Waste Bread Valorization Using Edible Filamentous Fungi
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Abstract
The present study is the first of its kind to use industrial waste bread for ethanol and food-grade filamentous fungal biomass production, with an ‘integrated-biorefinery’ approach for the existing wheat-based ethanol facilities. Four different food-grade fungi such as Neurospora intermedia, Aspergillus oryzae, belonging to ascomycetes and Mucor indicus, Rhizopus oryzae, belonging to zygomycetes, were screened. Initial screening for fungal cultures (without external enzyme saccharification) showed an ethanol yield maximum of 47.8 ±1.1 to 67.3 ±2.1, and 38.7 ±1.1 to 67.7±1.8 mg per g dry substrate loading from whole-grain bread and white-bread respectively, post the enzymatic liquefaction. Scale-up of the N. intermedia fermentation achieved using bench scale airlift bioreactor showed an ethanol yield maximum of 91.6 ±2.1 and 87.5 ±1.9 mg per g dry substrate loading for whole-grain bread and white-bread respectively.

Key words: Integrated-biorefinery; Filamentous fungi; Bread waste; Ethanol; Animal feed
1. Background
The use of food industry wastes or its intermediate by-products for generating value added products or biofuel is one of the most sustainable and economically feasible methods of waste-to-wealth process. It has been estimated that nearly 30-50% of the edible food is wasted each year globally which amounts to about 1.3 billion metric tons per year [1], part of which is mainly due to the inefficiency of Food Supply Chain (FSC) [2]. The direct economic cost of global food wastage of agricultural products (excluding fish and seafood) is estimated to be about USD 750 billion per year [3]. In the EU, food waste along the food supply chain has been estimated at approximately 89 million metric tons or 180 kg per capita per year, and is expected to rise to about 126 million metric tons a year by 2020 [1]. The production and disposal of EU food waste (89 million tons) leads to an estimated emission of 170 million metric tons of CO₂ and consumes 261 million metric tons of resources. Within the food products sector in the EU, the largest activity is with the manufacture of bread and cakes, comprising about two-fifth (39.8 % or EUR 28.6 billion) of the total food sector value [4]. Among other industrial food products, bread contributes to the major share of waste due its shorter shelf-life (4-7 days). Bread wastes have limited possibilities for reprocessing in the food industry and hence could be efficiently used as a potential energy substrate due to its high nutrients concentration [5, 6]. Annual global production of bread (i.e. white, mixed-grain or wheat-meal bread and bread roll/baguette) exceeds around 141 million metric tons with an estimated wastage of about 7–10%, making waste bread an easily available substrate for its valorization [5]. Research studies have shown that bread residues are high yielding bioenergy substrates mainly for ethanol and biohydrogen production [5, 7]. However, the available nutrients need to be hydrolyzed to utilizable forms, such as glucose and free amino nitrogen (FAN) prior its use by ethanol-fermenting or hydrogen-producing microorganisms. Enzymatic hydrolysis using α-amylase, gluco-amylase and proteases can efficiently facilitate this process [7], thereby increasing the overall process cost. In the present study an alternative method of waste bread valorization is reported by using it as a fermentation substrate for food-grade filamentous fungi, generating an array of value added products such as ethanol, glycerol, and animal feed components. Filamentous fungi are versatile microorganisms that are capable of growing on a wide range of substrates and have been traditionally recognized as the source of nutritious, highly palatable food products [8, 9]. Recent studies have unleashed their ethanol fermenting and enzyme production potential from various substrates of starch or lignocellulose origin [10-13]. However, the use of these edible fungi on an industrial food waste such as waste bread to produce value-added products has not been found in a complete review of the current literature. In this study we hence report a novel and first of its kind approach of a ‘integrated biorefinery’, using waste bread and edible filamentous fungi to produce ethanol and animal feed components at the existing wheat-based ethanol facilities.

2. Experimental
2.1. Fungal strains
Food grade- edible filamentous fungi such as Neurospora intermedia CBS 131.92 and Aspergillus oryzae var. oryzae CBS 819.72 (Centraalbureau voor Schimmelcultures, Netherlands) belonging to ascomycetes and Mucor indicus CCUG 22424 and Rhizopus

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oryzae CCUG 28958 (Culture Collection University of Göteborg, Sweden) belonging to zygomycetes, were used for the present study. Spore solution was prepared from PDA plates after 48h incubation at 35°C and served as an inoculum (concentration 4.7×10⁵ – 1.2 ×10⁷ spores/mL). Cells were grown in 100mL YPD broth media, and cell biomass was harvested after 48 h incubation at 35°C and used as the inoculum (25 to 31 mg).

2.2. Substrate and Enzymes
Two types of industrial waste wheat-bread such as wheat-flour bread (WB) and whole-grain bread (BB) and enzymes alpha-amylase and gluco-amylase were kindly provided by Lantmännen Agroetanol (Norrköping, Sweden). The substrates were dried for 12- 24h at 70°C hot-air oven, followed by particle size reduction (0.2 to 0.25 mm size) using a laboratory rotor beater mill (SM 100, Retsch Technology GmbH, Germany). Samples were autoclaved and stored in airtight containers at room temperature prior to use.

2.3. Enzymatic pretreatment and Fermentation
Liquefaction was carried out using thermostable alpha-amylase (Thermamy l 120L) at pH 5.2± 0.2 for a total treatment time of 4.5h, following a standardized protocol (data not shown). The fungal cultivations were made in 100mL volume (in 250ml Erlenmeyer flasks), for 120h in an orbital shaking water bath (Grant OLS-Aqua pro, UK) at 35°C and 125rpm (with an orbital shaking radius of 9mm and a flask diameter of 85mm), with samples taken every 24h. The pH was adjusted with 1M NaOH. All experiments and analyses were carried out in duplicate and results reported with error bars and intervals representing two standard deviations.

2.4. Scale-up in bench airlift reactor
Scale-up of the fermentation was achieved in a bench scale airlift bioreactor (4.5L) (Belach Bioteknik, Sweden), with a working volume of 3.5L. An internal loop with cylindrical geometry with a diameter 58mm, height 400mm and thickness 3.2mm was used to achieve the airlift-liquid circulation. Aeration at the rate 1.0 vvm (volumeair /volume_media /min) was maintained, using a sintered stainless steel air-sparger (90μm pore-size). Filtration of inlet air was achieved by using a membrane filter (0.1 μm pore size, Whatman, Florham Park, NJ, USA). The cultivation was carried out at pH 5.5±0.2, initially adjusted with 1M NaOH. The fermentation was carried out at a temperature of 35±2°C for 120h.

3. Results
Four food grade filamentous fungal strains were screened for their fermentation capabilities in waste bread with a moisture content of 25.3% (wheat flour bread- WB) and 28.2% (whole grain bread- BB). Liquefaction was carried out at varying total solid concentrations (TS) of 10 to 50% w/w. A solid concentration above 30% showed coagulation, with a maximum of 173.3 ±9.4 and 203.8 ± 7.5 g/L free fermentable glucose release from white bread and whole-grain bread respectively (30% w/w TS). Preliminary screening of the fungal strains N. intermedia, A. oryzae, R. oryzae and M. indicus was achieved in shake flask cultures post the liquefaction process (Figure 1) at a solid loading of 30% (w/w). Fermentation (120h) without external enzyme saccharification resulted in an ethanol yield maximum of 67.3 ±2.1, 64.2 ±2.8, 47.8 ±1.1, and 62.3 ±2.5 mg per g dry whole-grain bread substrate respectively.
However, while using fungal cultures on white-bread, the ethanol yield reduced to about 67.7±1.8, 46.1 ±1.2, 57.6 ±2.3, and 38.7 ±1.1 mg per g dry substrate loading respectively. Results are depicted in Figure 1. Scarification of both whole-grain and white bread using external fungal amyloglucosidase resulted in a maximum of 214.5 ±8.6 and 234.3 ±7.2 g/L glucose (respectively). However with the simultaneous fermentation (SSF) using *N. intermedia*, a reduced ethanol yield of 53.9 ±1.3 and 48.6 ±2.1 mg per g dry bread substrate loading was observed, attributing to the fermentation inhibition at high glucose. Scale-up of the fermentation experiments (post the liquefaction) using *N. intermedia* at 30% solid loading, resulted in a maximum of 27.3±0.8 and 26.2±1.2 g/L ethanol from whole-grain bread and white-bread respectively (Figure 2). At lower solid concentration (10%), an efficient airlift mixing was achieved, however the ethanol yield reduced by 51% while using white-bread. Results of the airlift experiments are depicted in Figure 2. Detailed results will be presented in the full-length paper.

**Figure 1.** Screening for fungal cultures, *N. intermedia* (●); *A. oryzae* (▲); *Mucor indicus* (♦) and *R. oryzae* (■) on (a) whole-grain bread and (b) white bread post enzymatic liquefaction.
4. Conclusion
Promising results were obtained with *N. intermedia*, which was further scaled up in a bench scale airlift reactor. The ease in availability of wheat bread, often wasted during the final consumption stage of the food-supply-chain, to the existing wheat-based ethanol facility could potentially make the current process economically feasible. Detailed results on the fungal fermentation, enzyme characteristics and scale-up of the present work will be presented in the full-length paper.

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