Research Article

Anaerobic co-digestion of waste yeast biomass from citric acid production and waste frying fat

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Abbreviations: COD, chemical oxygen demand; **CSTR,** continuous stirred tank reactor; **CYB,** concentrated yeast biomass; **LCFA,** long-chain fatty acids; **LR,** loading rate; **OLR,** organic loading rate; **SBY,** specific biogas yield; **SMY,** specific methane yield; **TN,** total nitrogen; **TOC,** total organic carbon; **TS,** total solids; **VFA,** volatile fatty acids (sum of concentrations of acetate, propionate and butyrate); **VS,** volatile solids; **WFF,** waste frying fat

Practical application

Recent trends in biotechnology favor a holistic approach to microbial processes, aiming at implementing a bio-based ‘circular flow’ economy that achieves multiple, resource-efficient uses of raw materials and material streams. By-products formerly regarded as waste are now regarded as secondary resources. Accordingly, anaerobic digestion is a structural component in the value chain. For example, waste yeast biomass from citric acid production can be re-used as a substrate for biogas production. This research work deals with the challenges and limitations of the anaerobic digestion of waste yeast biomass as a mono-substrate and co-substrate. It provides new insights into designing a novel bio-refinery system that uses organic residues from waste-based production processes to maximize the value derived from the residual biomass. These findings will be of particular interest to those engaged in the development of anaerobic digestion and waste-based production processes, and in bio-based sectors in general.

Abstract

The application of spent yeast for biogas production has been studied only in the context of breweries so far. This study is focused on the anaerobic digestion of concentrated yeast biomass (CYB), being a by-product of citric acid biosynthesis. Two experimental set-ups were used in order to test CYB as a mono-substrate and co-substrate for closing the loop in accordance with the 'bioeconomy' approach. The results show that CYB allows for obtaining a high biogas yield, with a maximum of 1.45 m^3_N/kg_{VS} produced when CYB was used as a mono-substrate. The average methane concentration was 66 ± 4%. However, anaerobic digestion of CYB alone was difficult to perform because of a tendency for over-acidification, meaning that the maximum possible organic loading rate was 1 kg/(m^3*d). Repeated clogging of tubes with coagulated biomass also disturbed continuous feeding.

In contrast, the co-digestion of CYB with waste frying fat at a ratio of 1:20 showed stable operation during a 70-day fermentation period. The biogas yield using the substrate mixture was 1.42 m^3/kg_{VS} at an organic loading rate of 2 kg/(m^3*d). The methane concentration reached 67 ± 4% and the acetate concentration did not exceed 30 mg/L during the entire fermentation.

1 Introduction

Citric acid is a bulk chemical that is generally produced in biotechnological processes using the fungus *Aspergillus niger* [1]. This production procedure is environmentally problematic because of the heavily polluted solid and liquid wastes that are by-products of citric acid production [2]. An alternative process that uses the "non-conventional" yeast *Yarrowia lipolytica* is more environmentally friendly. *Y. lipolytica* is a non-pathogenic ascomycetous yeast that is able to utilize a series of substrates such as hexoses, *n*-paraffins, fatty acids, fats and oils, organic acids and alcohols as sole carbon sources and can produce valuable products such as single-cell protein, lipases or organic acids (e.g. 2-ketoglutaric acid, citric acid, isocitric acid, and pyruvic acid) depending on the cultivation conditions [3, 4]. Our previous research concerned the optimization of citric acid production with *Y. lipolytica* H181 using glucose and sucrose (e.g. [5, 6]) and plant oils [7] as carbon sources. Other authors have reported on the utilization of various waste materials such as industrial fat [8], raw glycerol and crustacean waste [9], industrial raw molasses [10] and olive mill wastewater [11] for the production of citric acid, single cell oils, polyols and lipids with *Y. lipolytica*.

Current research into biotechnological processes is increasingly aiming at achieving holistic use of all process inputs and outputs, i.e. substrates and by-products. Taking the perspective of the 'bioeconomy' approach, new activities are focusing on the design of bio-refineries where production residues are considered as a secondary resource for subsequent production; the last step of residue conversion is biogas production for energy purposes [12]. Biogas is a mixture of mainly methane and carbon dioxide that is produced by anaerobic digestion degradation (also commonly referred to as anaerobic digestion) of biogenic materials – renewables or organic wastes. In the course of downstream processing after citric acid production, concentrated yeast biomass (CYB) is produced as a residue. There has been no research on *Y. lipolytica* digestion so far and only a few publications exist concerning anaerobic digestion from spent yeast from breweries [13-16]. Biogas yield of 0.45-0.72 m^3/kg_{VS} for brewery surplus yeast [13; 14] and average methane yield of 0.330–0.370 m^3_{CH4}/g_{COD} [15] were reported for batch fermentation tests. When an anaerobic sequencing batch reactor was used, a specific biogas productivity of more than 0.43 m^3/kg_{COD} was reached [16]. Moreover, it has been reported that the use of baker's yeast causes process upsets in full-scale anaerobic digesters such as the

formation of foam [17]. Foam formation can cause various problems in biogas plants such as blocking of gas pipes and disruption of measurement devices and recirculating pumps [18, 19, 20]. One possible method of preventing process imbalances is co-digestion with stabilizing substrates. The use of co-substrates with anti-foaming properties is appropriate for substrates that have a high foaming propensity. Kougias et al. [21] described the good anti-foaming properties of plant oils, especially rapeseed oil, during anaerobic digestion in overloaded manure-based biogas reactors. The use of WFF that is actually spent rapeseed oil that has been used for frying in a canteen kitchen would thus appear to be advantageous as a co-substrate for CYB.

Accordingly, the aim of this research work was to study the mono-digestion of CYB and co-digestion with WFF in semi-continuous fermentation in order to evaluate digestibility and process stability.

2 Materials and methods

2.1 Digestates and substrates

The substrates for the fermentation batch tests and the semi-continuous fermentation experiments were WFF (total solids (TS): 100% wet weight (WW), volatile solids (VS): 100% TS) from a canteen kitchen and a cross-flow filtrated thickened fermentation broth from the cultivation of a non-conventional *Yarrowia lipolytica* H181 yeast (abbr. CYB) (TS: 41.5% WW, VS: 91.4% TS, c (protein) = 0.18 g/L, c (fat) = 0.71 g/g_{TS}, TOC/TN = 70.1). The substrates CYB and WFF were used either in pure form or mixed at a ratio of 1:1 (w/w) or 1:20 (w/w). The inoculum (TS: 7.11% WW, VS: 91.2% TS) for the fermentation batch tests originated from the research biogas plant of the German Biomass Research Centre (DBFZ) (anaerobic digester utilizing maize silage). In the preliminary semi-continuous fermentation experiment, the digestate originated from the secondary digester of a biogas plant that is operated by an agricultural cooperative close to Leipzig, Germany. This digestate had a pH of 8.02, a TS content of 3.93% WW and a VS content of 66.9% TS. The digestate used in the main semi-continuous fermentations originated from the anaerobic digester of the Rosental waste water treatment plant in Leipzig and had a pH of 7.73, TS content of 2.44% WW and VS content of 62.4% TS.

2.2 Technical equipment

2.2.1 Batch fermentation test equipment

The batch fermentation test equipment consisted of twelve test jars. Each test jar was comprised of a 500-mL Schott bottle, a gas trap and a 2-L gas collection tube. Gas-tight pipes were used to transport the biogas produced to the collection pipe. A three-way stopcock was placed between the Schott bottle and the gas trap for taking gas samples in order to analyze the methane content in the produced biogas. The reaction mixtures in the Schott bottles were incubated in a water bath (GFL 1004, Gesellschaft für Labortechnik GmbH, Germany). The collection tubes contained 300 g L^{-1} sodium chloride and 50 g L^{-1} citric acid solutions as a sealing liquid. A syringe was used for pressure equalization at ambient pressure.

2.2.2 Continuous stirred tank reactor (CSTR)

CSTRs with a total volume of 40 L and working volume of 31 L were used for the continuous fermentation experiments. The process temperature was adjusted using a water-heated reactor jacket. A thermostat (Integral T 1200, Lauda, Germany) was used for continuous heating. The bioreactors were fitted with an insulating layer. A combination sensor (FU20, Yokogawa Deutschland GmbH, Germany) determined the pH and temperature levels in the digestate. Biogas production was measured by a drum gas meter (TG05-PVC, Dr.-Ing. RITTER Apparatebau GmbH & Co. KG, Germany). Online measured data, such as biogas production, temperature and pH, was recorded by a data logger. The motor of the stirrer (stirrer RZR 2101 control, Heidolph, Germany) was positioned above the reactor and the stirrer had a rotation speed of 35 rpm. A U-shaped tube filled with distilled water was used as an overpressure and underpressure safety device.

The automated feeding system consisted of a hose pump (REGLO Digital, ismaTec, Wertheim, Germany) and a time switch (93256 NK ZSU 4, Goobay, Braunschweig, Germany), which controlled the pump and Schott bottles with the substrates (CYB/water and/or WFF) that were continuously mixed by a magnetic stirrer. The substrate was supplied to the system over various time periods depending on the organic loading rate (OLR) – see experimental set-up for more information.

A sample of biogas was taken manually from the reactor once a week using a separate sampling device at the gas measuring section in order to determine the methane content in the biogas.

2.3 Experimental set-up

2.3.1 Determination of the specific biogas yield (SBY) using WFF and CYB as well as their mixtures in fermentation batch tests

Fermentation tests for the evaluation of the biogas yield were carried out in accordance with the VDI 4630 guideline [22]. The inoculum of the test system was sieved through a 5-mm sieve and incubated for 7 days at 37.5 °C prior to the start of the experiment.

Four different variants were tested: 1) CYB (VS substrate to VS inoculum ratio = 0.45), 2) WFF (VS substrate to VS inoculum ratio = 0.11), 3) CYB+WFF at a ratio of 1:1 (w/w) (VS substrate to VS inoculum ratio = 0.21), 4) CYB+ WFF at a ratio of 1:20 (w/w) (VS substrate to VS inoculum ratio = 0.45). Each variant was performed in duplicate. In addition, two bottles with inoculum served as zero samples. The reaction mixtures were incubated at 38 °C. The fermentation batch tests were performed until the termination criterion (i.e., daily biogas volume equivalent to less than 1% of the total volume of biogas produced up to that time) was met. The methane concentration in biogas was measured twice a week.

2.3.2 Anaerobic digestion of CYB and WFF in continuous fermentation experiments

2.3.2.1 Preliminary experiment

The mixed substrates of CYB and WFF at a ratio of 1:20 (w/w) were used in the preliminary anaerobic digestion experiment. The substrate supply to the fermenters was initially carried out once a day for 50 days. After 51 days, the automatic feeding system was installed and a cycle of 1.5 hours was selected for the timer for continuous addition of the substrate. The experiment was started at an organic loading rate (OLR) of 0.5 $kg_{VS}/(m^3 * d)$. After an adjustment period of 19 days, the OLR was increased to 1 $kg_{VS}/(m^3 * d)$ and, after 29 days, to 2 $kg_{VS}/(m^3 * d)$. In order to support the anaerobic digestion process, a nitrogen source, 66.4 g of urea, was added on day 57. After eleven more days, the OLR was increased

to 3 $kg_{VS}/(m^3*d)$. The hydraulic retention time was 60 days, and was adjusted by adding tap water to the feeding substrate. The preliminary experiment lasted 72 days.

2.3.2.2 Main continuous parallel experiments by use of CYB and CYB-WFF (1:20)

Substrate supply was carried out automatically from the very beginning in the main continuous experiments. Two CSTRs were run in parallel. The first CSTR was fed with the CYB-WFF substrate mixture at a ratio of 1:20 (w/w) just as in the case of the preliminary experiment. One Schott bottle was filled with CYB, tap water and later also with urea. Another substrate storage bottle contained only WFF. Sixteen times a day, the automatic feeding system pumped a defined volume of substrate adapted for the specific OLR into the reactor. After an adjustment period of 16 days at an organic loading rate (OLR) of 0.5 $kg_{VS}/(m^3*d)$, the OLR was increased to 1 $kg_{VS}/(m^3*d)$ and, after 45 days, to 1.5 $kg_{VS}/(m^3*d)$. In order to support the anaerobic digestion process, a nitrogen source, 0.5 g of urea (corresponding to a loading rate (LR) of 7.5 mg urea-N/(L*d)), was added from day 32 to 35. The urea addition was increased to 1.0 g of urea per day (LR=15.1 mg urea-N/(L*d)) in the period from day 36 to 43 and to 1.5 g urea per day (LR=22.6 mg urea-N/(L*d)) from day 44 to 49. From day 50, the daily urea addition was 2.0 g (LR=30.1 mg/(L*d)) and this was not increased any further until the experiment finished after 84 days. The hydraulic retention time was 100 days.

In the second CSTR, CYB (100%) was used as a substrate. One Schott bottle was filled with CYB, tap water and later also with urea. After an adjustment period of sixteen days at an OLR of 0.5 $kg_{VS}/(m^3*d)$, the OLR was increased to 1 $kg_{VS}/(m^3*d)$. The HRL was 100 days, adjusted by adding tap water to the feeding substrate bottle for CYB. The addition of urea had to be started on day 32 with 0.5 g of urea per day (LR=7.5 mg urea-N/(L*d)). After four more days, urea addition was increased to 1.0 g of urea per day (LR=15.1 mg urea-N/(L*d)) and, on day 44, to 1.5 g of urea per day (LR=22.6 mg urea-N/(L*d)). To adapt to the ammonium concentration, urea addition was reduced between days 64 and 72 from 1.5 g to 1.0 g of urea per day (i.e. decrease from 22.6 to 15.1 mg urea-N/(L*d)). On day 73, urea addition was increased to 1.5 g of urea per day (LR=22.6 mg urea-N/(L*d)) again until the end of the experiment. The experiment lasted 84 days.

Samples of digestates of all anaerobic digestion experiments were taken twice a week and analyzed as described below. The process temperature was maintained at 38 °C.

2.4 Analyses

The samples of CYB and digestates from the anaerobic digestion experiments described in 2.3.2 were analyzed directly after sampling.

2.4.1 Sample pre-treatment

COD, TS and VS were measured in the original samples without pre-treatment. The CYB sample was centrifuged for 5 minutes at 13.000 rpm (Eppendorf centrifuge 5415D, Hamburg, Germany) for the analysis of citric acid concentration. The protein concentration was measured in a filtrate (folded filter, pore size: 2 µm, No. 390, Filtrak, Germany). The fat amount was determined in the freeze-dried sample (Beta2-16, Christ, Germany) and milled by use of a pebble mill (MM301, Retsch, Germany).

The digestate samples were passed through a sieve with a mesh size of 0.75 mm. The sieved sample was centrifuged for 10 minutes at 5,300 rpm (Heraeus-Labofuge 200, Thermo Fisher Scientific GmbH, Dreieich, Germany) and filtered afterwards (pressure filtration device SM 16 249, Sartorius, Göttingen, Germany; nylon membrane filter: pore size 0.45 µm, Whatman, Germany) as well as analyzed for ammonium-nitrogen and volatile fatty acids (VFA).

2.4.2.2 Sample analyses

Once a week, a 20 mL sample of biogas was taken and the biogas composition (methane, hydrogen, nitrogen, and oxygen percentages) was determined by gas chromatography using an Agilent GC 6850 WLD wavelength detector (Agilent Technologies, USA) with an HP Plot separation column and argon as the carrier gas. A gas mixture of 49.8% methane and 50.2% nitrogen was used as a calibration gas.

3 Results

3.1 Fermentation batch tests

The aim of the fermentation batch tests was to explore the digestibility of the two substrates CYB and WFF and of their mixtures. The results that are described in Figure 1 and Table 2 were subsequently used for designing continuous fermentations on a technical laboratory scale.

Biogas production started with a delay in the case of WFF as compared to CYB (Figure 1). After two days, 0.02±0.01 m^3_N/kg_{VS} was produced using WFF, whereas 0.05±0.01 m^3_N/kg_{VS} was detected for CYB as substrate. Nevertheless, the produced biogas volume of 0.23 m^3_N/kg_{VS} was equal on the fifth day of fermentation, and the batch tests with WFF achieved a significantly higher SBY at the end of the fermentation than those with CYB as a substrate (Table 2). The methane end concentration reached the same value in both test variants.

In the case of substrate mixtures, both curves for biogas formation showed a typical diauxic trend due to the delayed digestion start of WFF (Figure 1). After the utilization of CYB with better bio-availability, a lag phase was observed before the WFF was utilized. It can be seen that this lag phase was longer in the case of higher percentages of WFF in the substrate mixture, with the result that the fermentation duration of 48 days was 25% longer than the other batch tests. Nevertheless, the results showed that the mixture of CYB and WFF at a ratio of 1:20 is more favorable than the 1:1 mixture of these two substrate components because in this case both the SBY and the SMY were higher.

3.2 Continuous fermentation of a substrate mixture of CYB and WFF

The aim of the experiment was to test the substrate combination of CYB and WFF at a ratio of 1:20 (w/w) based on VS in a continuous fermentation.

The limits of the usage of the given substrate mixture were tested in a preliminary experiment. The SBY and the profiles of the VFA concentrations are displayed in Figure 2A. During the first six days after the start of the fermentation, the SBY showed a large peak of 1.61 m^3_N/kg_{VS} on day 4, dropping again to 0.75 m^3_N/kg_{VS} on day 6. Further SBY decreased slightly until the increase of OLR to 1 kg/(m^3*d). Thereafter, the SBY showed a linear increase (R^2 = 0.998) until day 37. The substrate feeding period of once a day caused no problems during the first 38 days. However, after the increase of the OLR to 2 kg/(m^3*d) the SBY showed peaks with a very high amplitude of about 2 L_N/h between the daily maximum and minimum values (Figure 2B). In order to avoid strong fluctuations in biogas

production, automatically controlled pumping of both substrates was put into operation. The result shows an improvement of the biogas production curve. After increasing the OLR to 3 kg/(m^3*d), the pH dropped from 7.9 to 7.2 (data not shown). Substrate feeding was discontinued in order to achieve stabilization of the process. Thus, the SBY, which represents biogas production relative to the volatile solids added in substrates, was zero during this time (Figure 2A). SBY dropped very quickly and reached zero within four days. Despite the interrupted feeding, over-acidification proceeded with the result that the digestate contained 5.2 g/L of acetate at the end of the experiment.

The methane concentration was lower than 60% during the phase with an OLR of 0.5 kg/(m^3*d) and stabilized during OLRs of 1 and 2 kg/(m^3*d) at a methane content in biogas of 67.2 ± 2%. After the switch to an OLR of 3 kg/(m^3*d), the methane content decreased to 52.3% by the last feeding day (day 66).

The experience obtained in the preliminary experiment was applied to continuous fermentation using digestate from a WWTP as an inoculum and the CYB-WFF (1:20) substrate mixture. In this experiment, the continuous feeding strategy was used from the very beginning, the hydraulic retention time was extended from 60 to 100 days and the maximum OLR was set to 2 kg/(m^3*d) using smaller steps of 0.5 kg/(m^3*d).

Biogas production showed some fluctuations during the first two OLR periods (Figure 3A). As in the preliminary experiment, the SBY reached its maximum of 1.63 m^3_N/kg_{VS} on day 4, with a strongly declining tendency thereafter. There are drops in biogas production on the days when the OLR was changed due to the manipulation of the feeding pumps during their re-calibration. The drop in SBY on days 35 and 36 was due to a defective drum gas meter. In general, the biogas production curve flattened with advancing fermentation time. Any noticeable fluctuations occurred during an OLR of 2 kg/(m^3*d) and the SBY reached 1.32 ± 0.02 m^3_N/g_{VS} during this period. The methane concentration was stable at 67.0 ± 4.1% during the whole fermentation time. As regards VFAs, only acetate was detected in the digestate throughout the entire fermentation time and the acetate concentration did not exceed 30 mg/L (Figure 3A).

The nitrogen addition, pH value, TOC/TN ratio, and ammonium-nitrogen concentration in the course of the experiment are shown in Figure 3B. The pH value of 6.85 ± 0.12 was almost stable during the

fermentation showing some small fluctuations. The TOC/TN ratio increased from an initial value of 2.92 to 4.06 on the day when nitrogen supplementation started (day 32) and stagnated at a value of 3.74±0.12 thereafter.

The ammonium-nitrogen concentration dropped from almost 1 g/L to 0.6 g/L during the first 32 days. Thereafter, urea was added to the substrate mixture in order to prevent nitrogen limitation. As a result of this measure, the ammonium-nitrogen concentration stabilized and was 0.8 g/L at the end of the fermentation.

The TS content was stable at a value of 2.37 ± 0.15% of fresh mass during the whole experiment. The VS content was 65.6 ± 6.59% TS during the fermentation, with a slightly increasing tendency after the change to an OLR of 1.5 kg/(m^3*d).

There was one foaming event during the fermentation in the night between days 15 and 16, when the digestate foamed to the top of the digester. No countermeasures were necessary because of the rapid fall of the foam.

3.3 Continuous fermentation using CYB

The aim of this experiment was to test the possibility of using the substrate CYB in continuous fermentation with automated feeding. The SBY curve is shown in Figure 4A. During the first period with an OLR of 0.5 kg/(m^3*d), the biogas yield fluctuated initially and stabilized subsequently at values of about 0.70 m^3_N/kg_{VS}. After the increase of the OLR to 1 kg/(m^3*d), the SBY initially dropped to 0.42 m^3_N/kg_{VS} due to the recalibration of the substrate pump. The SBY stabilized at a value of 0.73 ± 0.04 m^3_N/kg_{VS} thereafter until the time of starting the addition of urea for nitrogen supplementation on day 32. Nitrogen addition was necessary due to the high C/N ratio of the substrate used (TOC/TN = 70.1). The mono-digestion of the CYB resulted in a shift in the TOC/TN ratio in the digestate from an initial value of 2.9 to 4.3 on day 32 (Figure 4B). After the urea addition, the TOC/TN ratio stayed almost constant and the biogas yield increased rapidly to values of up to 1.45 m^3_N/kg_{VS} as a maximum (days 69 and 77). The VFA concentrations reached their maximum of almost 4,139 mg/L (acetate: 3,959 mg/L, propionate: 66 mg/L, and butyrate: 116 mg/L) on day 39 and dropped rapidly thereafter. Unfortunately, the subsequent profile of the SBY curve showed certain

334 irregularities caused by clogging of hoses for the feeding pumps by coagulated yeast biomass. The
335 biomass coagulated on days 47, 58, and 77. pH and the ammonium-nitrogen concentration increased
336 after urea addition from initial values of 6.62 and 809 mg/L on day 32 to 7.43 and 1,053 mg/L on day
337 64, respectively (Figure 4A). In order to prevent ammonia inhibition that threat by a pH increase [23],
338 urea addition was decreased by one third. This led to a decrease in the ammonium-nitrogen
339 concentration to 1,020 mg/L, but also to an increase in the acetate concentration from 527 mg/L on
340 day 64 to 1,662 mg/L on day 67. The return of urea addition to 48 mg/(L*d) did not show the desired
341 effect and the volatile acids concentrations rose again until the end of fermentation (Figure 4A). The
342 end concentrations were 3,228 mg/L, 335 mg/L, and 248 mg/L in the case of acetate, propionate, and
343 butyrate, respectively. Nevertheless, the pH remained almost stable despite the high VFA
344 concentrations (Figure 4B). The methane concentration remained almost stable during the entire
345 fermentation period at 66.3±4.2%.
346 The TS content was 2.26 ± 0.26% of fresh mass and the VS content was 62.8 ± 2.34% TS during the
347 fermentation.
348 Foaming occurred on days 4 and 16 overnight, when the foam reached as far as the lid of the
349 fermenter. No countermeasures were necessary since the foam had disappeared by the morning check
350 and there were only foam traces recognizable on the sides of the fermenter.
351
352 **4 Discussion**
353 Biogas yield using pure CYB in a continuous experiment showed lower values than the fermentation
354 batch tests until urea addition (0.73 m^3_N/kg_{VS} in continuous fermentation versus 0.86 m^3_N/kg_{VS} in batch
355 tests). The nitrogen supplementation led to better usage of the CYB as a substrate. The biogas yield
356 increased by a factor of 2 and reached a maximum SBY of almost 1.45 m^3_N/kg_{VS} during phases with
357 no disturbances. This is similar as in the case of the co-digestion of CYB and WFF (1:20) where the
358 maximum SBY reached 1.42 m^3_N/kg_{VS} (not taking into account the SBY peak on day 4 that was
359 probably caused by the lag phase of the inoculum and thus sudden digestion of the WFF ratio of three
360 days at once). The SBY reached 1.32 m^3_N/kg_{VS} in the preliminary experiment during the period of
361 continuous feeding at 2 kg/(m^3*d), which is in accordance with the mean SBY of 1.32 m^3_N/kg_{VS}

362 during the same OLR period in the continuous co-digestion experiment. These data appear to be high
363 compared to the batch experiments (e.g. SBY (WFF+CYB, 1:20) = 1.06 ± 0.04 m^3_N/kg_{VS}). This is
364 probably due to the unequal degradation of the substrate, because the overall average SBY in the
365 continuous fermentation of the substrate mixture of CYB and WFF was 1.14 ± 0.25 m^3_N/kg_{VS}.
366 Furthermore, it should be noted that the described experiments ran only a part of their hydraulic
367 retention time that is not the usual approach. These attempts can be understood as some kind of proof
368 of principle; further long-term experiments are to be done in order to confirm the findings presented
369 here.
370 As regards the literature data, there are only a few publications on yeast anaerobic digestion, and these
371 mainly deal with spent yeast from breweries in co-digestion with wastewater [13-16]. Zupančič et al.
372 [13] observed a biogas yield of 0.45-0.72 m^3/kg_{VS} for brewery surplus yeast in batch fermentation
373 tests, which was lower than the SBY obtained in the batch experiment that is described in Table 2
374 (0.86 ± 0.04 m^3_N/kg_{VS}).
375 Even fewer publications can be found regarding the anaerobic digestion of waste frying fat. Labatut et
376 al. [24] described the anaerobic digestion of complex organic substrates, including vegetable oil. The
377 biogas yield curve showed similar behavior to the one in Figure 1. The authors explain that the initial
378 lag phase (of 12 days in their case) was caused by biochemical inhibition due to the accumulation of
379 long-chain fatty acids (LCFA) that are produced by the hydrolysis of neutral lipids. There are several
380 hypotheses regarding the inhibition mechanisms of LCFA in anaerobic digestion, e.g. sludge flotation
381 leading to washout, substrate, and product transport limitation from/to the cell due to the coating of the
382 cell by a layer of the LCFAs, and inhibition of methanogens (see [19] for an overview). The vegetable
383 oil used in the case of Labatut et al. [24] had a high methane potential of 0.68 m^3_{CH4}/g_{VS} when
384 compared with other measured substrates, and was somewhat lower than the SMY of WFF described
385 in Table 2 (0.71 ± 0.02 m^3_{CH4}/g_{VS}).
386 The methane content in the continuous fermentation of pure CYB was similar to the fermentation
387 batch test (66.3% in continuous fermentation versus 67% in the batch test).
388 In the case of the co-digestion experiments of the CYB+WFF substrate mixture, the SBY in batch tests
389 was lower than in continuous experiments (1.06 m^3_N/kg_{VS} in the batch test versus 1.32 m^3_N/g_{VS} in the

continuous experiment). This was probably caused by a worse distribution of the fat fraction in the batch tests that were mixed once a day. In both cases, the methane concentration was as high as 67%. The differences between the fermentation batch tests and continuous fermentation are in this case also probably related to urea addition, which has a stabilizing effect on biogas production [25].

There are contradictory statements with regard to the design of feeding in the case of an anaerobic digestion of fats in the literature [19]. According to Angelidaki & Ahring [26] the fats should be introduced gradually and fed continuously to biogas reactors to allow for the maintenance of a bacterial population capable of LCFA degradation and to prevent the accumulation of high concentrations of LFCA that have a limiting effect. In contrast, Coelho et al. [27] tested various operational conditions with a 6 h feeding time followed by a 6 h non-feeding time and a 3 h feeding time followed by a 9 h non-feeding time, with variable feeding flows for each case. The authors stated that an intermittent mode of feeding with longer feeding intermissions was more favorable for anaerobic digestion of fat containing substrates as the non-feeding periods allow the biomass to degrade the substrate adsorbed into the biomass during the feeding period. The results of the preliminary experiment presented in Figure 2 show that the shortening of the intermissions from 24 h to 1.5 h had a positive effect on biogas production from the substrate mixture of CYB+WFF.

Cavaleiro et al. [28] made an interesting observation in the case of the use of oleic acid as a substrate that a start-up strategy combining feeding phases and batch degradation phases promoted the development of microorganisms, which are capable of anaerobic digestion of LCFA. Indeed, the acclimation of the microflora was necessary in the fermentation experiments with a high ratio of WFF as a substrate, as can be seen in the lag phase of the batch tests (Figure 1). The biogas peaks on day 4 in the co-digestion experiments in CSTRs (Figures 2A and 3A) and the smaller biogas peak on day 6 in continuous fermentation of pure CYB (Figure 4A) imply that the substrate remained unused during the first days and all remaining fat was digested at once after the start of fat digestion.

The mean pH value of the digestate during the mono-digestion of CYB was higher than that of digestate from co-digestion of CYB+WFF (7.11 ± 0.27 versus 6.85 ± 0.12, respectively). This is surprising as the VFA concentration in CYB fermentation reached much higher values than in the case

of mixed substrate fermentation (the maximum VFA concentrations were 4,139 mg/L for CYB and 27 mg/L in the case of CYB+WFF).

The TOC/TN ratio in the case of the CYB+WFF fermentation was higher than in the case of sole CYB fermentation (the maximum TOC/TN was 5.1 in the case of CYB alone and 4.1 in the case of CYB+WFF fermentation). As described in Moeller et al. [29], the C/N ratio of digestate during the fermentation of C-rich substrates has an effect on the formation of foam. The authors showed that the application of certain nitrogen fertilizers can prevent foam formation in anaerobic digestion plants that utilize sugar beet as a substrate. The decrease of the C/N ratio in the substrate mixture has apparently a stabilizing effect on the biogas process with regard to foaming. Two foaming periods were observed during the fermentation of CYB. The first foaming that occurred during the anaerobic digestion of CYB+WFF can be regarded as typical for the start-up stage of anaerobic digestion, as has been described by anaerobic digestion plant operators [30]. The second foaming event occurred during the changeover of the OLR. However, the serious long-term foaming that is typical for substrates with high C/N ratios did not occur. It is possible that the digestate did not foam steadily because urea use caused the required stabilization of the digestate. Despite the absence of foaming, the mono-digestion of CYB was not stable enough to further increase the OLR.

In summary, the anaerobic digestion process with the CYB was not easy to perform due to its tendency for over-acidification to occur and due to the coagulation of the CYB in the feeding device. In contrast, the co-digestion of CYB with WFF ran in a stable manner up to an OLR of 2 kg/(m^3*d). Zupančič et al. [13] advise against the use of yeast as a mono-substrate in anaerobic digestion due to the ammonia inhibition that is caused by the high TN contents of 11-13 g/L. However, in the case of CYB fermentation the situation seems to be opposite, as the TN concentration in the yeast was only 1.92 g/L meaning that not only C/N ratios that are too low as in the case of Zupančič et al. [13] but also high C/N of 70.1 can cause problems during mono-fermentation. Neira & Jeison [15] investigated the co-digestion of surplus yeast and wastewater from a brewery. They described the anaerobic digestion process as feasible with no negative effects during 70 days of UASB reactor operation. Zupančič et al. [14] also observed no adverse effects up to 1.1% (v/v) of yeast and wastewater.

However, at concentrations over 2.8% (v/v) process failures were detected, such as biomass washout with the consequent diminished operating capacity.

Further research is needed in order to test the long-time stability of the co-digestion of CYB-WFF substrate mixtures. In addition, the stabilization of the yeast biomass with regard to coagulation is necessary in order to make the process more feasible for use in practice.

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Conflict of interest statement

The authors have declared no conflict of interest.

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584 **Tables**

585 **Table 1** Parameters and analytical methods for the evaluation of CYB and digestate samples

Parameter	Sample pre-treatment	Analytical methods
TS	none	DIN 12880
VS	none	DIN 12879
Protein concentration	filtered	Bradford reaction with Coomassie® Brilliant Blue R-250 (AppliChem GmbH, Germany) , photometric measurement with UV-1601 (Shimadzu, Japan) at 595 nm
Fat concentration	drying, milling	Determination by use of Soxhlet extraction apparatus (Soxtherm®/Multistat, C. Gerhardt GmbH & Co. KG, Gemany) by use of petroleum ether 30/40 as a solvent
NH_4-N concentration	filtered	DIN 38406 E5, Spektroquant® test kit (measuring range 0.01-3 mg L^{-1} NH_4-N, Merck, Germany), photometric measurement with MultiLab P5 (WTW, Weilheim, Germany)
Acetate, propionate, butyrate concentrations	filtered	High performance liquid chromatography (Shimadzu, Japan); detector: RID-10A; column: VA 300/7.8 Nucleogel Ion 300 OA; eluent: 0.01 N H_2SO_4

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Table 2 Results of the fermentation batch tests

CYB = concentrated yeast biomass, SBY = specific biogas yield, SMY = specific methane yield, WFF = waste frying fat

Substrate	SBY [m^3_N/kg_{VS}]	SMY [m^3_N/kg_{VS}]	Methane [%]
CYB	0.862 ± 0.04	0.573 ± 0.04	67 ± 1
WFF	1.08 ± 0.05	0.705 ± 0.02	67 ± 3
CYB + WFF (1:20)	1.06 ± 0.04	0.750 ± 0.00	71 ± 2
CYB + WFF (1:1)	0.85 ± 0.00	0.587 ± 0.01	69 ± 1

Figure legends

Figure 1: Specific biogas yields using substrates and substrate mixtures at two different ratios

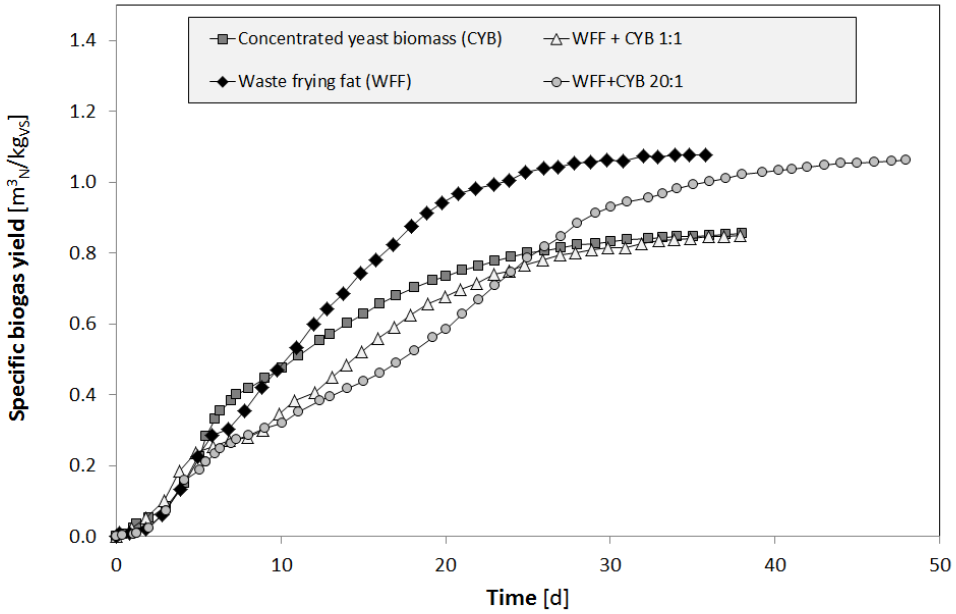
Figure 2: (A) Specific biogas yield, expected specific biogas yield according to the batch test results (Table 2), and volatile fatty acids concentrations (acetate, propionate and butyrate) as well as (B) hourly produced biogas during the preliminary experiment using the CYB-WFF substrate mixture (1:20).

Figure 3: (A) Specific biogas yield, expected specific biogas yield according to the batch test results (Table 2), and acetate concentration, and (B) nitrogen addition, pH value, TOC/TN ratio and NH_4-N concentration in the course of the co-digestion of CYB and WFF at a ratio of 1:20.

Figure 4: (A) Specific biogas yield, expected specific biogas yield according to the batch test results (Table 2), and volatile fatty acids (acetate, propionate and butyrate) concentrations, and (B) nitrogen addition, pH value, TOC/TN ratio and NH_4-N concentration during the mono-digestion of the concentrated yeast biomass.

(N added is the amount of the nitrogen added to the fermenter along with the substrate mixture per day related to the fermenter volume)

644 Figure 1: Specific biogas yields using substrates and substrate mixtures at two different ratios



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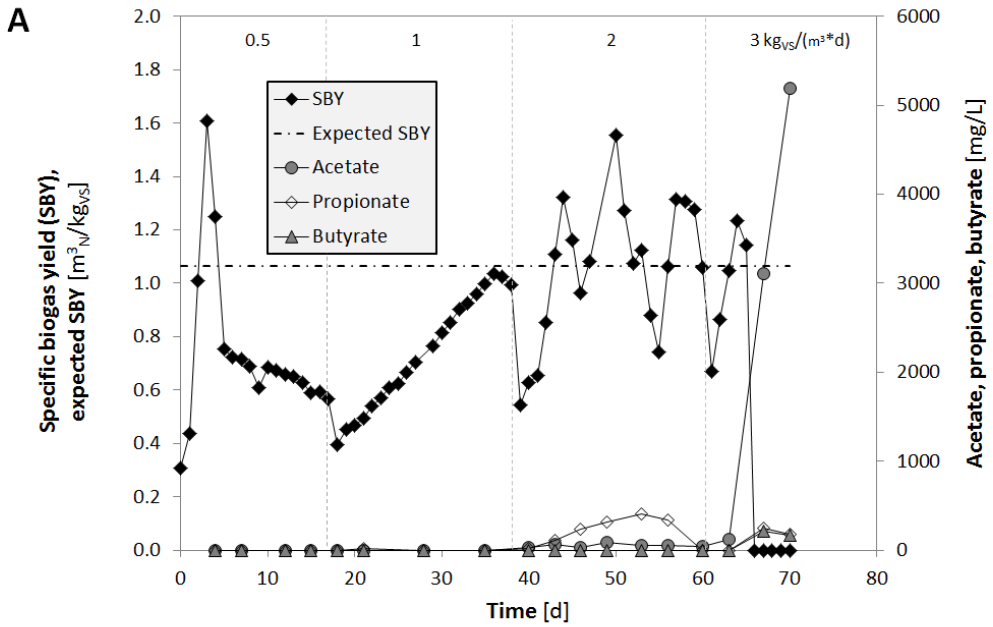

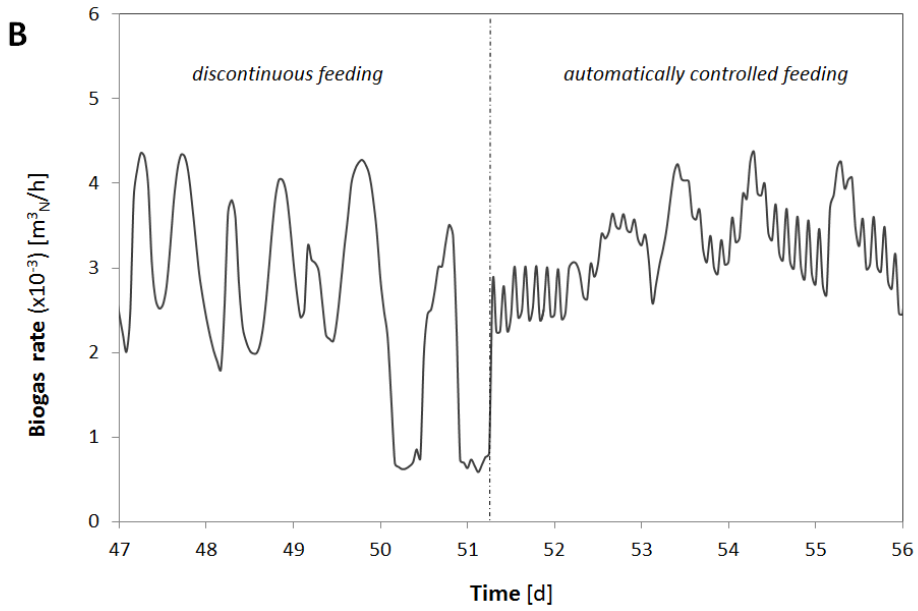

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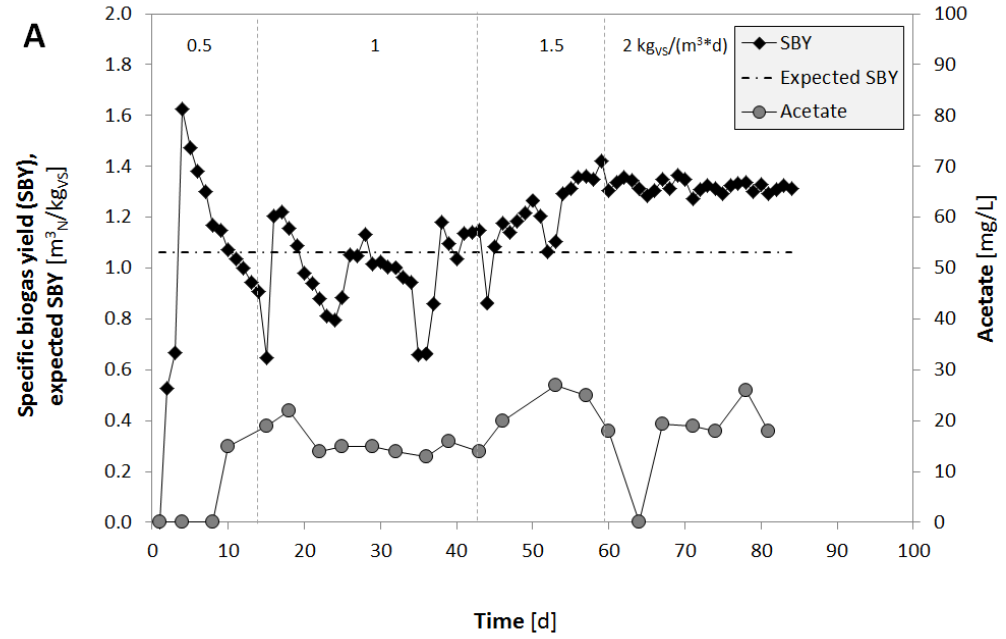

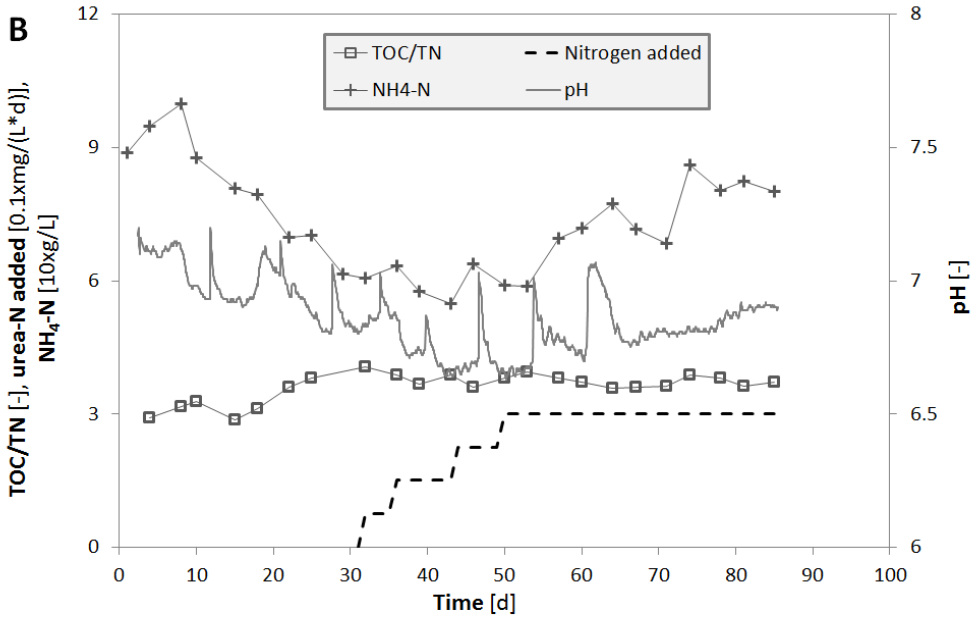

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