

1 Yeast single cell protein production from a 2 biogas co-digestion substrate

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12

13 **Abstract**

14 Biogas plants serve as hubs for the collection and utilization of highly nutritious waste
15 streams from households and agriculture. However, their outputs (biogas and
16 digestate) are of relatively low economic value. Here, we explore the co-production of
17 yeast single cell protein, a potentially valuable feed ingredient for aquaculture and
18 other animal producing industries, with biogas on substrate collected at a co-
19 digestion biogas plant, using three yeast species well suited for this purpose
20 (*Wickerhamomyces anomalus*, *Pichia kudriavzevii*, and *Blastobotrys adenivorans*).
21 All yeasts grew rapidly on the substrate, yielding 7.0–14.8 g l⁻¹ biomass after 12–15
22 h. The biomass crude protein contents were 22.6–32.7 %, with relatively favorable
23 amino acid compositions mostly deficient in methionine and cysteine. Downstream
24 biomethanation potential was significantly different between yeast species, with the
25 highest product yielding species (*Blastobotrys adenivorans*) also yielding the
26 highest biomethanation potential.

27

28 **Highlights**

- 29 • All yeasts grew well on the biogas substrate, with high growth rates.
- 30 • Produced biomass was of high nutritional value for use in fish feed
31 formulations.
- 32 • Downstream effects on methane potential were strain-dependent.
- 33 • Yeast biomass may be a viable biogas co-product.

34

35 **List of abbreviations**

36 μ_{\max} Maximum growth rate

37	AA	Amino acid(s)
38	AD	Anaerobic digestion
39	BMP	Biomethanation potential
40	CL	Crude lipid
41	CP	Crude protein
42	EAA	Essential amino acid(s)
43	FM	Fish meal
44	NS	Native substrate
45	OFMSW	Organic fraction of municipal solid waste
46	SCP	Single cell protein
47	TS	Total solids
48	VS	Volatile solids

49

50 **1 Introduction**

51 The organic fraction of municipal solid waste (OFMSW) is a major waste stream
52 which contains ample amounts of energy and nutrients available for microbial
53 conversion into higher value products. In the EU, policy changes such as the EC
54 Council Directive 1999/31/EC of 26 April 1999, by which member states are obligated
55 to reduce the amount of OFMSW being deposited on landfills, have increased
56 demands for alternative waste processing facilities such as anaerobic digestion (AD)
57 plants. AD plants convert waste streams into biogas and a post-digestion residue
58 mainly being used as fertilizer. OFMSW is typically co-digested with agricultural
59 residues such as manure, a practice which improves biogas yields as well as
60 reduces emissions of methane and odours from farms (Holm-Nielsen et al., 2009).

61 Moreover, biogas production by AD is in line with other policies aimed at improving
62 energy security and usage of renewable energy. In concert with the implementation
63 of several such policies, the installed capacity of AD in Europe has seen a steep
64 increase since the turn of the century (Scarlat et al., 2018), and future demand may
65 rise further if new uses such as biomethane-based power grid balancing, which
66 offsets energy fluctuations due to large parts of solar and wind in the energy mix, see
67 widespread deployment (Peters et al., 2018).

68
69 The widespread adoption of OFMSW as a substrate for AD has at least two main
70 implications that warrant considering AD plants as well suited for expanding their
71 product portfolios: first, the year-round and constant availability of readily
72 biodegradable substrate of relatively stable composition (Hartmann and Ahring,
73 2006), and, second, the presence of well-developed logistic networks for the
74 collection and transportation of substrate. Furthermore, AD substrate is typically
75 pretreated in several ways including removal of certain contaminants, size reduction,
76 and hygienization; unit operations which render the material more amenable to
77 microbial conversion (Carlsson et al., 2012).

78
79 Although biogas and digestate represent outputs that provide value, both are low-
80 value commodities. There are several recently proposed methods for improving the
81 financial proposition of biogas plants by altering the output streams towards more
82 valuable products (see e.g., Kleerebezem et al. (2015), Monlau et al. (2015)). One
83 possible value-added co-product of a biorefinery is yeast biomass intended as animal
84 feed. A particularly promising consumer of microbial protein feed sources is the

85 aquaculture industry. While outputs from marine fisheries are probably declining
86 (FAO, 2018), aquaculture today supplies 53 % of direct global fish consumption
87 (FAO, 2018), and as the fastest growing food producing sector over the last 40 years
88 it will play a crucial role in feeding the growing world population (Béné et al., 2015). A
89 major ingredient in aquaculture feed, especially for carnivorous fish species is fish
90 meal (FM), which is derived from wild-caught fish. With an ever-widening gap
91 between production of wild and farmed fish, availability of FM is becoming a major
92 limiting factor for further expansion of fish farming (Tacon and Metian, 2009).
93 Although feed ingredients of vegetable origin are used for replacing FM in feed
94 formulations, higher inclusion levels may negatively affect fish growth due to
95 antinutritional factors present in many vegetable feed sources (Francis et al., 2001).
96 Moreover, vegetable feed ingredients, as well as FM, may directly be at odds with
97 human food interests (Tacon and Metian, 2009).
98
99 Microbial biomass used for feed is commonly referred to as single cell protein (SCP),
100 and its use in animal feed is well established for both fish and other animals
101 (Goldberg, 1985). Targeting fish feed is especially beneficial as fish are able to
102 metabolize the nucleic acids that are present in large amounts in yeast biomass,
103 whereas high contents of nucleic acids in the diet can cause adverse health effects in
104 some other animals (Rumsey et al., 1992). Yeast biomass has been successfully
105 used in fish feed formulations, both at low inclusion levels for its appetite stimulating,
106 nutritional, or immunostimulatory properties, and at higher levels as FM replacement
107 where it is generally well tolerated up to a certain level (reviewed in Delgado and
108 Reyes (2018)). SCP production is desirable from a food security perspective, and the

109 demand for fish feed ingredients, including FM replacements, are likely to increase
110 due to the concomitant expansion of aquaculture and decrease in marine fish stocks.

111
112 Organisms suitable for SCP production on OFMSW should, first and foremost, be
113 able to utilize a large array of substrate molecules. Other desirable characteristics
114 include phytase production, as this may improve nutritional quality of the feed if
115 ingredients of vegetable origin are included in the formulation (Cao et al., 2007), as
116 well as the ability to outcompete other organisms due to the non-sterile nature of the
117 substrate. In this study, we evaluated three yeast species with suitable properties for
118 SCP production on typical co-digestion AD substrate: *Wickerhamomyces anomalus*,
119 a metabolically versatile species which has shown robustness to difficult growth
120 conditions, has been evaluated in fish feeding trials and which is known for its
121 biocontrol properties as well as being a phytase producer (Huyben et al., 2017;
122 Olstorpe et al., 2009; Passoth et al., 2006; Passoth et al., 2010; Schnürer and
123 Jonsson, 2010); *Pichia kudriavzevii*, another robust yeast known for its ability to grow
124 in the presence of inhibitory substances, and which has been reported to produce
125 extracellular phytase (Hellström et al., 2015; Olstorpe et al., 2009; Radecka et al.,
126 2015), and *Blastobotrys adenivorans*, known for its ability to utilize a large variety of
127 carbon and nitrogen sources, as well as for its high production of intra- and
128 extracellular phytase (Middelhoven et al., 1991; Olstorpe et al., 2009; Sano et al.,
129 1999).

130
131 The aim of this study was to investigate whether production of yeast biomass in
132 combination with AD could be a feasible option for further diversification of production

133 outputs from biogas plants. We have evaluated growth performance of yeasts on
134 biogas substrate obtained from a Swedish co-digestion biogas plant, characteristics
135 of the resulting biomass, and the effects of yeast cultivation on downstream chemical
136 composition and biomethanation potential (BMP).

137

138 **2 Materials and methods**

139 **2.1 Inoculum preparation and culture media**

140 Yeast strains (*W. anomalus* CBS 100487, *P. kudriavzevii* CBS 2062, and *B.*
141 *adenivorans* CBS 7377), stored in 50% glycerol stocks at -80°C , were inoculated
142 onto YPD agar (10 g l⁻¹ yeast extract (BD, Le Pont-de-Claix, France), 20 g l⁻¹
143 bacterial peptone (BD, Le Pont-de-Claix, France), 20 g l⁻¹ D-glucose (Merck,
144 Darmstadt, Germany), and 20 g l⁻¹ agar (BD, Le Pont-de-Claix, France)). Inoculum
145 cultures were prepared using the same medium, without agar, in 125-ml baffled
146 Erlenmeyer flasks (Thomson Ultra-Yield, Thomson Instrument Co., Carlsbad, CA,
147 USA), and cultivated on a rotary shaker for 24 h. Cells were harvested at 3000 × *g* for
148 5 min and washed with saline (NaCl, 9 g l⁻¹) using the same settings.

149

150 **2.2 Substrate preparation**

151 The biogas substrate was obtained directly from the inlet to the digester at a biogas
152 plant in Sweden, and consisted mainly of source-separated household waste,
153 organic waste from municipal kitchens, and liquid agricultural waste (swine and cattle
154 manure). Metals and plastics had been mechanically removed at the biogas plant,

155 and the substrate had been hygienized at 70°C for >1 h. This substrate will be
156 referred to as *native substrate (NS)*.

157

158 To be able to separate yeast biomass after culturing, and to reduce the risk of
159 contamination as substrate was collected through a non-sterile sampling port at the
160 biogas plant, the substrate was sterile-filtered. This was accomplished using an
161 Asahi Rexeed-25A hemodialyzer (Asahi Kasei Medical Co., Ltd., Tokyo, Japan)
162 connected to a peristaltic pump, with the filter replaced when the counterpressure
163 reached 0.6 bar. The filtered substrate was then sterile-filtered through a 0.2 µm
164 sodium acetate filter (Nalgene Rapid-Flow, Thermo Fisher Scientific, Waltham, MA,
165 USA) using a Büchner funnel.

166

167 **2.3 Bioreactor operation**

168 500-ml Infors HT Multifors CSTR bioreactors (Infors AG, Bottmingen, Switzerland)
169 were used for the cultivations. For each reactor, 400 ml of the sterile-filtered
170 substrate was inoculated at an initial OD₆₀₀ of 1.0. Reactor parameters were pH =
171 7.00 ± 0.10, stirrer = 500 rpm, and pO₂ = 0.2. pO₂ was maintained using stirrer
172 speed, with a minimum of 200 rpm and a maximum of 1200 rpm. pH was
173 automatically adjusted on-line using 5 M NaOH and 3 M H₃PO₄.

174

175 Fermenter temperature was set to 30°C for all cultivations, except for the evaluation
176 of *B. adenivorans* CBS 7377 growth performance. This strain exhibited better
177 growth at 37°C during initial growth assessment (results not shown), so growth was
178 evaluated at this temperature.

179

180 It was not possible to maintain a pO_2 value of 0.2 during the exponential growth
181 phase, due to the high oxygen consumption of the yeast. Cultivations were
182 terminated after the log phase was completed, as indicated by pO_2 readings.

183

184 To monitor the fermentations, viable cell counts were performed. Due to the
185 complexity and optical activity of the medium, plating was chosen instead of OD
186 measurements. Relevant dilutions, made with 1 g l^{-1} peptone water, were plated onto
187 YMC agar (3 g l^{-1} yeast extract, 3 g l^{-1} malt extract (BD, Le Pont-de-Claix, France), 5
188 g l^{-1} bacterial peptone, 10 g l^{-1} D-glucose, 100 mg l^{-1} chloramphenicol (Sigma-
189 Aldrich, Steinheim, Germany)). Plates were incubated at 30°C and colony forming
190 units (CFU) were counted when colonies were clearly visible.

191

192 After fermentation, bioreactor contents were centrifuged at $3000 \times g$ for 10 min. The
193 pellets, containing yeast biomass, were washed with deionized water using the same
194 centrifuge settings, and stored at -20°C .

195

196 **2.4 Biomethanation potential assay**

197 To assess downstream effects of yeast cultivation on biogas performance, spent
198 medium was collected from cultivations of each yeast. Cultivations were performed
199 largely as described in Section 2.3. In order to minimize confounding factors, all
200 cultivations were terminated at the same time (i.e., the time was determined by the
201 growth performance of the slowest growing yeast), and were run at the same
202 temperature (30°C). This was needed to ensure that evaporative losses of volatile

203 energy carriers, such as short-chain carboxylic acids, were similar between the
204 treatments. At the end of cultivation, yeast biomass was collected as described in
205 Section 2.3, and the supernatants, referred to as *spent media*, collected.

206
207 The BMP assay was conducted largely according to Angelidaki et al. (2009). In brief,
208 total solids (TS) and volatile solids (VS) of NS and spent medium (supernatants, post
209 yeast-treatment) were determined by drying the substrates at 105°C and incinerating
210 at 550°C in aluminum containers, noting the weights after each step. TS was
211 calculated as the quotient of dry matter divided by initial weight. VS was determined
212 as the difference between TS and ash content.

213
214 The assay was performed using untreated NS and fresh inoculum (collected from the
215 same biogas plant and degassed for 3 days at 37°C), contributing approximately 1.2
216 g VS and 3.6 g VS, respectively. The substrate control treatment contained only NS
217 and inoculum. Spent medium was added in the remaining treatments, so that the
218 mixtures contained, by weight, NS:spent medium in ratios of 10:1, 10:3, 2:1, and 1:1,
219 which corresponded to 9–50% spent medium in the final AD slurry. Due to the low
220 VS content of the spent medium, increases in VS due to supernatant additions were
221 modest, at most 20%, and it was assumed that this slight change in
222 inoculum:substrate ratio would not affect inoculum performance. Inoculum and
223 cellulose process controls were included. The inoculum control, used for
224 determination of background methane production, consisted of inoculum contributing
225 3.6 g VS. The cellulose control, needed for evaluating the function of the inoculum,
226 contained 3 g cellulose (medium fibers; Sigma-Aldrich, Steinheim, Germany) and 400

227 ml of inoculum. Tap water was added to each bottle to a final volume of 400 ml, and
228 each treatment was evaluated in triplicate.

229
230 To measure methane production, AMPTS II Automatic Methane Potential Test
231 Systems (BioProcess Control AB, Lund, Sweden) were used, detailed in Badshah et
232 al. (2012). Briefly, gas volume measuring was performed using a piston system, and
233 gas was upgraded by flushing it through 7 M NaOH. Samples were agitated using
234 stirrers attached directly to the bottles. The assay was performed at 37°C, as this
235 was the temperature used in the commercial digester from which the inoculum was
236 derived. Bottles were flushed with N₂ gas prior to initiating the experiment. Specific
237 methane potential was obtained by dividing the volume of methane produced by the
238 actual amount of VS in each sample. When less than one reading per day was
239 generated from all samples (i.e. the piston did not register any methane gas
240 emissions for 24 h), the assay was determined to be complete. Each AMPTS II
241 system contained an identical set of samples so that the response of each system
242 could be included in the statistical model (Section 2.6).

243

244 **2.5 Chemical analyses**

245 Chemical analyses were purchased from external labs. Crude protein (CP) content
246 was determined using the total nitrogen Kjeldahl method, and CP was calculated as
247 N × 6.25 (Nordic Committee on Food Analysis, 2003). Crude lipid (CL) content was
248 determined according to (The Commission of the European Communities, 1998).
249 Gross nutritional analyses were performed at the VHC lab (SLU, Uppsala, Sweden).
250 Amino acid (AA) analyses were performed according to the ISO 13903:2005 method

251 (Eurofins Food & Agro, Jönköping, Sweden). Micronutrient compositions of spent
252 medium (supernatants, yeast biomass removed), sterile-filtered medium, and NS
253 were analyzed using ICP-MS (Agrilab AB, Uppsala, Sweden). Dry matter was
254 determined by drying the samples at 105°C until constant weight was achieved.

255
256 When possible, analyses were performed in replicates. For the BMP assay, each
257 yeast treatment was carried out in a single bioreactor to ensure equal freshness and
258 composition of the spent medium, and the replicates represent aliquots of the
259 supernatants. For pellet (yeast biomass) characterization, four replicate
260 fermentations were carried out. Spent medium compositional analyses were
261 performed using material pooled from all bioreactors.

262

263 **2.6 Calculations and statistical analyses**

264 The maximum growth rate (μ_{\max}) was estimated by taking the greatest slope of the
265 growth curve

$$266 \quad y(t) = \frac{\ln N(t)}{\ln N(0)}$$

267 where $N(t)$ is the number of CFU at timepoint t . Final biomass productivity was
268 calculated by dividing final biomass concentration by the total cultivation time.

269 Maximum growth rates and final productivities were calculated independently for
270 each fermenter.

271

272 AA scores for the yeast biomass, reflecting the requirements of essential amino acids
273 (EAA), were calculated based on finfish requirements as reported in Tacon et al.
274 (2009).

275

276 To evaluate the downstream effects of spent substrate addition on biomethanation
277 potential, a linear model was fitted according to the formula

$$278 \quad y_{ijk} = a_i + b_j + c_k + (bc)_{jk} + \varepsilon_{ijk}$$

279 where y_{ijk} is the biomethanation potential of the sample, a is the effect of each
280 AMPTS II system, b is the effect of strain, c is the effect of spent medium at level k in
281 the reactor, (bc) is the interaction term for dose \times strain, and ε is the residual. The
282 model was fitted using the built-in function `lm()` in R version 3.6.0 (R Core Team,
283 2019). All graphs were generated using `ggplot2` version 3.1.1 (Wickham, 2009).

284

285 **3 Results**

286 **3.1 Yeast cultivation and biomass characterization**

287 Native biogas substrate (NS) was sterile-filtered and the three yeast strains were
288 cultured on this substrate in the fermenters. Fermentations were terminated after 12–
289 15 hours, yielding approximately 2 \log_{10} increases for strains *P. kudriavzevii* CBS
290 2062 and *B. adenivorans* CBS 7377, and approximately a 1.5 \log_{10} increase for *W.*
291 *anomalus* CBS 100487 (Figure 1), with high maximum growth rates (μ_{\max}) of 0.48–
292 0.76 h^{-1} (Table 1).

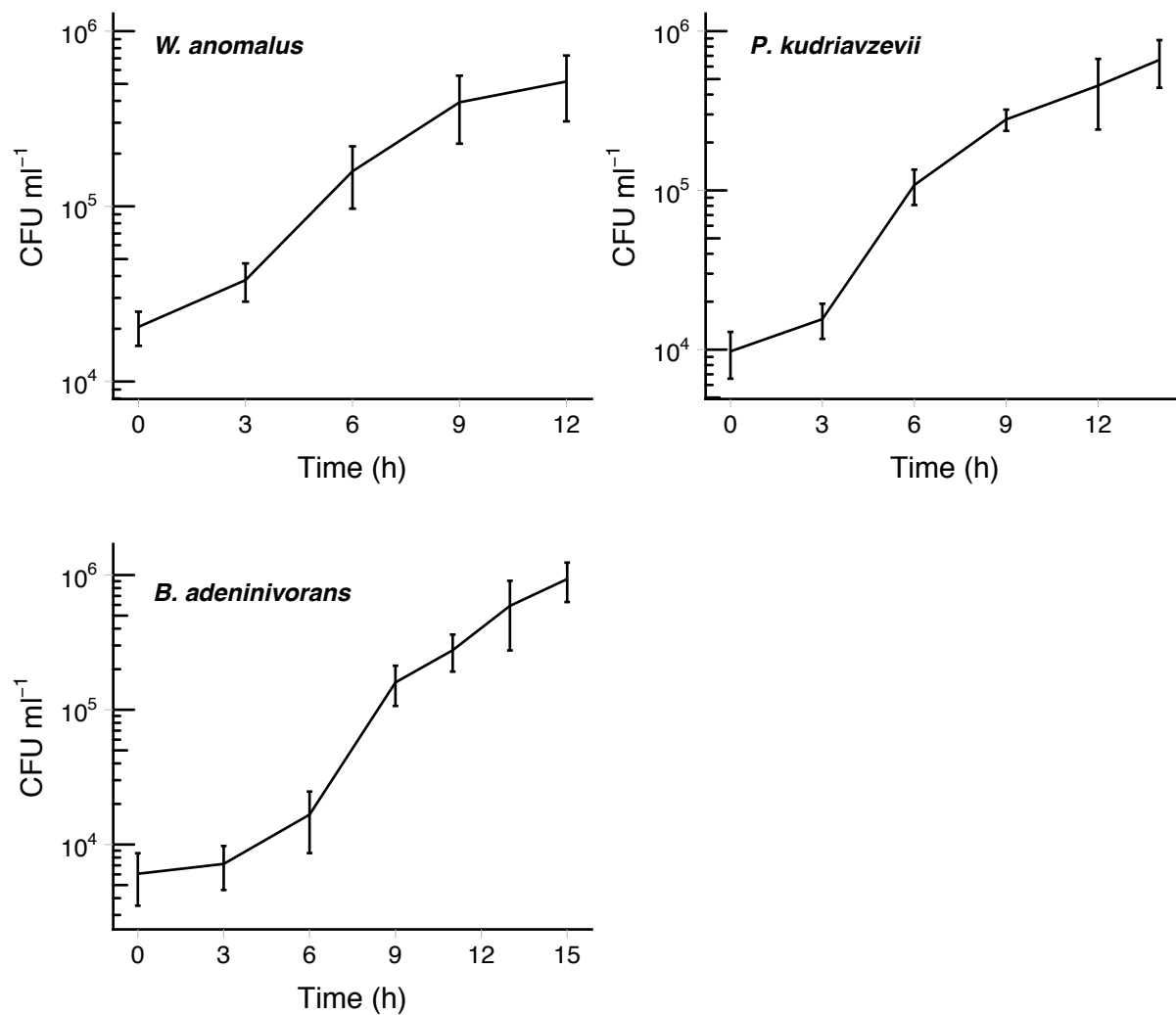
293

294 Final biomass concentrations ranged from 7.0 g l^{-1} (22.6% CP) for *W. anomalus* to
295 14.8 g l^{-1} (30.5% CP) for *B. adenivorans*. Final biomass productivities ranged from
296 0.53 $\text{g l}^{-1} \text{h}^{-1}$ for *P. kudriavzevii* to 0.99 $\text{g l}^{-1} \text{h}^{-1}$ for *B. adenivorans* (see Table 1 for
297 the kinetic parameters and gross nutritional characterization of the yeast biomass).

298

299 Protein content according to the AA analysis was largely in agreement with CP
300 values, indicating that most nitrogen present in the biomass originated from protein
301 (Table 2). Yeast biomass AA composition was similar for all three species, with *W.*
302 *anomalus* notably having a somewhat lower content of methionine and cysteine,
303 sulfur-containing EAA, compared to the other two yeasts. In general, yeast biomass
304 was deficient in arginine and in the sulfur-containing AA relative to the requirements
305 of finfish, whereas other AA were either in excess or at similar levels (Table 2). EAA
306 made up approximately half of the AA present in yeast biomass.

307



308

309 **Figure 1.** Growth curves for the yeast cultivation trials. Bars represent standard

310 deviation. *W. anomalus* and *P. kudriavzevii* were grown at 30°C, and *B.*

311 *adenivorans* at 37°C.

312 **Table 1.** Nutritional characterization of washed yeast biomass, on a dry matter basis.

313 Missing standard deviations indicate that measurements were performed on pooled

314 replicates

Parameter	<i>W. anomalous</i>		<i>P. kudriavzevii</i>		<i>B. adenivorans</i>	
	Value	SD	Value	SD	Value	SD
<i>N</i>	3 ^a		4		3 ^a	
<i>Biomass concentration (g/l)</i>	7.03	0.08	7.36	0.34	14.83	1.02
μ_{max} (h^{-1})	0.48	0.10	0.64	0.06	0.76	0.06
<i>Final biomass productivity (g/l·h)</i>	0.59	0.01	0.53	0.02	0.99	0.07
<i>Crude energy (MJ/kg)</i>	11.05	–	11.91	–	13.05	–
<i>Crude protein (%)</i>	22.59	0.35	32.71	0.54	30.46	1.22
<i>Ash (%)</i>	45.77	0.42	42.88	0.85	37.58	2.92
<i>Crude lipid (%)</i>	2.68	–	1.76	–	1.56	–
<i>Ca (g/kg)</i>	145.6	–	123.6	–	115.0	7.23
<i>K (g/kg)</i>	7.4	–	11.1	–	16.4	2.07
<i>P (g/kg)</i>	6.6	–	11.1	–	6.9	2.57
<i>Mg (g/kg)</i>	4.7	–	3.7	–	11.2	0.80
<i>Na (g/kg)</i>	83.3	–	85.3	–	76.7	0.93
<i>S (g/kg)</i>	3.0	–	3.6	–	4.3	0.40

315 ^a One sample was excluded due to equipment malfunction.

316 **Table 2.** Amino acid composition of dried yeast biomass. Tryptophan contents were not
 317 analyzed

	<i>W. anomalus</i>			<i>P. kudriavzevii</i>			<i>B. adenivorans</i>		
	Content (g/100 g)	Content (weight %)	AA score ^a	Content (g/100 g)	Content (weight %)	AA score	Content (g/100 g)	Content (weight %)	AA score
<i>Alanine</i>	1.55	8.8		1.71	6.4		1.93	7.7	
<i>Arginine</i>	0.87	5.0	85	1.35	5.0	82	1.36	5.4	95
<i>Aspartic acid</i>	1.88	10.7		3.32	12.4		2.42	9.7	
<i>Cysteine</i>	0.157	0.9	67	0.274	1.0	70	0.275	1.1	81
<i>Glutamic acid</i>	2.63	15.0		3.73	13.9		4.08	16.4	
<i>Glycine</i>	0.84	4.8		1.30	4.9		1.28	5.1	
<i>Histidine</i>	0.351	2.0	83	0.594	2.2	88	0.572	2.3	96
<i>Hydroxyproline</i>	<0.05	—		<0.05	—		<0.05	—	
<i>Isoleucine</i>	0.87	4.9	131	1.42	5.3	133	1.1	4.4	120
<i>Leucine</i>	1.36	7.7	114	2.11	7.9	110	1.84	7.4	110
<i>Lysine</i>	1.41	8.0	95	2.22	8.3	93	1.74	7.0	84
<i>Methionine</i>	0.242	1.4	50	0.529	2.0	69	0.417	1.7	63
<i>Ornithine</i>	<0.01	—		<0.01	—		<0.01	—	
<i>Phenylalanine</i>	0.84	4.8	100	1.31	4.9	97	1.09	4.4	94
<i>Proline</i>	0.75	4.3		1.06	3.9		1.44	5.8	
<i>Serine</i>	1.06	6.1		1.49	5.6		1.44	5.8	
<i>Threonine</i>	0.99	5.6	106	1.66	6.2	110	1.39	5.6	107
<i>Tyrosine</i>	0.76	4.4	134	1.15	4.3	125	1.00	4.0	125
<i>Valine</i>	0.96	5.5	115	1.58	5.9	117	1.55	6.2	132
<i>Total</i>	17.52	100.0		26.81	100.0		24.92	100.0	
<i>Total EAA</i>	8.81	50.3		14.20	53.0		12.33	43.0	

318
 319 ^a The AA score reflects dietary requirements of essential amino acids in finfish, with
 320 values below 100 indicating deficiency. Essential amino acid requirements of finfish
 321 are according to Tacon et al. (2009).

322 **3.2 Characterization of spent medium**

323 Filtration of the substrate removed a considerable amount of nutrients, presumably
324 contained in particulate matter. Compared to NS, filtered substrate contained 5-fold
325 less VS, 2.1-fold less total N, and 4.5-fold less total C (Table 3). Compared to the
326 filtered supernatant, spent medium contained less TS, VS, C and N, and generally
327 reduced levels of micronutrients. The exceptions were Na, which increased in all
328 treatments, and P, which increased in two out of three treatments. This is most likely
329 due to the use of NaOH and H₃PO₄ for pH control. Treatment with *P. kudriavzevii*
330 yielded the lowest reduction in TS and VS.

331

332 **Table 3.** Chemical composition of substrate before (native) and after filtration, and
 333 after yeast fermentation

	Native	Filtered	Post-fermentation supernatants		
			<i>W. anomalus</i>	<i>P. kudriavzevii</i>	<i>B. adenivorans</i>
<i>TS (%)</i>	7.8	2.1	1.5	1.6	1.2
<i>VS (% of TS)</i>	86.3	67.2	49.8	63.9	47.9
<i>Total N (g kg⁻¹)</i>	2.7	1.3	0.6	0.5	0.3
<i>Organic N (g kg⁻¹)</i>	2.2	0.8	0.2	0.4	0.2
<i>NH₄⁺-N (g kg⁻¹)</i>	0.5	0.4	0.4	0.2	0.1
<i>Total C (g kg⁻¹)</i>	36.3	8.1	5.0	5.1	3.4
<i>C/N ratio</i>	13.3	6.3	8.5	9.4	11.0
<i>P (g kg⁻¹)</i>	0.37	0.20	0.15	0.74	0.39
<i>K (g kg⁻¹)</i>	1.37	1.25	1.19	1.14	0.96
<i>Mg (g kg⁻¹)</i>	0.24	0.19	0.08	0.06	0.09
<i>Ca (g kg⁻¹)</i>	1.77	1.23	0.06	0.07	0.03
<i>Na (g kg⁻¹)</i>	0.52	0.51	2.09	1.27	1.70
<i>S (g kg⁻¹)</i>	0.24	0.08	0.05	0.06	0.04

334

335 **3.3 BMP assay**

336 The BMP assay was terminated after 40 days. The methane potential of native
337 substrate with addition of spent substrate was significantly different depending on
338 strain choice ($p = 0.0009$), with no effect of the dose of spent medium added or
339 interaction of strain and dose observed (Table 4). Native substrate with spent
340 medium had BMP levels of 342.0–380.5 ml CH₄/g VS (Table 5). Native substrate had
341 a BMP of 323.0±17.0 ml CH₄/g VS. Cellulose controls yielded 392.3±31.0 ml CH₄/g,
342 indicating that the inoculum performed adequately, and inoculum controls produced
343 70.7±21.3 ml CH₄.

344

345 **Table 4.** ANOVA table for effects of strain choice and spent medium dosage on
346 downstream biomethanation potential

Factor	DF	Sum sq.	Mean sq.	F value	p-value
<i>AMPTS system</i>	2	7595.2	3797.6	10.1089	0.0005
<i>Strain</i>	2	6890.2	3445.1	9.1707	0.0009
<i>Dose</i>	1	14.3	14.3	0.0381	0.8467
<i>Strain:Dose</i>	2	2284.8	1142.4	3.0410	0.0644
<i>Residuals</i>	27	10143.0	375.7		

347

348 **Table 5.** Biomethanation potential (BMP) of native substrate with addition of spent
349 medium from the yeast cultivations according to the fitted linear model

Strain	BMP (ml CH₄/g VS)	SE
<i>W. anomalus</i>	350.7	13.0
<i>P. kudriavzevii</i>	342.0	17.5
<i>B. adenivorans</i>	380.5	17.2

350

351 **4 Discussion**

352 In this study, we have produced yeast biomass from the liquid fraction of a substrate
353 from a Swedish co-digestion biogas plant. Depending on the yeast strain, biomass
354 concentrations reached 7.0–14.8 g/l after 12–15 h. By including the spent medium
355 from yeast fermentation in the AD slurry, we were also able to assess the relative
356 effects of each yeast strain on downstream methane potential.

357
358 For yeast-based SCP to be a viable co-product at a biogas plant, the organism must
359 be able to utilize the substrate used at the plant. The species evaluated here grew
360 well on the biogas substrate, with the highest final biomass productivity being 0.99 g
361 l⁻¹ h⁻¹, with only minor efforts made to optimize cultivation conditions. Initial
362 experiments, carried out in shake flasks and in aerated 96-well deep-well plates,
363 were conducted to determine suitable fermentation parameters, but no subsequent
364 optimization was conducted. Furthermore, fermentations were performed at the same
365 pH for all strains, in order to get representative spent substrates for the BMP assay. It
366 is worth noting that none of the yeast strains were able to grow at a pH of below 6,
367 likely due to the presence of weak acids in the substrate. The substrate otherwise
368 proved non-toxic to the yeast and no dilution was necessary. This is important for a
369 biogas plant, as further addition of water would lower the organic loading rate and
370 hydraulic retention time, compromising methane production.

371
372 Crude protein contents of the yeast biomass were lower than what has been reported
373 in the literature for fodder yeast, which range from 34.3–48.2 on a dry weight basis
374 (Tacon et al., 2009), whereas the levels in this study ranged from 22.6 to 32.7.

375 However, much of the crude protein was accounted for by the amino acid analysis,
376 indicating low levels of non-protein nitrogen sources. Amino acid compositions of the
377 yeast biomass were similar to those found in the literature for other yeasts used as
378 fish feed (Tacon et al., 2009), with methionine being the most limiting amino acid.
379 Notably, lysine levels were close to the requirements of finfish. Lysine is commonly a
380 limiting amino acid, especially in feed ingredients of vegetable origin but also in many
381 species of yeast (Tacon et al., 2009). With further optimization, it is likely that the
382 protein content could be increased. Rajoka et al. (2006), in a study using *Candida*
383 *utilis*, found that true protein contents increased during the first 24 hours of
384 cultivation. Likewise, for *Saccharomyces cerevisiae* it was shown that protein
385 contents were highest after 36 h, for two out of three strains evaluated (Novak,
386 2007). In the present study, however, cultivations were terminated after 12–15 h,
387 suggesting an obvious route for optimization.

388
389 The choice of yeast strain proved to be important both for product yields as well as
390 for downstream BMP. The best-performing yeast strain, *B. adenivorans*, produced
391 approximately twice the amount of biomass compared to the other two species, with
392 a similar protein content. Accordingly, final biomass productivity was also
393 approximately double compared to the two other strains. It is possible that this is due
394 in part to the higher cultivation temperature used for this strain. Rajoka et al. (2006)
395 found that temperature had a large impact on crude protein contents of yeast
396 biomass, which they attributed to increased transport of nutrients over the cell
397 membrane. Interestingly, *B. adenivorans* also had the highest BMP when mixing its
398 spent substrate with native biogas substrate, suggesting a synergistic effect of this

399 yeast treatment on the AD process and highlighting the importance of yeast strain
400 choice for overall financial viability. It is worth mentioning that the production of yeast
401 biomass in all cases exceeded the reduction of TS in the substrate during cultivation.
402 This finding is an artifact of the way TS and VS are measured, which discounts all
403 compounds with a boiling point below 105°C, such as several organic acids
404 abundantly present in household waste.

405
406 Although the results of this initial study are promising, future scale-up studies are
407 needed need to address a number of limitations, some of which are summarized
408 below. First, cultivations should be performed on minimally filtered substrate. In this
409 study, sterile-filtered substrate was used in order to facilitate analyses, such as
410 biomass dry matter concentration and CFU counts. Despite the substrate being
411 hygienized (70°C for at least one hour), it cannot be regarded as sterile and it
412 remains to be seen whether this proves a challenge in scaling up. Second, efficient
413 product recovery will be essential for the financial outcome of the process, but was
414 not investigated in the present study. Third, the downstream effects on methane
415 production should be more rigorously characterized. Whereas the present study used
416 batch tests for assessing methane potential, a more thorough study would use a
417 continuous biogas reactor with continuous feeding of spent substrate. Further,
418 although the results suggest an increase in methane potential over the native
419 substrate, it is fair to assume that sterile filtration of the substrate acts as a
420 pretreatment by removing the more recalcitrant particulate matter. Thus, future
421 studies, using the setup described above and including the retentate in the biogas
422 reactor, could better assess the effects of yeast cultivations on the economics of the

423 integrated process, preferably by examining effects on different types of biogas
424 substrates using several inocula.

425

426 **5 Conclusions**

427 Biogas plants are in several ways well suited for production of products other than
428 methane and digestate. In this initial investigative study, we demonstrate that co-
429 production of yeast SCP on biogas co-digestion substrate is feasible, with high
430 growth rates attained during the batch cultivations. Furthermore, yeast biomass
431 amino acid profiles were similar to yeast SCP already in use in the aquaculture
432 industry. Due to the presence of logistics networks and year-round substrate
433 availability, co-production of yeast SCP and biogas may be an attractive option for
434 diversifying biogas plant outputs. Further optimization of cultivation parameters is
435 likely to improve product yield and productivity; however, scale-up experiments are
436 required to assess the financial and technological viability of this integrated process.

437

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447

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