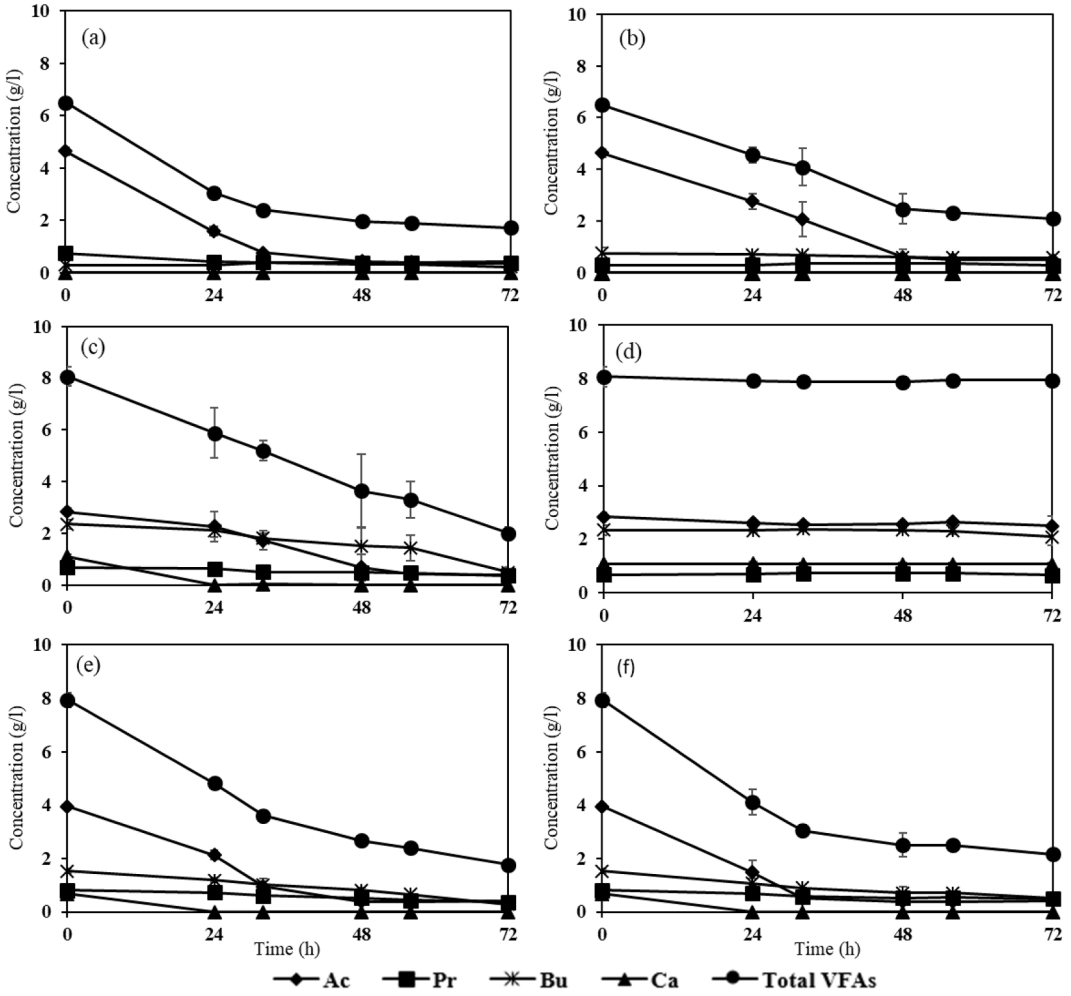

Fig. 6. VFAs consumption trend by *A. oryzae* in cow manure-derived VFAs effluents with (a) as-received pH and (b) pH adjustment to 6, food waste-derived VFAs effluents (c) as-received pH and (d) pH adjustment to 6, and mixture of cow manure and food waste-derived VFAs effluents (e) as-mixed pH and (f) pH adjustment to 6.

Table 4
Summary of VFAs consumption profile, biomass, yields, ammonium, and protein content during *A. oryzae* cultivation in VFAs.

Substrate	Conditions	Initial VFAs (g/L)	Final VFAs (g/L)	Biomass (g/L)	Yields (g dry biomass/gVFAs$_{fed}$)	Consumption rate (g VFAs/L.h)	Initial NH_4^+-N (mg/ L)	Final NH_4^+-N (mg/L)	Protein content (%)
Cow manure (CM)	CM as-received pH 8	6.49 ± 0.12	2.10 ± 0.13	0.86 ± 0.12	0.13 ± 0.01	0.06 ± 0.00	380 ± 0.00	155 ± 35.4	39%
	CM pH ajd. to 6	6.49 ± 0.12	1.72 ± 0.02	1.48 ± 0.09	0.23 ± 0.00	0.06 ± 0.00		215 ± 7.1	37%
Food waste (FW)	FW as-received pH 5.5	8.06 ± 0.38	7.93 ± 0.08	0.00 ± 0.00	0.00 ± 0.01	0.00 ± 0.02	358 ± 28.3	320 ± 42.4	0%
	FW pH ajd. to 6	8.06 ± 0.38	1.99 ± 0.25	2.01 ± 0.10	0.26 ± 0.03	0.08 ± 0.01		120 ± 28.3	41%
Mixture of CM + FW (1:1)	FW as-received pH 6.2	7.93 ± 0.28	2.17 ± 0.17	1.71 ± 0.01	0.22 ± 0.01	0.09 ± 0.00	360 ± 14.1	120 ± 0.00	39%
	FW pH ajd. to 6	7.93 ± 0.28	1.77 ± 0.12	1.74 ± 0.05	0.22 ± 0.01	0.08 ± 0.01		165 ± 21.2	40%

growth inhibition is more sensitive to pH than acids concentration. Regarding VFAs consumption prioritization, in FW-derived VFAs effluents with pH 6, the fungi preferably consumed acetic acid (86%) and then caproic acid (complete consumption). Consumption of butyric and propionic acids came in the third and fourth place by 79% and 46%, respectively. Although butyric acid concentration in FW was much higher than in CM effluent, it was observed that the consumption of butyric acid in food waste effluent was nearly 4-times higher compared to CM effluent. No significant difference was observed for propionic and acetic acids consumption in both FW and CM effluents. Similar result is reported in a study by Wainaina et al. (2020a), on cultivation of *R. oligosporus* on FW-derived VFAs. They pointed out that the *R. oligosporous* utilizes only the even-chained acids regardless the concentration of VFAs. In this regard, *A. oryzae* showed minor consumption of odd-chained acids such as propionic acid. Besides, *A. oryzae* also showed the ability in consuming lactic acid which is an odd-chained acid produced by lactic acid bacteria (Mahboubi et al., 2017). Narendranath et al. (2001), also reported the simultaneous consumption of acetic acid (even chain) and lactic acids and their synergic effect of yeast growth rate. They concluded that the increase in concentration of either acetic acid or lactic acid slowed yeast growth rate. However, the fact that *A. oryzae* could slightly consume propionic acid could be due to the presence of propionic acid at low concentration in medium.

In order to overcome the issue of off-the-optimum pH of the effluents (CM 8.0 and FW 5.5) in this phase of study, CM and FW mixture of 1:1 was used as the starting medium. The cultivation condition was the same as applied previously, however, due to high buffering capacity of CM, the pH value of the mixture became 6.2. This mixture was used for fungal cultivation at its original pH and also in a treatment where the pH was adjusted to 6 every 24 h. As can be seen in Fig. 6 e and f, the *A. oryzae* was capable to grow in both medium with original pH and pH adjusted. The performance in both cases was rather similar, as the results showed that the fungi utilized 78% and 73% of total VFAs in 72 h for the original and adjusted pH, respectively. The yield of biomass in this case was about 0.22 g dry biomass/gVFAs$_{fed}$ containing about 40% protein for both conditions (Table 4). Moreover, up to 90% acetic, 54% propionic, 81% butyric and 100% caproic acids were used within the same cultivation period.

The consumption of acetic and propionic acids in mixture of FW and CM did not significantly differ (P-value $>$ 0.05) for either FW or CM effluents. However, FW effluents and mixture of FW and CM experienced an enhanced consumption of butyric acid comparing to CM. In the cultivation with the as-received effluent, the pH increased to 8 after 8 h hours of cultivation and further to 9 in 72 h. Although this increase in pH apparently reduced the rate of acetic acid assimilation, it did not bring fungal cultivation to a halt. This phenomenon indicates that the initial pH of 6.2 was the determining factor for the initiation of fungal growth rather than pH adjustment along the way. The presence of other nutrients (high content of ammonium nitrogen, and other macro- and micronutrients) and minerals in both CM and FW could have assisted the results acquired. This could be confirmed by the findings when *A. oryzae* was cultivated in the synthetic mixed VFAs, where only acetic and caproic acids could be consumed while pH 8 inhibited acetic acid consumption.

The protein content in produced *A. oryzae* fungal biomass from VFAs effluents derived from anaerobic digestion of CM and FW ranged between 37 and 41% and was similar to the protein content in *R. oryzae* and *A. oryzae* biomass produced from starch processing wastewater in the study done by Souza Filho et al. (2019) and Jin et al. (1999). Wainaina et al. (2020a) also reported comparable results when FW-derived VFAs was used for *R. oligosporus* cultivation. The achieved results proved promising for the produced biomass, as the protein content in commercial animal feed generally ranges between 30 and 50% (Banaszkiewicz, 2011; Świątkiewicz et al., 2016). Therefore, VFAs streams derived from anaerobic digestion that can be converted into protein-rich edible fungal biomass such as A. oryzae are a sustainable nutrient source that

can contribute to meet protein demand in animal feeding.

4. Conclusion

In this novel study, the use of VFAs solution derived from anaerobic digestion of cow manure and food waste as sole carbon source, proved to be suitable substrates for the cultivation of *A. oryzae* biomass. The combination of KH_2PO_4, $MgSO_4{\cdot}7H_2O$ and $CaCl_2{\cdot}2H_2O$ enhanced fungal growth at control concentrations. Fungal growth depended on the type of acids (mainly acetic acid), acids concentrations and pH. In waste-derived VFAs effluents, the maximum biomass yield of 0.26 ± 0.03 g dry biomass/ $gVFAs_{fed}$ with protein content ranging between 37 and 41% was achieved. Furthermore, the initial pH was the determining factor for the fungal growth initiation.

CRediT authorship contribution statement

Clarisse Uwineza: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Amir Mahboubi:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Supervision. **Amelia Christina Atmowidjojo:** Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Alya Nur Ramadhani:** Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Steven Wainaina:** Conceptualization, Methodology, Writing - review & editing, Supervision. **Ria Millati:** Writing - review & editing, Supervision. **Rachma Wikandari:** Writing - review & editing, Supervision. **Claes Niklasson:** Writing - review & editing, Supervision. **Mohammad J. Taherzadeh:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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