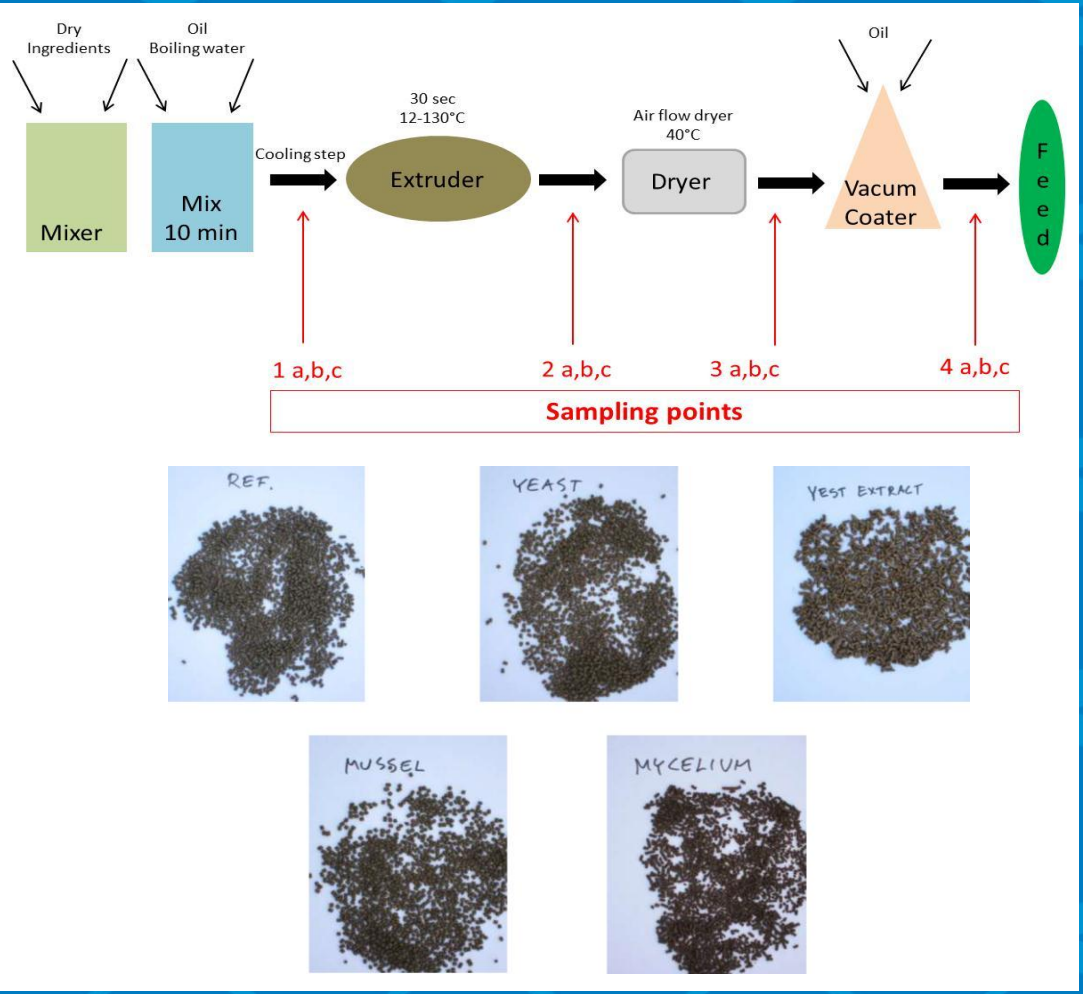


# A technical report on the production of microbial protein

Matilda Olstorpe, Aleksandar Vidakovic, David Huyben, Anders Kiessling

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# Description

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**Abstract**
A screening method was developed to elucidate the ability of different yeast strains to utilise waste products as growth substrate. Selected yeasts were grown in fermenters on waste products to generate biomass for single cell protein (SCP) for incorporation into fish feed. The viability of yeast cells during feed manufacture and passage through the fish intestinal tract was evaluated. The yeast *Saccharomyces cerevisiae* grown on molasses was used as SCP source. The yeast was viable during the whole manufacturing process of fish feed and was re-isolated and identified using rRNA sequencing. Viable *S. cerevisiae* in the feed were ingested by the fish. However, only few S. cerevisiae cells were found in faeces collected in the distal intestine. The yeast population was similar in both control (simulated commercial feed) and experimental diets, and the dominating species were *Debaryomyces fabryi, Debaromyces hansenii, Candida zeylanoides* and *S. cerevisiae*. However, the presence of yeast in the distal intestine was increased in fish fed the Baltic Blend diet compared to the control.

**Keywords**
Yeast, *Saccharomyces cerevisiae*, waste products, fermentation, rRNA identification, Intestinal yeast flora

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# Contents

# 1. Introduction

According to FAO estimates, aquaculture will double in the next 18 years (FAO 2014). Today's fish feed relies heavily on fisheries to obtain protein (fish-meal) and oil ingredients, although there is a strong drive to replace these with high-value plant resources. Different plant materials (e.g. soy) have been used as alternative protein sources, but the presence of bioactive compounds in common plant-derived protein sources such as oilseeds and legumes limits their use in aquaculture feeds (Gatlin et al., 2007), especially in diets for salmonids (Olli et al., 1994, Zhang et al., 2012). Furthermore, the growing human population limits the use of agricultural land for production of animal feed, including plant-derived feeds for aquaculture (Brown, 2012). Also, both soy and fishery products are of human-food quality, creating tensions between human food security and fish feed production. Fish meal is commonly used as a protein source in fish feeds, but a decline in wild catch from fisheries has reduced its availability (FAO, 2014). This stresses the importance of finding alternatives to fish and soya meal that are sustainable, both economically and environmentally.

Research to develop microorganisms suitable for inclusion in fish feed is ongoing, and trials on Rainbow trout, Eurasian perch and Arctic charr, among others, show promising results (Øverland et al., 2011; Olstorpe et al., 2013; Langeland 2014). Microorganisms have a protein and lipid composition of high nutritive value to higher animals. However, they also contain high levels of nucleic acids that in parity with a high meat diet can lead to gout and kidney stones in humans if eaten in larger quantities. Fish on the other hand, can naturally metabolise these organisms, at least during a part of their life cycle. Therefore, fish are genetically adapted to handle high levels of nucleic acid, and microorganisms can be utilised to produce either live feed (suitable to specific fish species and life stages) or microbial meal, which can be incorporated as a protein source in commercial feeds.

There are discussions whether fish can digest and utilise the nutrients from whole yeast cells compared to cells with disrupted walls from which nutrient have been released. Protein digestibility is shown to be lower in whole cells compared to yeast extract. However, growth rates did not differ between fish fed on whole yeast cells vs yeast extract (Langeland. 2014).

Yeasts have a long tradition of use as feed components, due to a high protein content and favourable essential amino acid profile (Oura 1983). Furthermore, yeasts such as *Rhodotorula* spp. and *Pfaffia rhodozyma* can contain carotenoids, (Whyte & Sherry 2001); *Wickerhamomyces anomalus* or *Blastobotrys adeninivorans* can degrade phytate (Olstorpe et al., 2009) with the concomitant increased availability of phosphorus; and, W. anomalus can inhibit moulds and *Enterobacteriaceae* and may thus decrease the risk of contamination of the produced feed with potential pathogens (Olstorpe & Passoth 2011; Olstorpe et al., 2012). Yeast also has the potential to produce high levels of lipids both for feed and biofuel when grown on various organic residues as substrate. Such substrates are inexpensive and can include side-streams and waste from the food chain, the wood-pulp industry, and manure from fish and poultry.

Via aquaculture, resources unfit for human consumption can be transformed via microorganisms into yeast meal, and then converted into fish, a high-value food. In addition, development of alternative protein sources reduces the reliance of aquaculture on arable land and irrigation to produce feed ingredients. Aquaculture based on new feed sources, themselves based on recaptured and recycled nutrients, can become a net contributor to human food rather than a direct threat to human food secu-

rity. This report presents results from screening and matching of yeast strain to feedstock, and evaluation of yeast cell survival during feed production and through the intestinal tract of the fish.

# 2. Material and methods

## 2.1. Yeast strains

The yeast strains used in this study (*Blastobotrys adeninivorans* J562, *Blastobotrys adeninivorans* J564, *Candida tropicalis* J279, *Cryptococcus cerealis* J595, *Holtermaniella takashimae* J596, *Issatchenkia orientalis* J550, *Kluyveromyces lactis* J469, *Kluyveromyces marxianus* J186, *Phaffia rhodozyma* J552, *Pichia angusta* J549, *Wickerhamomyces anomalus* J475, *Wickerhamomyces anomalus* J121, *Wickerhamomyces anomalus* J379, *Pichia jadinii* J556, *Pichia stipitis* J563, *Rhodotorula glutinis* J195, *Saccharomyces bayanus* J482, *Saccharomyces boulardii* J551, *Saccharomyces cerevisiae* J545, *Saccharomyces cerevisiae* J546, *Sporobolomyces roseus* J466, *Torulaspora delbrueckii* J352, *Yarrowia lipolytica* J134) were accessioned into the culture collection of the Department of Microbiology, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, held as 50% glycerol stocks at –70°C. The yeasts were selected based on prior use in fish feed trials or known characteristics desirable in feed.

## 2.2. Growth media

Waste products tested as growth substrate were: liquid waste whey (A) and whey (B) (Milko, Bollnäs, Sweden); wet wheat distillers' grain liquid from the consumption ethanol industry (Absolut Spirits, Åhus, Sweden) (C); condensate and secondary condensate from bioethanol (fuel) production from wheat (Agroetanol, Norrköping Sweden) (D and E, respectively); wet wheat distillers' grain liquid, light wet wheat distillers' grain liquid, and starch fraction from wet wheat distillers' grain liquid from the ethanol industry (Reppe, Lidköping, Sweden) (F, G and H, respectively); waste water in spent grain from dark lager, ecologic pale ale and pilsner beer production (Slottskällan, Uppsala, Sweden) (I, J and K, respectively); and, three different production lines (1, 2/3 and 3) of Thermo Mechanical Pulp (TMP) from the paper industry (Hallstaviks pappersbruk, Holmen, Hallstavik, Sweden) (L, M and N, respectively). All waste products were centrifuged at 4500 g for 5 min (Beckman J2-21, Beckmans Instruments Inc., Palo Alto, USA). The pellet was discarded and the supernatant was filter sterilised (Filtropur S, 0.45 µm, Sarstedt, Nümbrecht, Germany). Growth substrate (TMP 2/3, L) used in the 300 L continuous culture experiment was received in 1000 L tanks. The material was left to sediment for 2 hours. The substrate was then filtered through a 1 µm filter before injection into the fermenter; approximately 18 % of organic material had been removed from the substrate.

## 2.3. Preparation of yeast cells for growth experiments

Yeast inocula were prepared (unless otherwise stated) from cultures grown in 10 mL yeast extract–peptone–D-glucose (YPD) broth (yeast extract, 10 g liter$^{-1}$ [Oxoid Ltd., Basingstoke, Hampshire, England]; bacteriological peptone, 20 g liter$^{-1}$ [Oxoid Ltd., Basingstoke, Hampshire, England]; and D-glucose, 20 g liter$^{-1}$ [VWR International Ltd., Poole, England]) on a rotary shaker at 25 $^{o}$C overnight.

Cells were harvested by centrifugation at 3000 g for 5 min (Beckman J2-21, Beckmans Instruments Inc., Palo Alto, USA), and washed three times with 10 mL sterile NaCl solution (9 g $L^{-1}$).

## 2.4. Microtiterplate growth test in liquid cultures

The final yeast suspension from 2.3, was diluted to an OD at 540 nm (OD540) of approximately 2.0 prior to inoculation in microtiterplates. For each yeast strain, cells were inoculated in triplicate into 200 µL of each growth substrate in the wells of microtiterplates (Greiner, Bio-one, Frickenhausen, Germany). The wells were inoculated with 10 µl of the washed and diluted cell suspension (see above) to an initial $OD_{540}$ of approximately 0.01. Growth at 25 $^{o}$C was measured every 15 min in a Hidex Chameleon, Multilabel Detection Platform (Chroma Technology Corp., Rockingham, USA) for 48 h. Prior to the OD measurements, the plate was agitated for 3 s with an amplitude of 3 mm. The mean values of the triplicates were calculated and growth curves were generated for yeasts on each substrate.

## 2.5. Yeast production in 100 ml to 300 L

Selected yeasts were pre-cultured according to 2.3 and then inoculated in Erlenmeyer flasks containing 100 ml of selected substrates. Samples were incubated on a rotary shaker at 25 $^{o}$C for 24 hours. Thereafter, the cells were harvested by centrifugation and freeze dried for further analysis.

Growth in 5 L fermenters was initiated by calibration of the pH electrode and the pO2 electrode in each fermenter prior the start of the experiment. The fermenters (Belach Bioteknik AB) have a total capacity of 5 L, and in this experiment, a starting volume of 3L was used. Pre-culture was done accordingly 2.3. The cells from the pre-culture were suspended in a small volume of NaCl at 9 g/L solution and injected into the fermenter. The initial OD was 1. The fermenters were run in batch mode for 48 h. The fermenter had a constant stirring speed of 200 rpm, a temperature of 30 °C and a pH of 5. Thereafter, the cells were harvested by centrifugation and freeze dried for further analysis.

To produce larger quantities of yeast, a 300 L continuous fermenter (Belach Bioteknik AB) was used. The pH and $pO_2$ were set as described above. Instead of stirring in the large fermenter, agitation was achieved via air inlets in perforated silicon membranes on elevated platforms in the bottom of the fermenter. Continuous harvesting of the cells was performed by filtering. Thereafter, the cells were harvested by centrifugation and freeze dried for further analysis.

## 2.6. Chemical analysis of yeast and faeces

Faecal dry matter was analysed by drying at 103 °C for 16 hours. Crude protein was determined by Kjeldahl. Mineral content was measured according to the Nordic Committee on Food Analysis (1991). Yeast cells were preserved by freeze-drying according to Melin et al. (2007) and stored at −20 ◦C before analysis.

## 2.7. Feed production and sampling points

Two feeds were used during the feeding experiments. A novel Baltic Sea-sourced feed, hereafter called the Baltic Blend, BB, and a fishmeal-based feed, equivalent to a commercial feed formulation, as control feed. See Carlberg et al (2014) for formulation and composition of the feed. The diets were produced by extrusion on a twin-screw extruder (3 mm die, BC-45 model, Clextral, Creusot Loir, Fran-

ce), while the lipids were added by a vacuum coater (Pegasus PG-10VC, Dinnissen, Sevenum, Netherlands), at the Finnish Game and Fisheries Research Institute (Laukaa research station, Finland). To evaluate the viability of the cells during feed manufacturing, samples were taken at the following stages: after mixing of the ingredients (dry ingredients are mixed with boiling water and part of the fish oil for 10 minutes and then left to cool) (1); directly after extrusion (the feed is subjected to 120-130 °C for 30 seconds) (2); after drying (air flow dryer set at 40 °C over night) (3); and after oil inclusion (vacuum coater) (4). The feed ingredient yeast meal (5) was also tested prior to mixing the feed ingredients.

## 2.8. Microbial quantification in feed material

Aliquots of 20 g of yeast and feed collected at different stages during feed manufacturing. were diluted with 180 ml sterile peptone water [Bacteriological peptone 2 g $l^{-1}$ (Oxoid LTD, Basingstoke, Hampshire, England), supplemented with 0.15 g $l^{-1}$ Tween 80 (Kebo AB Stockholm, Sweden) and homogenized for 120 seconds at normal speed in a Stomacher 400 Laboratory blender (Seward Medical, London, England). Samples were then serially diluted in peptone water and 100 µl portions were spread on YPD agar plates (yeast extract, 10 g $liter^{-1}$ [Oxoid Ltd., Basingstoke, Hampshire, England]; bacteriological peptone, 20 g $liter^{-1}$ [Oxoid Ltd., Basingstoke, Hampshire, England]; and D-glucose, 20 g $liter^{-1}$ [VWR International Ltd., Poole, England], technical agar 15 g $liter^{-1}$ [Oxoid LTD, Basingstoke, Hampshire, England]), supplemented with 100 µg ml-1 chloramphenicol (Sigma-Aldrich Inc, St Louis, USA). YPD plates were incubated at 25°C for 2 to 4 days and colony forming units per gram feed (cfu $g^{-1}$) were calculated.

## 2.9. Microbial quantification in faeces

Faeces were collected for microbial activity-analysis in September and December (sampling II and III, see figure 1). Four fish was taken from each of three replicate tanks of both control and BB feed, resulting in 12 faecal samples per feed treatment (control, BB). The fish were netted and anaesthetised with 100 mg $L^{-1}$ MS-222 solution. Each fish was euthanised by a blow to the head and then cervical dislocation. An incision was made along the ventral side of the fish and the distal intestine was cut before the anus. Forceps were used to push faeces from the distal intestine outwards into a collection tube. Faecal samples were immediately refrigerated. Portions of 100 mg of faeces was mixed with 100 µL of peptone detergent solution and then streaked on YPD agar plates. YPD plates were incubated at 25°C for 3 days and cfu $g^{-1}$ faeces were calculated.

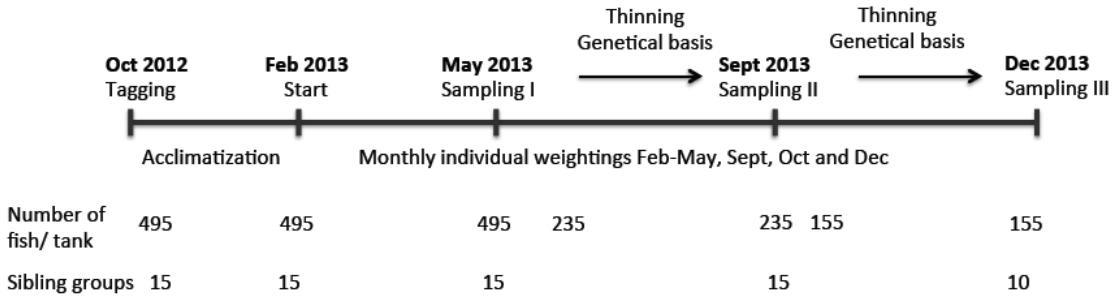


Figure 1. Schematic description of the experimental protocol from Carlberg *et al* 2014.

## 2.10. Microbial identification of yeast isolates

Yeasts isolated from feed and faeces were identified according to the method of Olstorpe et al. (2008). Briefly, up to 10 representative colonies of yeast from each sample were purified and identified by sequence analyses of selected yeasts, the D1-D2 region (approximately 600 bp) in the 25S rRNA gene was amplified using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). Reaction conditions were initial denaturation at 94°C for 2 min followed by 29 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min, with a final extension step at 72°C for 5 min. Amplification products were visualised by electrophoresis in 1% agarose gel in 0.5-Tris-borate-EDTA buffer, 5.1 V cm-1 and 80 mA for 120 min. Amplicons were sequenced at Macrogen, Korea, and isolates were identified by sequence comparison against the Genbank database.

# 3. Results

There was a large variation in $OD_{540}$ values among different substrates and yeast species, and also among strains within the same species. Figure 2 shows an example of growth curves for three different yeasts on waste water from Dark Lager production.

Efficacy of growth could not be predicted based on either yeast strain or substrate, and each interesting waste stream needs to be evaluated against all selected yeasts to identify the most promising yeast. Several yeast candidates showed very pronounced growth characteristics on specific waste products Most yeast had not entered stationary phase at 24 hours growth and an increase in OD was noted in most samples measured at 48 hours growth (Table 1, 2). In the next stage, selection of waste products to continue evaluation of growth in larger fermenters was done according to availability of large quantities of that substrate at the time of experiment.

Table 1. Compilation of measured OD540 values at 24 hours growth in microtiterplates for tested yeast species and strains on respectively substrate

| | Substrate | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yeast /Subs. | A[b] | B[b] | C[b] | D[b] | E[b] | F[b] | G[b] | H[b] | I[b] | J[b] | K[b] | L[b] | M[b] | N[b] |
| J596[a] | - | - | - | 0,013 | 0,001 | - | - | - | - | - | - | - | - | - |
| J595[a] | 0,091 | 0,226 | - | 0,036 | 0,044 | 0,034 | 0,035 | - | 0,522 | 0,449 | 0,330 | 0,063 | 0,218 | 0,036 |
| J564[a] | 0,087 | 0,140 | - | 0,062 | 0,029 | 0,041 | 0,039 | 0,010 | 0,163 | 0,176 | 0,143 | 0,064 | 0,193 | 0,047 |
| J563[a] | 0,040 | 0,197 | - | 0,030 | 0,020 | 0,022 | 0,020 | - | 0,905 | 0,527 | 0,649 | 0,035 | 0,198 | 0,013 |
| J562[a] | 0,109 | 0,147 | - | 0,034 | 0,037 | - | - | - | 0,565 | 0,379 | 0,598 | 0,081 | - | - |
| J556[a] | 0,098 | 0,148 | 0,005 | 0,022 | 0,079 | 0,677 | 0,018 | - | 0,902 | 0,554 | 0,958 | 0,073 | 0,196 | 0,103 |
| J552[a] | 0,035 | 0,102 | - | 0,073 | 0,035 | 0,041 | 0,052 | 0,026 | 0,142 | 0,158 | 0,127 | 0,057 | 0,095 | 0,050 |
| J551[a] | 0,065 | 0,150 | - | 0,030 | 0,047 | 0,018 | 0,020 | - | 0,787 | 0,385 | 0,600 | 0,047 | 0,154 | 0,094 |
| J550[a] | 0,062 | 0,133 | - | 0,028 | 0,061 | 0,429 | 0,025 | 0,002 | 0,687 | 0,385 | 0,736 | 0,093 | 0,172 | 0,075 |
| J549[a] | 0,108 | 0,193 | 0,018 | 0,067 | 0,061 | 0,058 | 0,060 | 0,047 | 0,821 | 0,310 | 0,668 | 0,125 | 0,173 | 0,107 |
| J546[a] | 0,017 | 0,083 | - | 0,053 | 0,069 | 0,028 | 0,030 | 0,019 | 0,248 | 0,142 | 0,281 | 0,042 | 0,064 | 0,064 |
| J545[a] | 0,112 | 0,089 | 0,021 | 0,031 | 0,054 | 0,039 | 0,033 | 0,017 | 0,966 | 0,629 | 1,064 | 0,038 | 0,177 | 0,110 |
| J482[a] | 0,073 | 0,035 | - | 0,011 | 0,022 | - | - | - | 1,031 | 0,673 | 0,892 | - | 0,065 | 0,031 |
| J475[a] | 0,096 | 0,294 | - | 0,008 | 0,001 | - | - | - | 0,232 | 0,386 | 0,192 | - | 0,024 | - |
| J469[a] | - | 0,048 | - | 0,012 | - | - | - | - | 0,432 | 0,349 | 0,404 | - | 0,013 | - |
| J466[a] | 0,039 | 0,104 | - | 0,002 | 0,005 | - | - | - | 1,093 | 0,648 | 1,254 | 0,052 | 0,112 | 0,021 |
| J379[a] | - | 0,130 | - | - | 0,013 | - | - | - | 0,831 | 0,408 | 0,438 | - | 0,054 | - |
| J352[a] | - | 0,094 | - | - | - | - | - | - | 0,889 | 0,568 | 0,489 | - | 0,173 | 0,087 |
| J279[a] | 0,041 | 0,153 | - | - | - | - | - | - | 0,673 | 0,366 | 0,772 | - | 0,088 | - |
| J195[a] | 0,106 | 0,172 | - | - | 0,037 | - | - | - | 0,702 | 0,537 | 0,644 | - | 0,064 | - |
| J186[a] | 0,344 | 0,593 | - | 0,017 | 0,027 | 0,007 | - | - | 0,673 | 0,433 | 0,726 | 0,010 | 0,142 | 0,042 |
| J134[a] | 0,058 | 0,067 | - | - | 0,010 | - | - | - | 1,029 | 0,769 | 1,088 | 0,012 | 0,056 | - |
| J121[a] | 0,135 | 0,230 | - | - | 0,062 | - | - | - | 0,783 | 0,595 | 0,786 | 0,062 | 0,188 | 0,055 |

[a] species number see 2.1, [b] substrate definition see 2.2 ,- No growth

Table 2. Compilation of measured OD540 values at 48 hours growth in microtiterplates for tested yeast species and strains on respectively substrate.

| | Substrate | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yeast /Subs. | A[b] | B[b] | C[b] | D[b] | E[b] | F[b] | G[b] | H[b] | I[b] | J[b] | K[b] | L[b] | M[b] | N[b] |
| J596[a] | - | - | - | 0,003 | - | - | - | - | - | - | - | - | - | - |
| J595[a] | 0,187 | 0,407 | - | 0,021 | 0,085 | 0,026 | 0,012 | - | 0,795 | 0,523 | 0,593 | 0,176 | 0,295 | 0,167 |
| J564[a] | 0,065 | 0,176 | 0,009 | 0,060 | 0,026 | 0,034 | 0,014 | - | 0,251 | 0,198 | 0,200 | 0,050 | 0,292 | 0,046 |
| J563[a] | 0,215 | 0,380 | - | 0,016 | 0,044 | 0,008 | - | - | 0,932 | 0,573 | 0,728 | 0,144 | 0,310 | 0,184 |
| J562[a] | 0,158 | 0,167 | - | - | - | 0,034 | 0,010 | - | 0,197 | 0,223 | 0,521 | - | 0,050 | - |
| J556[a] | 0,170 | 0,221 | 0,776 | 0,009 | | 0,946 | - | - | 0,917 | 0,577 | 0,979 | 0,096 | 0,256 | 0,174 |
| J552[a] | 0,079 | 0,128 | 0,016 | 0,071 | 0,026 | 0,032 | 0,023 | 0,017 | 0,259 | 0,162 | 0,453 | 0,046 | 0,173 | 0,075 |
| J551[a] | 0,161 | 0,27 | - | 0,013 | 0,074 | 0,003 | - | - | 0,847 | 0,416 | 0,656 | 0,095 | 0,247 | 0,238 |
| J550[a] | 0,117 | 0,196 | 0,385 | 0,018 | 0,107 | 0,475 | - | - | 0,747 | 0,601 | 0,786 | 0,164 | 0,261 | 0,092 |
| J549[a] | 0,177 | 0,322 | 0,022 | 0,057 | 0,103 | 0,046 | 0,032 | 0,037 | 0,810 | 0,337 | 0,732 | 0,180 | 0,271 | 0,169 |
| J546[a] | 0,059 | 0,104 | - | 0,044 | 0,073 | 0,015 | 0,001 | 0,013 | 0,271 | 0,155 | 0,303 | 0,033 | 0,084 | 0,091 |
| J545[a] | 0,176 | 0,081 | 0,014 | 0,025 | 0,071 | 0,027 | 0,069 | 0,016 | 0,985 | 0,636 | 1,085 | 0,069 | 0,243 | 0,159 |
| J482[a] | 0,111 | - | - | 0,006 | 0,023 | - | - | - | 1,069 | 0,673 | 0,868 | - | 0,109 | 0,073 |
| J475[a] | 0,160 | 0,353 | - | 0,001 | - | - | - | - | 0,509 | 0,386 | 0,188 | - | 0,109 | 0,010 |
| J469[a] | - | 0,257 | - | 0,005 | 0,011 | - | - | - | 0,488 | 0,349 | 0,428 | - | 0,106 | - |
| J466[a] | 0,127 | 0,216 | - | - | 0,010 | - | - | - | 1,093 | 0,648 | 1,235 | 0,057 | 0,177 | 0,051 |
| J379[a] | 0,166 | 0,204 | - | - | 0,028 | - | - | - | 0,831 | 0,408 | 0,525 | 0,011 | 0,161 | - |
| J352[a] | 0,245 | 0,401 | - | - | 0,076 | - | - | - | 0,565 | 0,379 | 1,000 | 0,172 | 0,296 | 0,028 |
| J279[a] | 0,261 | 0,252 | - | - | - | - | - | - | 0,771 | 0,491 | 0,798 | - | 0,207 | 0,018 |
| J195[a] | 0,144 | 0,392 | - | - | 0,036 | - | - | - | 0,864 | 0,535 | 0,685 | - | 0,046 | - |
| J186[a] | 0,535 | 0,770 | - | 0,005 | 0,035 | - | - | - | 0,756 | 0,508 | 0,770 | 0,094 | 0,231 | 0,154 |
| J134[a] | 0,164 | 0,065 | - | - | 0,033 | - | - | - | 1,061 | 0,783 | 1,108 | 0,036 | 0,173 | 0,026 |
| J121[a] | 0,221 | 0,465 | - | - | 0,109 | - | | - | 0,917 | 0,705 | 0,850 | 0,160 | 0,265 | 0,194 |

[a] species number see 2.1, [b] substrate definition see 2.2, - No growth

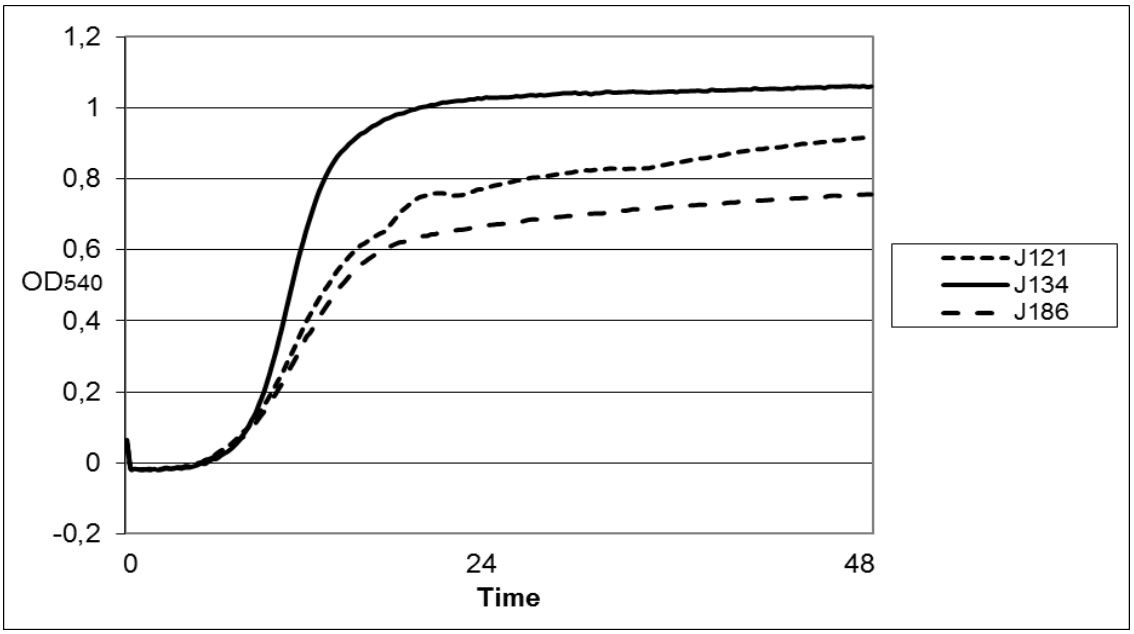


Figure 2. Growth curves of *W. anomalus* J121, *Y. lipolytica* J134 and *K. marxianus* J186 on waste water from Dark Lager beer production.

Thermo Mechanical Pulp (TMP) and waste water from Pilsner Malt beer production were selected for further evaluation. Most yeast grew slowly and the transition between exponential phase and lag phase was not pronounced on TMP. *W. anomalus* grew satisfactory and two strains, J121 and J379, were selected for further growth trials. When a mixture of TMP and Pilsner Malt GW was used, *B. adeninivorans* strains J562 and J564 showed satisfactory growth in Erlenmayer flasks. Crude protein and amino acid composition of the yeast meal is presented in table 3.

Yeast cultivation in 5 L fermenters yielded varying protein content in the yeast meal depending on duration of growth, shown in Table 4 as crude protein content in *W. anomalus* J121 and J379.

A 300 L continuous fermenter was further used to scale up the production of SCP, as sufficient cell material for analysis and feed trials could not be produced in small scale growth vessels. The fermentation was done with TMP alone as substrate since waste water from beer couldn't be allocated to the facility. Due to the design of the aeration system, yeast cells could not be harvested satisfactorily. Cells were trapped under the aeration pads and production per L substrate could not be calculated. Cells which could be harvested were in late exponential phase with low crude protein contents. *W. anomalus* J121 had a crude protein content of 257 ± 23 g $kg^{-1}$ dm (n=4) and *W. anomalus* J379, 221 ± 15 g $kg^{-1}$ dm (n=3). Mineral composition of the substrate and yeast meal is presented in Table 5.

Table 3. Crude protein and amino acid composition in *B. adeninivorans* and *W. anomalus* yeast meal grown on a 50/50 mixture of Thermo Mechanical Pulp and waste water from Pilsner Malt beer production.

| g /kg dm | J562 | J564 | J121 | J379 |
|---|---|---|---|---|
| Crude protein | 391 | 343 | 269 | 453 |
| Cysteine | 2.97 | 2.45 | 2.17 | 3.31 |
| Methionine* | 5.45 | 4.41 | 3.04 | 5.52 |
| Aspartic acid | 27.71 | 22.06 | 23.48 | 37.57 |
| Threonine* | 15.84 | 12.75 | 13.48 | 20.44 |
| Serine | 16.83 | 12.25 | 14.35 | 20.44 |
| Glutamic acid | 32.67 | 25.98 | 23.48 | 40.33 |
| Proline | 12.87 | 10.78 | 9.57 | 16.02 |
| Glycine | 15.35 | 11.76 | 12.61 | 19.89 |
| Alanine | 22.28 | 17.16 | 15.65 | 27.62 |
| Valine* | 18.81 | 15.2 | 14.78 | 23.76 |
| Isoleucine* | 15.84 | 12.25 | 14.35 | 22.1 |
| Leucine* | 28.71 | 23.53 | 20 | 33.7 |
| Tyrosine | 10.4 | 6.67 | 9.57 | 16.02 |
| Phenylalanine* | 12.87 | 9.8 | 11.3 | 18.23 |
| Histidine* | 7.43 | 5.39 | 5.65 | 8.84 |
| Ornithine | 5.94 | 5.39 | 3.91 | 6.08 |
| Lysine* | 16.83 | 12.25 | 19.13 | 25.97 |
| Arginine | 9.41 | 6.37 | 6.96 | 13.81 |
| Hydroxyproline | 0.1 | 0.1 | 0.1 | 0.1 |
| Ammonia | 9.41 | 8.33 | 6.96 | 11.6 |

Table 4. Dry weight of yeast cells (gram per 3 L substrate) after 24 and 48 hours of growth on Thermo Mechanical Pulp [TMP] and waste water [ww] from Pilsner Malt as TMP:ww 50:50 or pure ww. Amount of crude protein (gram per kilogram dry matter) in the harvested cells after 24 and 48 hours of growth.

| | Substrate | | | |
|---|---|---|---|---|
| Yeast | TMP / ww 24 | TMP / ww 48 | ww 24 | ww 48 |
| J121 | 18.61 [a] | 29.69 [a] | 30.56 [a] | 34.7 [a] |
| J379 | 23.47 [a] | 24.39 [a] | 33.41 [a] | 52.19 [a] |
| J121 | 285 | 188 | 325 | 252 |
| J379 | 340 | 259 | 281 | 192 |

Extensive growth of contaminant bacteria in the substrate was observed. Due to inadequate equipment to facilitate transfer of sterile medium to the fermenter directly from the paper mill, production of SCP on TMP was ended. As no other suitable waste product could be received in sufficiently large quantities for feed trials, commercially available *S. cerevisiae* grown on molasses and dried without carrier material by fluidised bed methods was used in the experimental feed Baltic Blend.

Yeast cells, *S. cerevisiae*, remained viable during the entire fish feed production process. Yeast cfu $g^{-1}$ feed varied depending on where in the production line the samples were taken. The cfu was markedly lowered after extrusion but increased at later stages of production processes. The yeast meal alone was also evaluated prior to mixing the feed ingredients (Table 6). All isolates from the feed were identified by sequence analyses of the D1-D2 region in the 25S rRNA gene using primers NL1 and NL4. All isolates were *S. cerevisiae*.

The presence of yeast in the distal intestine of fish fed on BB was always higher than in fish fed with the Control diet (Table 7).

The major yeast populations in the distal intestinal of fish fed Control and BB diets comprised *Debaryomyces fabryi* and *Debaromyces hansenii* (Table 8). The yeast composition did not differ substantially, however, the amount of yeast cells enumerated was higher in fish fed the experimental diet BB than those fed the control diet (Table 7). One fish had a completely different yeast population than all other tested fish, namely, the population was comprised solely of *Rhodotorula glutinis*.

Table 5. Chemical composition of TMP and yeast cells grown on TMP substrate.

| | TMP mg $L^{-1}$ | Yeast mg $kg^{-1}$ ts |
|---|---|---|
| Cadmium | 0.00441 | 0.418 |
| Chromium | <0.02 | 6.9 |
| Copper | 0.453 | 186 |
| Mercury | <0.002 | 0.0584 |
| Nickel | <0.02 | 6.2 |
| Lead | 0.0201 | 2.73 |
| Zinc | 1.35 | 93.1 |

Table 6. Yeast cfu $g^{-1}$ faeces, samples taken on yeast meal prior to feed mix, after mixing of all ingredients, after extrusion, after drying, and after oil vacuum coating of the pellet.

| | [a] Cfu $g^{-1}$ dm material |
|---|---|
| Yeast meal | $1.00*10^{10}$ |
| Ingredient mix | $1.63*10^{9}$ |
| Extrusion | $1.60*10^{2}$ |
| Dryer | $1.48*10^{3}$ |
| Vacuum coating | $1.49*10^{5}$ |

Table 7. Yeast cfu $g^{-1}$ faeces. Samples taken from the distal intestine at sampling point II and III see Figure 1.

| | [a] Cfu $g^{-1}$ dm material | |
|---|---|---|
| | Sampling point II | Sampling point III |
| Control | $8.75*10^1$ | $7.93*10^1$ |
| Baltic Blend | $1.03*10^3$ | $1.93*10^3$ |

Table 8. Dominating yeast species identified by rRNA sequencing in faeces collected from the distal intestine in fish fed Baltic Blend and Control diets, sampling points II and III, see Figure 1.

| | Sampling point II | | Sampling point III | |
|---|---|---|---|---|
| Yeast species | Control | Baltic Blend | Control | Baltic Blend |
| *Candida zeylanoides* | 10 | 8 | 5 | 10 |
| *Cryptococcus carnescens* | 2 | 0 | 0 | 0 |
| *Cryptococcus victoriae* | 2 | 0 | 5 | 0 |
| *Debaryomyces fabryi* | 60 | 66 | 65 | 65 |
| *Debaryomyces hansenii* | 14 | 12 | 20 | 15 |
| *Rhodotorula glutinis* | 5[a] | 0 | 0 | 0 |
| *Saccharomyces cerevisiae* | 7 | 14 | 5 | 10 |

Number represents percentage of species identified out of the total isolated yeasts. Five yeast isolates from each fish were identified with a total of 12 fish per diet.

a *Rhodotorula glutinis* was only isolated from one fish, in which it was the sole yeast isolated

# 4. Discussion

Several waste products from the food, bioethanol and paper industries could be used as substrates to grow yeast for incorporation as a protein replacement in fish feed. It is just a matter of matching the right yeast to the available substrate. The amount of protein in the yeast cells was affected by growth substrate, yeast species and strain, as well as the time of growth. Due to lack of suitable equipment and access to sufficient volumes of a reproducibly homogeneous waste stream, yeast production could not be established on a larger scale for any of the tested substrates.

The yeast *S. cerevisiae* was partially used as a protein replacement instead of fish meal in the BB diet. It was demonstrated that the yeast cells remained viable during the entire feed production process, including several heating and drying steps, as well as during the feeding trial. However, only a very low number of viable cells of *S. cerevisiae* were found in the distal intestine. Andlid et al. (1995) discovered that *S. cerevisiae* is present naturally in the gut of rainbow trout, and inclusion of this yeast in the diet increased its intestinal concentration. We also isolated *S. cerevisiae* in fish fed both control and BB diets, but at very low numbers; the dominating flora instead consisted of *D. fabryi*, *D. hansenii* and *C. zeylanoides*. Since the number of viable *S. cerevisiae* cells was very low in the distal intestine, we assume that the yeast included in the diet was metabolised. However, the BB diet did affect the

yeast gut flora, by apparently increasing the presence of yeasts in the intestine, as indicated by the increased number of yeast cells enumerated in the faeces.

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