Screening of intact yeasts and cell extracts to reduce Scrapie prions during biotransformation of food waste

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Abstract

Yeasts can be used to convert organic food wastes to protein-rich animal feed in order to recapture nutrients. However, the reuse of animal-derived waste poses a risk for the transmission of infectious prions that can cause neurodegeneration and fatality in humans and animals. The aim of this study was to investigate the ability of yeasts to reduce prion activity during the biotransformation of waste substrates—thereby becoming a biosafety hurdle in such a circular food system. During pre-screening, 30 yeast isolates were spiked with Classical Scrapie prions and incubated for 72 h in casein substrate, as a waste substitute. Based on reduced Scrapie seeding activity, waste biotransformation and protease activities, intact cells and cell extracts of 10 yeasts were further tested. Prion analysis showed that five yeast species reduced Scrapie seeding activity by approximately 1 log10 or 90%. Cryptococcus laurentii showed the most potential to reduce prion activity since both intact and extracted cells reduced Scrapie by 1 log10 and achieved the highest protease activity. These results show that select forms of yeast can act as a prion hurdle during the biotransformation of waste. However, the limited ability of yeasts to reduce prion activity warrants caution as a sole barrier to transmission as higher log reductions are needed before using waste-cultured yeast in circular food systems.

Keywords: Food waste, Prions, Protease, Yeast

Findings

Prions are misfolded proteins that can cause transmissible spongiform encephalopathies (TSEs) that result in fatal neurodegeneration [1, 2]. The infectious form occurs when the cellular membrane bound prion protein (PrPc) is converted into a partially protease-resistant pathologic form (PrPSc) [3]. Infectious prions can cause diseases such as Creutzfeldt–Jakob Disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, Scrapie in sheep and goats, and chronic wasting disease (CWD) in deer [2, 4]. Human dietary exposure to BSE prions resulted in the emergence of a new form of human TSE (variant CJD) and has led to feeding bans of specific risk material, such as brain and spinal cord, to other animals [5, 6]. However, prion transmission still occurs in livestock farms, such as prevalence of Scrapie in Greek sheep [6], and in wild populations, such as the recent CWD outbreak in Norwegian reindeer [7], that poses a substantial risk to the safety of food and public health.

There is increasing pressure to produce more food with fewer resources to feed the growing human population in spite of the depletion of phosphorus, limited arable land and negative effects of climate change. One promising solution to this challenge is to use microorganisms, such as bacteria and fungi, derived from sediment, soil...
and lichens can degrade PrPSc using their proteolytic enzymes [9–11]. Species of yeast have been shown to convert organic waste to high-quality animal feed [12, 13] and exhibit high protease activity [14]. These combined properties may represent a solution to tackle the prion-associated risk during recycling of animal-derived waste while producing feed. In this study we characterized the impact of intact yeast cells and yeast cell extracts on the activity of Classical Scrapie prions during the biotransformation of casein as a waste substitute.

Yeast strains were obtained from the Department of Molecular Science, SLU (Uppsala, Sweden) and precultured from freezer stocks on media of yeast peptone dextrose (YPD) [15]. After pre-culture, yeasts were cultured in YPD broth at 25 °C in a shaker at 120 rpm for 24 h. Yeasts were centrifuged at 3000g, washed twice with phosphate buffer saline and diluted to an OD600nm of 2.0. Separately, yeast extracts were prepared by centrifugation at 5000g. 0.2 μm filter sterilization of supernatant and 20-fold concentration with 10 kDa centrifugal filters (Merck, Solna, Sweden) [16]. Total protease activity was measured by incubating intact and extracted yeasts with casein labelled with fluorescein isothiocyanate for 1 h at 37 °C and measuring fluorescence, according to the manufacturer (Sigma-Aldrich, Stockholm, Sweden).

The Classical Scrapie isolate was derived from experimentally VRQ/VRQ affected sheep (PG127) and end titrated from inoculated tg338 mice at the terminal stage (107.6 ID50/g) [17, 18]. The brain homogenate was serially diluted with glucose to a 10% solution.

Initial screening of yeast with Scrapie was performed at the Swedish National Veterinary Institute (SVA; Uppsala, Sweden) where risk group 3* agents are handled according to Swedish legislation. Aliquots of 100 μL of each yeast isolate (Table 1) were incubated with 800 μL of casein substrate (10 g/L tryptone casein, 10 g/L dextrose, 1 g/L yeast nitrogen base and 0.66 g/L ammonium sulphate) and spiked with 100 μL of Scrapie (final dilution of 106.6 ID50/g) in a permeable deep-well microflask (Applichon, Foster City, CA, USA). Positive and negative controls (presence/absence of yeast and/or Scrapie) at 0 and 72 h of incubation were included. Microflasks were incubated at 20 °C for 72 h in a shaker at 300 rpm to mimic yeast production conditions. Each reaction was stopped by adding yeast protease inhibitor (Roche, Basel, Switzerland) and stored at −80 °C.

Protein misfolding cyclic amplification (PMCA) was used to measure the level of prion seeding activity by amplifying minute amounts of PrPSc at ENVT/INRA (Toulouse, France) [19]. The reaction product was serially diluted 1:10 and used as a seed in PMCA where 5 μL was added to 45 μL of 10% tg338 brain homogenate and amplified using two rounds of 96 cycles of 10 s sonication with 14 min and 50 s of rest at 38.5 °C (QSonica, Newtown, CT, USA) [20]. Presence of PrPSc was detected by Dot blotting using a microfiltration apparatus according to the manufacturer (Bio-Rad, Hercules, CA, USA) and immunoblotting with Sha31 anti PrP antibody according to [20]. Scrapie presence in each sample was compared to the positive-Scrapie control at 72 h to account for reductions in seeding activity from 0 h due to incubation with the yeast substrate.

Initial screening indicated that 19 out of 30 intact yeast isolates resulted in 1–2 log10 reduction of the Scrapie seeding activity (Table 1). Among these isolates, 10 were selected for further testing based on different criteria. Some strains, like Diutina catenulata (J598) and Kluyveromyces marxianus (CBS 1089) were selected due to their reported ability to culture on plant and dairy wastes, their high protease activity and their ability to reduce Scrapie activity. Others like Kluyveromyces lactis (CBS 2359), Phaffia rhodozyma (J552) and Scheffersomyces stipites (CBS 5774) were excluded because of their low reported protease activity and their lack of Scrapie reduction.

The 1:10 dosage of Scrapie spiked in the yeast cultures was observed to inhibit yeast growth, thus the next experiment used a 1:100 dilution. For intact yeast, incubation was repeated as previously described, except 890 μL of casein substrate was spiked with 10 μL of Scrapie (final dilution of 105.6 ID50/g) and incubated with 100 μL of intact yeast. For yeast extracts, 135 μL was incubated with 15 μL of Scrapie for 24 h at 30 °C in a micro-titre plate.

These new tests indicated that that the processing of the substrate by five out of the 10 intact and/or cell extracts resulted in a 90% decrease (approximatively 1 log10) of the PG127 Scrapie seeding activity (Table 2). Both the intact cells and extracts of Cryptococcus laurentii reduced Scrapie activity. Intact forms of D. Catenulata and Wickerhamomyces anomalus and cell extracts of Blastobotrys adeninivorans and Debaryomyces hansenii also reduced Scrapie seeding activity. Interestingly, the three yeast proteases that reduced Scrapie also had the highest level of protease activity, which was especially high for Cr. Laurentii. In contrast, protease activity was lowest for intact yeast species of D. Catenulata and W. Anomalus, while they were still able to reduce Scrapie activity.

More than 5 log10 reduction was needed to safely eliminate prions in this study, thus 1 log10 reduction was insufficient. Moreover, even higher inactivation is needed depending on the source material and its infectivity [21, 22]. Therefore, these findings indicate the potential of yeast biotransformation as one hurdle, but the limited reduction indicates additional hurdles, such as heat...
treatment, are needed before using waste-cultured yeast as animal feed or food [2, 23].

This study is the first attempt to determine the ability of yeasts to reduce prion activity. However, there are several ways this approach could be improved. First, the number of yeast isolates tested could be expanded and even filamentous fungi should be included since they too have been used in animal feed. Second, the period of yeast–Scrapie incubation (i.e. 72 h) could be increased, such as 8 days used previously with soil microbes [11], to increase the likelihood of Scrapie reduction. Third, the pH and temperature could be increased, such as pH 10 and > 30 °C used previously with bacterial proteases [9, 24, 25], although these conditions may impact yeast growth and jeopardise their use as animal-grade feed. Fourth, different techniques could be used to produce cell extracts, such as acetone extraction as described previously [10], that may improve prion reduction.

Scrapie reduction by two intact yeasts with low protease activity was unexpected (Table 2) since prion

| Yeast species | Reference strain | SLU strain | Waste substratea | Protease activityb | Scrapie reductionc | Highest detectiond |
|---------------|------------------|-----------|-----------------|-------------------|-------------------|-------------------|
| *Aureobasidium pullulans* | NA | J126 | NA | NA | 2/2 | $10^{-5.5}$ |
| *Blastobotrys adeninivorans* | CBS 8244 | J562 | Plant, dairy | Low | 1/2 | $10^{-5.0}$ |
| *Cyberlindnera jadinii* | CBS 621 | J556 | Plant, dairy | High | 1/2 | $10^{-5.0}$ |
| *Debaryomyces hansenii* | CBS 1962 | J136 | Dairy | Low | 1/2 | $10^{-6.0}$ |
| *Debaryomyces hansenii* | CBS 6958 | J187 | Dairy | Low | 0/2 | $10^{-6.0}$ |
| *Debaryomyces hansenii* | NRRL 7268 | J345 | Dairy | Low | 1/2 | $10^{-6.0}$ |
| *Diatina catenulata* | NA | J598 | Plant, dairy | High | 2/2 | $10^{-5.0}$ |
| *Kluveromyces lactis* | CBS 2359 | J469 | Plant, dairy | NA | 0/2 | $10^{-6.0}$ |
| *Kluveromyces marxianus* | CBS 6556 | J137 | Dairy | High | 1/2 | $10^{-6.0}$ |
| *Kluveromyces marxianus* | CBS 1089 | J186 | Dairy | High | 2/2 | $10^{-5.0}$ |
| *Kluveromyces marxianus* | CBS 1555 | J367 | Dairy | High | 0/2 | $10^{-6.0}$ |
| *Ogataea polymorpha* | CBS 4732 | J549 | Plant, dairy | Low | 1/2 | $10^{-5.5}$ |
| *Pichia kudriavzevii* | CBS 2062 | J550 | Plant, dairy | NA | 1/2 | $10^{-5.5}$ |
| *Saccharomyces cerevisiae* | CBS 2978 | J122 | Plant, seafood | Low | 0/2 | $10^{-6.0}$ |
| *Saccharomyces cerevisiae* | NA | J545 | Plant, seafood | Low | 1/2 | $10^{-5.5}$ |
| *Saccharomyces cerevisiae* | NA | J546 | Plant, seafood | Low | 1/2 | $10^{-5.0}$ |
| *Scheffersomyces stipitis* | CBS 5774 | J563 | Plant, dairy | Low | 0/2 | $10^{-6.0}$ |
| *Torulaspora delbrueckii* | NA | J352 | Plant, dairy | NA | 1/2 | $10^{-5.5}$ |
| *Wickerhamomyces anomalus* | CBS 100487 | J121 | Plant, dairy | Low | 0/2 | $10^{-6.0}$ |
| *Wickerhamomyces anomalus* | CBS 1947 | J379 | Plant, dairy | Low | 0/2 | $10^{-7.0}$ |
| *Wickerhamomyces anomalus* | CBS 100487 | J475 | Plant, dairy | Low | 0/2 | $10^{-6.0}$ |
| *Yarrowia lipolytica* | CBS 6114 | J134 | Plant, dairy, seafood | High | 1/2 | $10^{-5.5}$ |

| Yeast species | Reference strain | SLU strain | Waste substratea | Protease activityb | Scrapie reductionc | Highest detectiond |
|---------------|------------------|-----------|-----------------|-------------------|-------------------|-------------------|
| *Cryptococcus laurentii* | CBS 6473 | J463 | NA | Low | 2/2 | $10^{-5.5}$ |
| *Holtermanniella takashimae* | CBS 11174 | J596 | Plant | NA | 1/2 | $10^{-5.0}$ |
| *Naganishia cerealis* | NA | J595 | Plant, dairy | NA | 2/2 | $10^{-4.5}$ |
| *Phaffia rhodozyma* | NA | J552 | Plant, dairy | NA | 0/2 | $10^{-7.0}$ |
| *Rhodotorula glutinis* | NA | J195 | Plant, dairy | High | 1/2 | $10^{-5.5}$ |
| *Sporidiobolus pararoseus* | CBS 4216 | J466 | Plant, dairy | High | 0/2 | $10^{-6.0}$ |
| *Sporidiobolus roseus* | NA | J104 | NA | 0/2 | $10^{-6.0}$ |
| *Sporidiobolus salmonicolor* | CBS 490 | J360 | NA | NA | 1/2 | $10^{-5.5}$ |

| Yeast species | Reference strain | SLU strain | Waste substratea | Protease activityb | Scrapie reductionc | Highest detectiond |
|---------------|------------------|-----------|-----------------|-------------------|-------------------|-------------------|
| *Basidiomycota phyla* | CBS Central Bureau for Fungus Cultures (Utrecht, Netherlands); NRRL Northern Regional Research Laboratory (Peoria, IL, USA); NA not available; SLU Swedish University of Agricultural Sciences (Uppsala, Sweden) | | | | | |

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* Food grade yeasts that have been cultured on these waste substrates [12, 13]
* High indicates that > 50% of isolates were reported to produce protease and low indicates < 50% [14]
* Number of positive tests that showed 1–2 log10 reduction in Scrapie activity after incubation for 72 h
* Mean dilution of highest positive detection of Scrapie after immunoblotting

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Table 1: Ability of intact yeast isolates to grow on waste, produce proteases and reduce Scrapie activity
Table 2 Reduction of Scrapie activity by intact and extracted yeasts and their corresponding protease activity

| Yeast species                        | Scrape reductiona and highest detectionb | Protease activityc |
|-------------------------------------|------------------------------------------|-------------------|
|                                     | Intact yeast                             | Extracted yeast   | Intact yeast | Extracted yeast |
| A. pullulans (Saccharomyces cerevisiae) | 0/4 10−4.1                               | 0/2 10−3.3        | 18.6 (4.9)  | 13.6 (16.7)    |
| B. adeninivorans                    | 0/4 10−3.9                               | 1/2 10−3.8        | 16.2 (3.8)  | 144.7 (60.1)   |
| C. laurentii                        | 3/4 10−3.3                               | 2/2 10−2.8        | 33.4 (48.9) | 286.5 (6.6)    |
| D. hansenii                         | 0/4 10−4.8                               | 2/2 10−3.8        | 42.3 (11.0) | 61.7 (41.8)    |
| D. catenulata                       | 2/4 10−3.4                               | 0/2 10−3.8        | 16.7 (2.8)  | 22.5 (1.7)     |
| H. takashimae                       | 0/4 10−4.1                               | 0/2 10−3.5        | 19.8 (0.6)  | 19.7 (0.1)     |
| K. marxianus                        | 0/4 10−3.6                               | 0/2 10−3.8        | 39.8 (46.3) | 48.7 (1.1)     |
| N. cerealis                         | 0/4 10−4.3                               | 0/2 10−4.8        | 7.1 (2.1)   | 43.4 (63.0)    |
| S. cerevisiae                      | 0/4 10−3.5                               | 0/2 10−4.8        | 18.6 (2.3)  | 23.6 (2.6)     |
| W. anomalous                        | 3/4 10−3.1                               | 0/2 10−3.5        | 12.9 (3.1)  | 21.5 (0.1)     |

a Number of positive tests that showed 0.5–1.0 log10 reduction in Scrapie after incubation compared with a control

b Mean dilution of highest positive detection of Scrapie after immunoblotting

c Analysed by the fluorescence detection of trypsin (ng) from casein substrate after 60 min incubation at 37 °C

degradation has been shown to be mediated by serine proteases [10]. In a similar study, 199 food-borne bacterial isolates were screened for Scrapie reduction and six were positive, while one isolate had low protease activity [16]. These findings suggest that total protease activity is not the only defining aspect that enables yeasts to degrade prions. In addition, the aforementioned study noted the bacterial isolates represent four different genera and suggested that their enzymes may share specific properties that allow them to effectively degrade Scrapie [16]. In comparison, four out of five yeast isolates in the present study belong to the phylum Ascomycetes, order Saccharomycetales, while the most promising isolate, Cr. laurentii, belongs to the phylum Basidiomycetes, order Tremellales. More research is needed to compare the enzymatic properties of different groups of yeast and determine the underlying mechanism that enables yeast to degrade Scrapie prions.

In conclusion, these results indicate that some yeast species, both as intact cells and cell extracts, have the potential to reduce the transmission of prions while converting organic waste to high-quality animal feed, thereby becoming a prion hurdle. This study is an important first step, although additional hurdles are required to prevent the transmission of prions into circular food systems.
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