FISH FEED FROM WOOD

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Increased demand of fish in combination with overexploitation of the fish stocks of the oceans has led to an increased production of fish through aquaculture. Today, fishmeal is the main protein source in fish feed for most aquaculture species. However, fishmeal is soon expected to fall short of demand as its production is associated with environmental problems. This shortage must therefore be met by sustainable alternative protein sources. Protein-rich microorganisms (i.e. Single cell protein) is an interesting option as a fishmeal substitute in fish feed which, in addition, can be produced as an important co-product in wood-based biorefineries. In the current study, four different microorganisms were cultivated on five different residual streams from Swedish wood-based biorefineries. Screening experiments were carried out in shake flasks, optimization experiments in benchtop bioreactors, and scale-up experiments were performed in a 50-litre pilot bioreactor. In addition, a demo-scale experiment was carried out in the Swedish Biorefinery Demo Plant. Microbial biomass from the scale-up experiments was collected and used for production of different fish feed formulations which, in turn, were used in feeding trials of the freshwater fish Tilapia. Fishes fed with feed, in which part of the fishmeal had been substituted with Single cell protein, showed similar or better growth than fishes fed with a fishmeal-based control feed.

Keywords: Single cell protein, biorefinery residual streams, fish feed, tilapia, *Fusarium venenatum*, *Paecilomyces variotii*

INTRODUCTION

The world's population is projected to reach about 9 billion in 2050.[1] This will lead to a strong demand for food. The production of fish must increase significantly in order to meet the increasing demand, while today about 80% of the fish stocks of the oceans are fully exploited, overexploited or depleted.[2] Intensifying the fishing even further does not appear as a possible solution. Therefore, a large proportion of the fish production must come from aquaculture in the future. Today, aquaculture stands for about 50% of the fish supply for human consumption. However, fishmeal derived from wild fish is the preferred protein source for many aquaculture species. Aquaculture based on fishmeal is therefore not the solution to the problem with decreasing fish stocks. Fishmeal production has been fairly stable with around 5-7 million tons per year during the last 40 years, while aquaculture production has grown by an average of about 8% since the 1970s.[2,3] This has led to a large increase in the fishmeal price in recent years. The increased demand of fishmeal for fish feed production and the high price have caused a need for alternative protein sources. However, most available protein sources are of plant origin and they can, in general, only be used in limited amounts due to a different amino acid composition compared to fishmeal protein and the presence of anti-nutritional substances that can be detrimental for the fish.[4] An interesting alternative is Single cell protein (SCP). SCP consists of microorganisms such as yeast, bacteria, algae and filamentous fungi. Many species have high protein content and some have amino acid profiles that are very similar to that of fishmeal. In addition, SCP can be produced using the residual stream from the forest industry. This offers an attractive concept of turning forest raw material into a protein-rich component in fish feed.

In this study, four different microorganisms (*Candida utilis*, *Rhizopus oryzae*, *Fusarium venenatum* and *Paecilomyces variotii*) were evaluated for SCP-production using five different residual streams found at the industrial site of Domsjö (Örnsköldsvik, Sweden). *R. oryzae* and *P. variotii* are filamentous fungi that previously have been considered for SCP production from

various residual streams for feed purposes.[5-7] The possibility of producing the yeast *C. utilis* from wood-derived sugars for feed and food applications has a long history and it was described already in the 1930s.[8] The filamentous fungus *F. venenatum* is currently being produced from glucose in a commercial plant in the UK and constitutes the basis for the human food product that is sold under the brand name Quorn.[9]

EXPERIMENTAL

Microorganisms and residual streams

Four different microorganisms were used, *C. utilis* (CCUG 28186), *R. oryzae* (CCUG 28958), *F. venenatum* (ATCC-20334), and *P. variotii* (CCUG 26807). Four residual streams from the Domsjö Biorefinery (Örnsköldsvik, Sweden) and one hemicellulose hydrolysate of straw from the Swedish Biorefinery Demo Plant (Örnsköldsvik, Sweden), produced by Sekab E-technology, were evaluated. The residual streams from the Domsjö plant were spent sulfite liquor (SSL), spent sulfite liquor permeate (SSL-permeate), fiber sludge, and the residual generated after the first ethanol evaporation in the SSL-ethanol plant. The fiber sludge was converted to a glucose-rich hydrolysate by enzymatic hydrolysis prior to use.

Cultivation experiments at lab and pilot scale

The microorganisms and residual streams were first evaluated in cultivation experiments using 250-mL shake flasks. The most promising combinations of residual streams and microorganisms were selected for cultivation experiments in a 300 mL multi bioreactor (Sixfors, Infors, Switzerland) to optimize the conditions for biomass production. Different additions of nutrients and trace elements, as well as the fed-batch cultivation mode, were evaluated.

P. variotii grown on SSL-permeate and *F. venenatum* grown on fiber sludge hydrolysate were selected for scale-up experiments in a 50-L pilot bioreactor (Belach Bioteknik, Sweden). The residual streams were diluted with water prior to use to reach optimal sugar concentrations in aspect of biomass yield (g biomass produced per g consumed sugars). The cultivations were performed at 30 °C, pH 6.0, and the stirring was increased with increasing culture broth viscosity to maintain a sufficient oxygen level. The pilot reactor was equipped with two pitched-blade impellers, three baffles and a ring sparger to enable satisfactory oxygen transfer rates. The cultivations were carried out in "fed-batch fill and draw" mode. The starting volume in the reactor was 10 L. After 24 h batch cultivation, the reactor was fed continuously during 24 h with the carbon source (i.e. SSL-permeate or fiber sludge hydrolysate) to a volume of 50 L. 40 L was then harvested and a new feeding sequence was started and maintained for 24 h until a volume of 50 L was reached again. The fill and draw procedure was maintained for up to ten harvests before starting over with a fresh inoculum. Samples were withdrawn during the cultivation for analyses of biomass, monosaccharides, oligomeric and polymeric carbohydrates and aliphatic acids. The harvested biomass was separated from the liquid fraction through vacuum filtration. The resulting filter cakes were washed with two volumes of distilled water and dried in an oven at 75 °C. The dried filter cakes were then ground to a powder in a mill with a 0.5 mm screen (Hosokawa Alpine, Germany). The resulting biomass powder was stored in a freezer at -80 °C until further use.

Cultivation experiments at demo scale

P. variotii grown on SSL-permeate was selected for an experiment in the Swedish Biorefinery Demo Plant. The same cultivation conditions as in the pilot experiments were applied. One 400-L inoculum bioreactor and one 10 000-L bioreactor were used for the experiment. The 10 000-L reactor was equipped with two concave disc impellers (CD-6) and four baffles. A 20 L inoculum was prepared in the pilot bioreactor and added to the 400-L inoculum reactor that had been filled to a volume of 180 L (SSL-permeate, nutrients, and trace elements). The cultivation was maintained for about 24 h and the 200 L culture broth was used as inoculum for the cultivation in the 10 000-L reactor. The starting volume in the reactor, including inoculum, was 2000 L. After 24 h of batch cultivation, the reactor was fed continuously during approximately 12 or 24 h to reach a final volume of 7000 L. 5000 L was harvested and a new feeding sequence was started. Samples were withdrawn during the cultivation for analyses of biomass, monosaccharides, and aliphatic acids. The harvested biomass was separated from the liquid fraction using a filter press.

Feed production

Based on chemical analyses of the SCP, test diets were formulated for feeding trials of the fish Tilapia. The aim of the formulation was to make comparable diets with different SCP. Two different concentrations were tested for each type of SCP, 9.5% and 19% of the total feed. The degree of fishmeal substitution was 38% and 66% for the feed containing *P. variotii* and 39% and 68% for the feed containing *F. venenatum*. The total content of protein in all feed formulations was 35%. The control feed contained fishmeal proteins instead of the SCP proteins. The feed was produced by mixing all ingredients, drying the dough and making feed crumbles.

Feeding experiments

Tilapia larvae were obtained from the company Íslensk Matorka. 528 larvae of 0.45 grams average weight were divided into 20 tanks to get equal biomass

in each tank. The number of fish in each tank varied from 25 to 31. Around 100 fishes for each feed type were divided in four different tanks. The volume of each tank was 20 litres and a starting volume of 10 litres of water was used. After the first week, the water levels were lowered to around 5 litres to reduce territorial behaviour of the fish. Fresh aerated water was supplied in a flow-through system at the temperature of 26 °C. The fish growth was followed during the experiment by gravimetric methods.

Analyses

Analyses of monosaccharides, formic acid and acetic acid were carried out using an ICS-5000 high-performance anion-exchange chromatography (HPAEC) system (Dionex, Sunnyvale, CA, USA).[10] The analysis of oligomeric and polymeric carbohydrates was performed by MoRe Research Örnsköldsvik AB (Örnsköldsvik, Sweden) using Size Exclusion Chromatography (SEC). Biomass concentration was determined gravimetrically.

The nutritional analysis of the fungal biomass and the analysis of mycotoxins were performed by Eurofins Food & Agro Testing Sweden AB (Lidköping, Sweden). Protein content determination was carried out according to the Dumas method (N×6.25). Fat content was analyzed by the SBR method (SLV VF 1980). The content of carbohydrates was estimated by using the methods SLVFS 1993:21 and AOAC 985.29. The water and ash contents were determined by NMKL 23 and NMKL 173, respectively. All amino acids, except for tryptophan, were determined according to SS-EN ISO 13903:2005. Tryptophan was determined by SS-EN ISO 13904:2005. The presence the aflatoxins (B1, B2, G1, and G2) was determined according to EN 14123, mod. Determination of deoxynivalenol, HT-2, T-2, livalenol, and zearalenone was carried out according to Eurofins in-house method 210. Fumosin (B1 and B2) and ochratoxin were determined by a Eurofins internal method and NMKL 143, respectively.

RESULTS AND DISCUSSION

Cultivation experiments at lab and pilot scale

The cultivations at lab and pilot scale showed that the limiting factor for the biomass concentration was the culture broth viscosity rather than the concentration of the carbon source. The maximum biomass concentration for *P. variotii* grown on SSL-permeate and *F. venenatum* grown on fiber sludge hydrolysate was about 9 and 25 g/L, respectively. The residual streams were therefore diluted with water to enable high biomass yields. *P. variotii* was able to consume glucose, mannose, xylose, galactose, and arabinose simultaneously. Biomass yields >0.5 g/g consumed monosaccharides were achieved for *P. variotii* grown on SSL-permeate. This indicates that the fungus can also consume other carbon sources present in the SSL-permeate, which have been also proposed in previous studies.[11] Consumption of acetic acid and formic acid was noted, as well as decreased levels of polymeric and oligomeric carbohydrates (data not shown). About 5 kg of *P. variotii* biomass and 5 kg *F. venenatum* biomass were produced for the feed and feeding experiments. The protein content of the *P. variotii* and *F. venenatum* biomass was 51% and 58% (g/g dry biomass), respectively (Table 1). No significant levels of 12 common mycotoxins were found for any of the two microorganisms (Table 2).

The European food and Safety Authority has set guidance values for mycotoxin content in animal feed: aflatoxin B1: 5-20 µg/kg,[12] deoxynivalenol: 900-12 000 µg/kg, zearalenone: 100-3 000 µg/kg, ochratoxin: 50-250 µg/kg, and fumonisin B1+B2: 5 000-60 000 µg/kg.[13] The content of important amino acids was very similar to that of a fishmeal with similar protein content for both species (Table 3).

Cultivation experiments at demo scale

The demo experiment was started with feed and harvest intervals of 24 h. However, the growth showed to be faster than in the pilot experiments and therefore the feed and harvest intervals were decreased to about 12 h. Figure 1A shows the consumption of monosaccharides and the production of biomass during a fed-batch fill and draw experiment with four feed and harvest sequences. The biomass yield was higher than or equal to about 0.5 g/g consumed monosaccharides. The level of protein was fairly stable at about 55 g/100 g dry biomass throughout the experiment (Figure 1B). Filter pressing showed to be a convenient method to separate the biomass from the liquid fraction.

Feed production and feeding experiments

During the trial period (28 days), the fish increased their weight up to tenfold. The specific growth rate (SGR, %/day) was 8.50, 8.84, 8.60, 8.51, and 8.30 for the control, *P. variotii* 38% sub., *P. variotii* 66% sub., *F. venenatum* 39% sub., and *F. venenatum* 68% sub., respectively. The average fish growth per fish tank was similar to or better than the control (Figure 2A). The fishes fed with *P. variotii* 66% sub. resulted in about 12% better growth than the control. The largest growth per fish was noted for the fishes

fed with *P. variotii* 38% sub. (Figure 2B). The fish mortality (data not shown) was normal and not related to the dietary treatments.

Table 1
Biomass nutrient composition

	P. variotii (g/100 g biomass)	*F. venenatum* (g/100 g biomass)
Protein	48	53
Fat	5	7
Carbohydrate	37	26
Water	5	9
Ash	5	5

Table 2
Levels of 12 common mycotoxins

Mycotoxin	*P. variotii* (µg/kg biomass)	*F. venenatum* (µg/kg biomass)
Aflatoxin B1	<0.1	<0.1
Aflatoxin B2	<0.1	<0.1
Aflatoxin G1	<0.1	<0.1
Aflatoxin G2	<0.1	<0.1
Ochratoxin	0.1	<0.1
Deoxynivalenol	<10	<10
HT-2 toxin	<10	<10
Nivalenol	<10	<10
T-2 toxin	<10	<10
Zearalenone	<10	<10
Fumonisin B1	<20	<20
Fumonisin B2	<20	<20

Table 3
Amino acid profiles of *P. variotii*, *F. venenatum* and a fishmeal with similar crude protein content (*i.e.* 52%)[14]

Amino acid	*P. variotii* (g/100 g biomass)	*F. venenatum* (g/100 g biomass)	Fish meal India[14] (g/100 g biomass)
Arginine	2.7	2.5	2.6
Threonine	1.8	2.0	1.8
Isoleucine	2.0	2.1	1.9
Leucine	3.1	3.1	3.3
Lysine	2.9	3.0	3.0
Methionine	0.7	0.8	1.1
Valine	2.4	2.5	2.3
Tryptophan	0.6	0.5	0.5

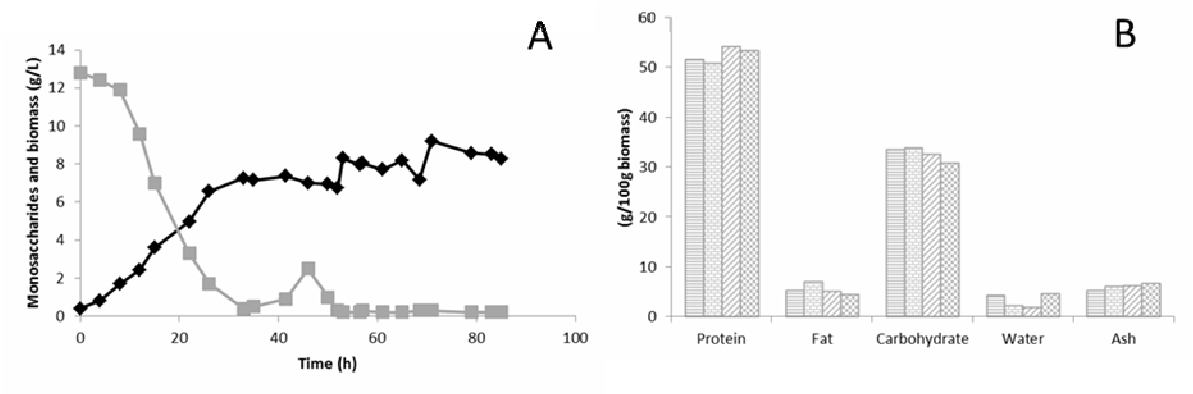

Figure 1: (A) Consumption of total monosaccharides (squares) and biomass production (diamonds) during a fed-batch fill & draw experiment at demo scale. Feeding of carbon source was started at 15 h, the biomass was harvested at 35 h, 57 h, 69 h, and 85 h. A new feed sequence was started after every harvest. (B) Nutrient composition of *P. variotii* biomass of the four harvests

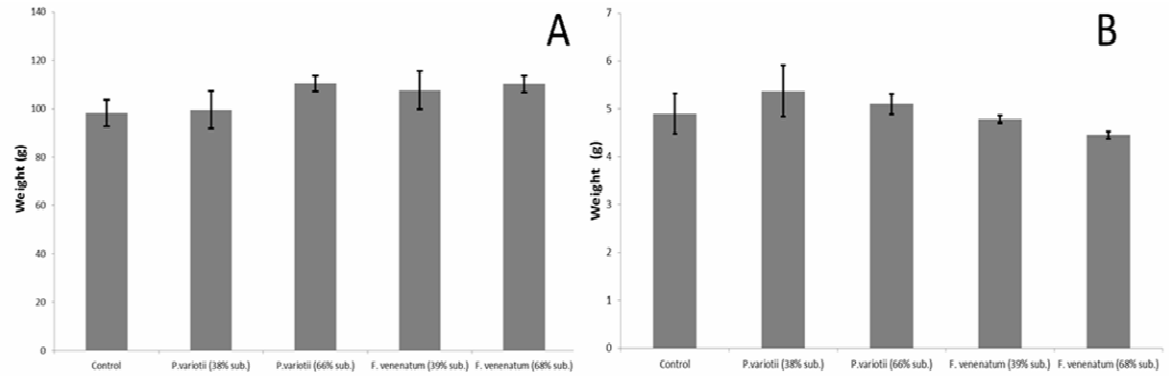

Figure 2: Fish weight of fishes fed with different feeds after 28 days; (A) Average fish weight per fish tank; (B) Average weight per fish. The main protein source in the control feed consisted of fishmeal. In the *P. variotii* and *F. venenatum* feed, the fishmeal was substituted to different degrees with SCP. The degree of substitution is given in percent. Error bars indicate standard deviations

CONCLUSION

This study demonstrates the concept of turning wood into food. Single cell protein produced from industrial residual streams is an interesting potential co-product for the forest industry and an interesting alternative protein source in fish feed for the aquaculture industry.

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