RESEARCH ARTICLE

Utilization of salt-rich by-products from the dairy industry as feedstock for recombinant protein production by *Debaryomyces hansenii*

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Funding information
European Cooperation in Science and Technology, Grant/Award Number: CA-18229; Novo Nordisk Fonden, Grant/Award Number: NNF17SA0031362 and NNF19SA0057794

Abstract

The dairy industry processes vast amounts of milk and generates high amounts of secondary by-products, which are still rich in nutrients (high Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) levels) but contain high concentrations of salt. The current European legislation only allows disposing of these effluents directly into the waterways with previous treatment, which is laborious and expensive. Therefore, as much as possible, these by-products are reutilized as animal feed material and, if not applicable, used as fertilizers adding phosphorus, potassium, nitrogen, and other nutrients to the soil. Finding biological alternatives to revalue dairy by-products is of crucial interest in order to improve the utilization of dry dairy matter and reduce the environmental impact of every litre of milk produced. *Debaryomyces hansenii* is a halotolerant non-conventional yeast with high potential for this purpose. It presents some beneficial traits – capacity to metabolize a variety of sugars, tolerance to high osmotic environments, resistance to extreme temperatures and pHs – that make this yeast a well-suited option to grow using complex feedstock, such as industrial waste, instead of the traditional commercial media. In this work, we study for the first time *D. hansenii*'s ability to grow and produce a recombinant protein (YFP) from dairy saline whey by-products. Cultivations at different scales (1.5, 100 and 500 ml) were performed without neither sterilizing the medium nor using pure water. Our results conclude that *D. hansenii* is able to perform well and produce YFP in the aforementioned salty substrate. Interestingly, it is able to outcompete other microorganisms present in the waste without altering its cell performance or protein production capacity.

INTRODUCTION

The food industry is the majorly responsible for food and water waste among industrial activities (Ravindran & Jaiswal, 2016). One example is the dairy industry. From cheese production, vast amounts of a green-yellowish effluent (whey) are obtained, which is one of the most environmentally damaging wastes generated by this industry if disposed to the environment (Papademas & Kotsaki, 2019). The global production of whey is estimated to be around 190 million tons per year, being Europe the highest cheese consumer (approximately 10 million tons of cheese consumed annually) and whey producer (Asunis et al., 2020; Papademas & Kotsaki, 2019).

Whey retains up to 55% of the milk nutrients (Macwan et al., 2016) and it is composed of lactose (60–70% of the dry fraction), proteins (e.g., β-lactoglobulin, α-lactalbumin, or immunoglobulin), lipids and small amounts of minerals and vitamins (e.g., calcium, phosphorous, sodium, or potassium) (Nishanthi et al., 2017; Zaccheria et al., 2017).

The waste treatment needed to sufficiently reduce the Chemical Oxygen Demand (COD) and the Biological Oxygen Demand (BOD) levels is a tedious and expensive process; therefore, most companies are looking for sustainable alternatives to revalue this waste. Currently, it is possible to recover the high-value proteins from the whey by ultrafiltration, and part of the lactose present in the whey permeate can be crystallized and revalorized as a food ingredient (Zaccheria et al., 2017). The final by-product obtained after the crystallization is the delactosed whey permeate (DLP). This by-product is still rich in nutrients, as it contains around 10–20% of whey lactose (Shen et al., 2019).

Salt (NaCl) is one of the main components of cheese since it contributes to its flavour and preservation (Guinee, 2004). Hence, aside from whey, the dairy industry also generates vast amounts of a waste effluent with a very high salt concentration (up to 2 M NaCl). Treating this residue to reduce the salt concentration under the legal dumping limits is also challenging, so new alternatives to revalue high-salt effluents also need to be studied (Chen et al., 2018, 2019).

One possibility to give a 'second life' to the DLP and salty waste (SW) is to use them as a substrate for other processes, where new valuable bioproducts, such as food additives, chemicals, enzymes or even therapeutical proteins could be produced. Several authors have studied the biological revalorization of DLP to produce bioproducts (Banger et al., 2021; Cervantes et al., 2020; Parashar et al., 2016). Shen et al. (2019) studied the production of ethanol through bacterial fermentation of this waste. However, they had to use a recombinant *Corynebacterium glutamicum* strain, as the wild-type bacteria lacked lactose metabolism. Moreover, they needed to add additional cations (e.g., Mn^{2+}, Fe^{2+}) to the media to obtain optimal cell densities.

Yeast could also be used for this purpose. However, the considered as one of the best yeast workhorses, *Saccharomyces cerevisiae*, requires genetic modifications to metabolize lactose (Rubio-Texeira et al., 1998; Sreekrishna & Dickson, 1985) and has strong difficulties growing in moderate osmotic media (Norkrans & Kylin, 1969). In contrast, other species like some non-conventional yeasts (e.g., *Kluyveromyces lactis*, *Kluyveromuces marxianus* or *Candida pseudotripicalis*) have the inherent ability to assimilate and ferment lactose, but none of them can survive in high salinity environments (Karim et al., 2020; Sampaio et al., 2020; Shen et al., 2019). Therefore, the biological revalorization of high osmotic waste streams is challenging, as

most microorganisms cannot thrive under this harsh external osmotic pressure.

Debaryomyces hansenii (*D. hansenii*) is a non-conventional yeast with high potential for the transition to the so-called green biotechnology (Navarrete et al., 2022; Prista et al., 1997). It is a halotolerant, osmotolerant and xelotolerant yeast that can be isolated from environments with low water activities, such as seawater, Atlantic and Artic glaciers, cheese, cured meat or soil (Andreu et al., 2022; Prista et al., 2005).

Since the early 70s, *D. hansenii* has been a model for studying salt tolerance mechanisms in eukaryotic cells (Adler et al., 1985; Adler & Gustafsson, 1980; Gezelius & Norkrans, 1970; Lucas et al., 1990). Many studies have demonstrated that *D. hansenii* metabolism is enhanced when moderate-high concentrations of salt (1 M) are found in the media, even though it can tolerate up to 4 M of salt (Breuer & Harms, 2006). Recently, Navarrete, Frost, et al. (2021) performed a physiological characterization of *D. hansenii* in controlled bioreactors testing different salt concentrations (either NaCl or KCl) and assessing cell performance. Moreover, they described a novel survival strategy of this yeast in high salinity environments: when the salt concentration increases from 1 M (optimal for *D. hansenii*) to 2 M or more, the yeast changes its metabolism from growing as fast as possible and limiting the nutrients for the competitors, to grow as much as possible and overpopulate the area (Navarrete, Frost, et al., 2021).

The global molecular mechanism involved in the natural halotolerant behaviour of *D. hansenii* is still far from being fully understood. However, several transport systems involved in glycerol, K^+ and Na^+ fluxes have been hypothesized to play an important role. They include: (i) H^+-ATPases: DhPma and DhVma2 (Prista et al., 2005); (ii) pumps to uptake K^+: Hak1 and Trk1 (Martínez et al., 2011; Prista et al., 2007); (iii) pumps to extrude Na^+: Nha1, Ena1, Ena2, Nhx1 and Kha1 (Almagro et al., 2001; Carcía-Salcedo et al., 2007; Montiel & Ramos, 2007; Velkova & Sychrova, 2006); (iv) and glycerol or arabinitol accumulation as compatible solutes (Adler et al., 1985; Gustafsson & Norkrans, 1976). In 2021, our group performed the first multi-omics analysis of *D. hansenii* when growing in chemostats cultivations and in the presence of 1 M NaCl or KCl (Navarrete, Sánchez, et al., 2021). Surprisingly, we found a novel and uncharacterized transmembrane transporter (B5RUG0) that could also be involved in the long-term salt-tolerant character of *D. hansenii*.

Salt tolerance is not the only quality that makes *D. hansenii* an attractive host for the industry, as it presents some other interesting features. For example, it can assimilate and ferment a broad range of carbon sources apart from glucose, such as lactose, galactose or glycerol (Laadan et al., 2008). In addition, we recently found that in the presence of salt, pentose catabolism is also stimulated, allowing this yeast to

metabolize sugars such as arabinose, maltose and xylose (Navarrete, Sánchez, et al., 2021).

Moreover, in complex culture media, the yeast can synthesize a killer toxin (mycocin), which is described to be effective against different microorganisms and other yeast species, however innocuous to humans (Marquina et al., 2001; Santos et al., 2002).

The presence of salt in the media is not only needed for an optimal performance of *D. hansenii*, but also confers a protective effect to the cell against different abiotic stresses. For example, Dyerberg et al. (2022) demonstrated that concentrations of 1 M NaCl relieve the stress caused by fermentation inhibitors often released from the lignocellulosic biomass pretreatment, such as furfural or hydroxymethylfurfural. Moreover, Navarrete, Sánchez, et al. (2021) also observed an overexpression of two genes (TPO1 and PDR5), which are usually involved in drug resistance, when high sodium concentrations were present in the media (Navarrete, Sánchez, et al., 2021). Salt also protects the yeast when growing at high temperatures (up to 35 degrees) and extreme pH (less than 4 or more than 8) (Almagro et al., 2000; Papouskova & Sychrova, 2007). Last but not least, sodium also confers protection against oxidative stress, such as the one caused by the presence of H_2O_2 (Ramos-Moreno et al., 2019). All these inherent beneficial traits that *D. hansenii* displays make it a well-suited platform for revalorizing industrial by-products, including dairy waste.

In this work, we analyse *D. hansenii*'s performance when growing in two different by-products from a Danish dairy industry (Arla foods®/Arla Food Ingredients P/S Denmark) as a feedstock: delactosed whey permeate (DLP) and an effluent containing 1.5–2 M of salt (SW). Fermentations using the Arla by-products as feedstock at different scales (1.5, 100 and 500 ml) were performed, avoiding the traditional media sterilization step, which is one of the most expensive costs of a bioprocess. As expected, both by-products contain some inherent microorganisms derived from the dairy production process. Therefore, skipping the media sterilization also generates two main challenges: (i) *D. hansenii* has to compete with other species for nutrients; and (ii) methods commonly used to measure biomass, such as the Optical Density (OD) or the Dry Weigh quantification (DW), are not accurate in independently monitoring the growth of the yeast in complex and mixed cultures. Although they can still be used to follow the evolution of the global biomass during cultivation, the measurement will quantify *D. hansenii* biomass but also the rest of the microorganisms present in the by-products. Moreover, the dairy by-products are thick and contain some fat aggregates that can also interfere with the OD and DW measurements. To solve these challenges, pasteurizing both by-products as a more cost-efficient alternative to the sterilization process is studied, and the recombinant Yellow Fluorescent Protein (YFP) was introduced into the yeast and used as a reporter protein to exclusively monitor its growth among the other microorganisms present in the dairy by-products.

Although *D. hansenii* has already been used to naturally produce xylitol, trehalose and fat-soluble vitamins (López-Linares et al., 2018; Prista et al., 2016), this is the first time that *D. hansenii* is used to produce a recombinant protein (YFP) from a non-conventional feedstock. Hence, this study is a proof of concept of both, the potential that *D. hansenii* has as a microbial cell factory for revalorizing salt-rich industrial waste, and its ability to produce recombinant products.

EXPERIMENTAL PROCEDURES

Strains and standard culture conditions

Debaryomyces hansenii strain IBT22 from the *Institut for Bioteknologi* (IBT) at the Technical University of Denmark was used in this work. Previous studies performed in our group showed a better performance of this strain when salt is present in the media than the standard *D. hansenii* CBS767 (Navarrete, Frost, et al., 2021). From a glycerol stock (18–20% final glycerol concentration) stored at –80°C, YPD plates containing 1% yeast extract, 2% peptone, 2% agar (Sigma-Aldrich, Germany) and 2% of D-(+)-glucose monohydrated (VWR Chemicals, Germany) were used to grow the cells at 28°C. Liquid precultures of the yeast in 500 ml baffled Erlenmeyer shake flasks (culture volume 100 ml) were performed for at least 24 h, at 28°C, and 150 rpm, in 6.7 g/L of synthetic complete Yeast Nitrogen Base (YNB) medium (Thermo Fisher) plus 2% of D-lactose monohydrated (VWR Chemicals, Germany). The pH of the medium was adjusted to 6.0 with NaOH. All the solutions were autoclaved at 121°C for 20 min.

A transformant *D. hansenii* IBT22 strain expressing the Yellow Fluorescent protein (YFP) was also used for this study (see below). In this case, both the YPD (for agar plates) and YNB (for precultures) media were supplemented with 100 μg/ml of the antibiotic Nourseothricin after sterilization (NTC; Werner BioAgents).

Finally, *Escherichia coli* DH5α strain was used for the maintenance of the plasmid encoding the YFP. From a glycerol stock (18–20% final glycerol concentration), cells were grown in agar plates containing Lysogeny Broth (LB) medium, 100 mg/L ampicillin (Sigma) and 2% agar. Then, cells were precultured in liquid LB medium with 100 mg/L ampicillin.

YFP expression by *D. hansenii*

A transformant *D. hansenii* IBT22 strain expressing the YFP was constructed for this study. For this purpose, the plasmid pDIV213 provided by Mortensen group (DTU

Bioengineering) was used. It contains the encoded YFP gene sequence between a Sc_TEF1 promoter and a Sc_synthetic terminator, and the resistance marker to NTC to select the transformed cells. A GeneElute Plasmid MiniPrep Kit (Sigma-Aldrich) was used to purify the plasmid from *E. coli*. Then, it was cut using the restriction enzymes PacI, SpeI and BglI to separate the desired fragment (containing only the YFP gene and the NTC resistance marker with their corresponding promoter and terminator) from the rest of the plasmid. The different obtained pieces were separated by gel electrophoresis (2% agarose), and the desired band was purified using the Illustra Gel Band Purification kit (GE health care). Finally, *D. hansenii* was transformed with the desired fragment that was integrated into the genome of the cell in a random locus (random integration) following the non-homologous end-joining (NHEJ) native DNA repair pathway.

The transformation was performed following the electroporation protocol described by Strucko et al. (2021), using the MicroPulser electroporator (BioRad) on manual settings at 2.3 kV. YPD + NTC agar plates were used for the selection of the transformant cells. Each positive colony integrated the YFP fragment into a different locus and with a different number of copies (unknown). Among the obtained positive colonies, 16 were selected and isolated in new YPD + NTC plates to further analyse their phenotype. A test with different media was performed to choose the best transformant strain and to ensure that no essential gene had been interrupted by the integration of the YFP into the genome. To do that, the 16 transformant strains were spotted from liquid YNB precultures into agar plates containing YNB (6.7 g/L), agar (2%), carbon source (2% of D-glucose monohydrated or D-lactose monohydrated), and NaCl (0 M or 1 M) using the ROTOR HDA colony picker (Singer Instruments) in technical quadruplicates. The plates were incubated at 28°C for 144 h. Pictures of the plates were taken using the Phenobooth (Singer Instruments) to compare the size of the colonies (Figure S1). All transformant strains could grow in the conditions tested similarly to *D. hansenii* WT, with minor differences between them. Three transformants (T4, T6 and T8) were selected for further analysis. Growth profiles of the three transformants and the WT strains were performed in 500 ml shake flasks (100 ml working volume). Two different media were analysed: (i) synthetic YNB (6.7 g/L), D-lactose monohydrated (2%), and 0.75 M NaCl, and (ii) a mix of DLP and SW in a proportion of 1:1 (v/v) supplemented with YNB (6.7 g/L). The pH was adjusted to 6.0 in both media. The shake flasks were inoculated with the corresponding *D. hansenii* strain at an initial OD of 0.1, and were incubated at 28°C, 150 rpm for 120 h. Samples to measure the OD_{600} were taken

over the cultivation. The experiment was performed in biological duplicates.

T4 was the transformant strain that showed the most similar behaviour to the WT when growing in the dairy by-products (Figure S2) and was thus selected to be used for the experiments performed in this work.

Arla foods/Arla foods ingredients dairy by-products streams

Two different effluents from the cheese production provided by the Arla foods®/Arla Foods Ingredients company (Denmark) were used as a feedstock in this study: delactosed whey permeate (DLP), and an effluent with 1.5–2 M of salt (SW). The main chemical composition of both by-products is summarized in Table S1. The waste streams were stored at –20°C for preservation.

When required, both effluents were pasteurized. The pasteurization process consisted on warming them in a water bath at 63°C during 30 min.

Microfermentation cultivations

Microfermentation experiments were performed in biological triplicates in the BioLector II (m2p Labs, Germany) using a 48-well FlowerPlate (MTP-48-B, m2p Labs, Germany) with a working volume of 1.5 ml.

Precultures of both *D. hansenii* IBT22 strains (*D. h* WT and *D. h* expressing the YFP) were prepared in 500 ml shake flasks (50 ml culture volume) and incubated at 28°C, 150 rpm, for at least 24 h. Then, the cells were washed twice with ddH$_2$O, and inoculated in the corresponding wells to have an initial OD_{600} 0.1 in 1.5 ml.

Four different culture media were analysed (Table 1). All of them were supplemented with 6.7 g/L of YNB and the pH was adjusted to 6.0 with NaOH. Potassium phthalate monobasic ≥99.5% (20.4 g/L, Sigma-Aldrich, Germany) was also added and used as a pH buffer. In some cases, the media was pasteurized.

Two filters were used in the BioLector II: biomass (gain 1) and mVenus (gain 7). The humidity was maintained at >85%, the temperature was set at 28°C and the agitation speed at 1000 rpm. The oxygen supply was the atmospheric air (20.8 5%). Biomass formation (light scattered units, LSU) and the fluorescence produced (arbitrary units) were monitored for 90 h and analysed using R version 2.12.1.

In order to compare the fluorescence produced during microfermentation experiments (monitored with the BioLector) with the fluorescence produced during larger scale cultivations (monitored using the CLARIOstar Plus microplate reader), the fluorescence

TABLE 1 Composition of the media used in this study.

Medium	AFI DLP	Arla SW	Pure lactose	Pure NaCl	YNB (g/L)	Final lactose concentration	Final NaCl concentration (M)
i	50% of the volume	50% of the volume	—	—	6.7	6%	0.75
ii	50% of the volume	—	—	50% of the volume ($ddH_2O + 0.75\,M\ NaCl$)	6.7	6%	0.75
iii	—	50% of the volume	50% of the volume ($ddH_2O + 6\%$ pure lactose)	—	6.7	6%	0.75
iv	—	100% of the volume	6%	—	6.7	6%	1.5

emitted in each well at the end of the microcultivations (endpoint fluorescence) was also measured with the CLARIOstar Plus equipment.

Finally, *D. hansenii*-specific growth rate values (μmax) were calculated based on the LSU data for all the conditions tested.

Shake flask cultivations

Shake flasks cultivations were carried out in biological triplicates using 500 ml baffled Erlenmeyer shake flasks, with a working volume of 100 ml.

The transformant *D. hansenii* IBT22 expressing the YFP was precultured in 500 ml shake flasks (50 ml culture volume) for at least 24 h at 28°C and 150 rpm. Then, the cells were washed twice with ddH_2O and inoculated to the shake flasks at an initial OD_{600} 0.1.

A combination of both by-products (DLP and SW) in a proportion of 1:1 (v/v), and supplemented with synthetic YNB (6.7 g/L), was used as a culture medium (Table 1, medium i). The pH was adjusted to 6.0 with NaOH, and the medium was pasteurized. A control was also included in this experiment, which consisted in only the medium, without being inoculated.

Cells were incubated at 28°C, 150 rpm for 90 h. Optical density (OD_{600}) and fluorescence of the cultures were monitored during the cultivation. Finally, *D. hansenii* -specific growth rate values (μmax) were calculated based on the biomass profiles.

Bioreactor cultivations

Batch fermentations were perfomed in biological triplicates in 1 L Biostat Qplus bioreactors (Sartorius Stedim Biotech, Germany), with a working volume of 500 ml.

A mix of both Arla streams (DLP and SW) in a proportion of 1:1 (v/v), and supplemented with synthetic YNB (6.7 g/L), was used as a feedstock for the fermentations (Table 1, medium i). The pH was

adjusted to 6.0 with NaOH. When required, the media was pasteurized.

Precultures of either *D. hansenii* WT or *D. hansenii* expressing the YFP were incubated in at 28°C, 150 rpm, for at least 24 h in 500 ml shake flasks (50 ml culture volume). Then, the cells were washed twice with ddH_2O and inoculated in the bioreactors to have an initial OD_{600} 1.

The temperature in the bioreactors was kept at 28°C, the stirring was set at 150 rpm and the pH was controlled by pH sensors (Model EasyFerm Plus K8 160, Hamilton) and maintained at 6.0 by the automatic addition of 2 M NaOH/ 2 M H_2SO_4. The volumetric flow rate (aeration) was set at 0.5 vvm and dissolved oxygen (DO) sensors (Model OxyFerm-FDA 160, Hamilton) were used to measure the DO concentration.

Samples for fluorescence measurement were taken throughout the fermentation, and the Carbon Dioxide Evolution Rate (CER) was calculated from the CO_2 produced.

Fluorescence analysis

YFP fluorescence was measured with the CLARIOstar Plus microplate reader (BMG Labtech, Germany), using black Nunc 96-well microplates (Nunc 167008, Sigma-Aldrich). The excitation and the emission wavelengths were set at 510 and 545 nm, respectively, and the plate was shaken at 100 rpm during 15 s before each measurement.

Carbon dioxyde evolution rate (CER) calculation

The CO_2 and the O_2 concentrations were monitored in real time during the cultivations, as the off-gas line of the bioreactors is connected to a mass spectrometer (model Prima PRO Process MS, Thermo Scientific,

UK). CER [mmol/L/h] was calculated from the off-gas CO_2 emission using the following equation:

$$CER \left[\frac{mmol}{L*h} \right]$$
$$= \frac{\frac{Aeration \left[\frac{L}{h} \right]}{Working\ volume\ [L]} * \frac{CO_2\ emission\ (t)\ [\%]}{100} * Pressure\ [atm]}{Ideal\ gas\ constant \left[\frac{L*atm}{K*mol} \right] * Gas\ temperature\ [K]}$$

where the aeration was 30 L/h; the working volume 0.5 L; the pressure 1 atm; the ideal gas constant 0.082 [L*atm/K*mol]; the gas temperature 303.15 K; and the CO_2 emission detected by the mass spectrometer at each time.

Statistical analysis

Debaryomyces hansenii-specific growth rate values (μmax) were statistically compared by the Tukey's test ($p < 0.05$ confidence) using OriginPro (OriginLab Corporation, version 2022, Northampton, MA, USA). μmax values were classified in letter groups and those labelled with the same letter did not present any statistically significant difference.

RESULTS

Determination of the optimal lactose concentration as carbon source

DLP contains a high lactose concentration, approximately 11–12% of this carbon source (Table S1). Hence, preliminary microfermentation experiments were performed to determine which concentration of sugar resulted in a better performance of the yeast. The results showed a better growth of *D. hansenii* when the initial lactose concentration in the media was between 6% and 8% (Figure S3). However, 6% was the concentration chosen to prepare the media for the following experiments (Table 1), as it can be achieved by diluting

the DLP by half with ddH$_2$O or by mixing the DLP with the SW in a proportion of 1:1 v/v, revalorizing both by-products equally (same volume of each one).

These experiments also demonstrated that an additional nitrogen source needs to be added to the DLP for optimal performance of the yeast (Figure S3). Therefore, synthetic complete Yeast Nitrogen Base (YNB) was supplemented to the media as a nitrogen source for the yeast culture (Table 1).

Performance of *D. hansenii* in both Arla by-products

As this study aimed to evaluate the use of both Arla by-products as fermentation feedstock (DLP and the SW), their role in the performance of *D. hansenii* was investigated. The growth of the yeast and fluorescence emitted were evaluated in each individual by-product (combined with pure lactose or NaCl) as well as in a mix of both effluents. In this regard, different media combinations were tested, as presented in Table 1. Specific growth rate values (μmax) were calculated based on the biomass profiles obtained (Table 2). In order to have comparable data with larger-scale processes (shake flask and 1 L bioreactor experiments), the fluorescence emitted at the end of the microfermentations (measured with the BioLector) was also quantified using the CLARIOstar Plus microplate reader, and the results are summarized in Table 2 (Endpoint fluorescence).

As shown in Figure 1, *D. hansenii* grew and produced recombinant YFP in all the media tested, even though some differences were observed. Higher μmax values were obtained when the yeast grew in a mix of SW and pure lactose (media iii and iv), even when the initial concentration of NaCl was higher (1.5 M) (Figure 1). However, the maximum growth rate was achieved when the SW was diluted by half with ddH$_2$O (medium iii), resulting in a final NaCl concentration of 0.75 M. On the other hand, the highest YFP production (fluorescence) was not achieved in these two particular conditions (Table 2).

TABLE 2 Maximum specific growth rate values (μmax) determined by LSU data, and fluorescence intensity emitted at the end of the BioLector cultivations measured with CLARIOstar plus equipment (endpoint fluorescence).

Medium	μmax (h^{-1}) ± SD	Tukey test	Endpoint fluorescence ± SD
(i) DLP + SW (0.75 M)	$0.202 \pm 7.47 \times 10^{-3}$	A	$1.21 \times 10^6 \pm 73,229$
(ii) DLP + pure NaCl (0.75 M)	$0.131 \pm 1.50 \times 10^{-3}$	B	$8.49 \times 10^5 \pm 37,501$
(iii) SW (0.75 M NaCl) + pure lactose	$0.276 \pm 5.41 \times 10^{-3}$	C	$6.43 \times 10^5 \pm 15,288$
(iv) SW (1.5 M NaCl) + pure lactose	$0.157 \pm 1.64 \times 10^{-3}$	D	$4.83 \times 10^5 \pm 18,196$

Note: The data shown are mean values of three biological triplicates ± standard deviation. μmax values are classified in different statistically significant groups (letters) according to Tukey's test. Groups shearing the same letter are statistically no different between them, whereas groups with different letters are statistically different ($p < 0.05$ confidence).
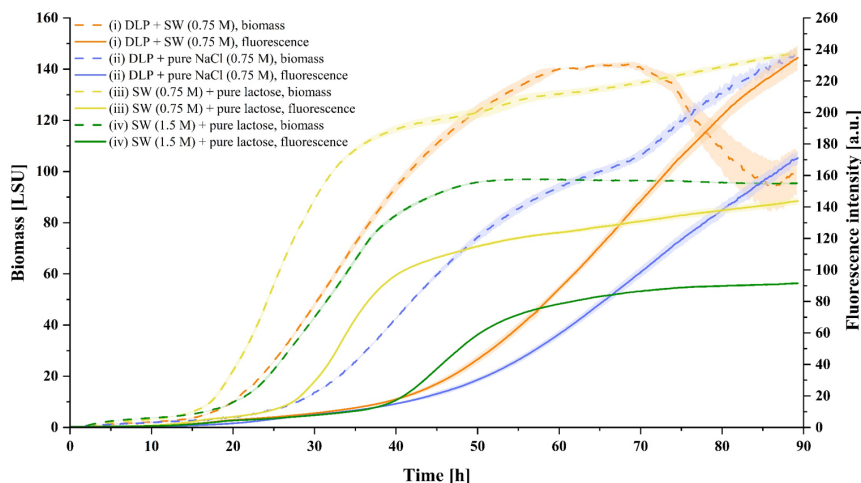
FIGURE 1 *Debaryomyces hansenii*'s biomass and YFP production (fluorescence) profiles obtained throughout micro-fermentation experiments performed using four different media. For each media, the biomass profile is represented in Light Scattered Units (LSU) in a discontinuous line and the fluorescence profile in a continuous line. Each profile (biomass and fluorescence) represents the average from three independent biological replicates, with their corresponding standard deviation (in shadow).

The lowest growth rate was found using medium ii as a feedstock (DLP and pure NaCl). In addition, a longer lag phase was observed in this medium compared with the other ones (≈ 25 h vs. 15–18 h) (Figure 1). Nevertheless, high levels of YFP fluorescence were detected at the end of the fermentation for this specific condition (Table 2).

The best performance in terms of biomass formation and YFP production was detected in medium i, when both by-products were combined (Figure 1). In this case, the fluorescence increased 2.5-fold, whereas the biomass improved by 1.5-fold at the end of the stationary phase compared with the worst-performing medium (medium iv). Therefore, medium i was the chosen one for further experiments.

In general terms, higher fluorescence emission was detected when the growth of the yeast was slower. For the same concentration of salt (media i, ii, and iii), using pure lactose as a carbon source (medium iii) allowed *D. hansenii* to grow faster and, consequently, less YFP was detected at the end of the culture. In contrast, when DLP was used as a carbon source (media i and ii), regardless of the salt source used (SW or pure NaCl), *D. hansenii*'s growth was slower, which was translated to a higher final biomass formation and YFP production at the end of the fermentation (Figure 1).

Finally, the production of YFP was coupled to the growth of *D. hansenii*, as it was produced constitutively by the yeast during the exponential growth. The fluorescence levels followed the same trend as the biomass, reaching a plateau at the end of the cultivation soon after the cells entered the stationary phase. Nevertheless, a delay of at least 10–20 h (depending on the media) was observed between the beginning of the yeast's exponential growth and the start of YFP detection in all the conditions tested.

Effect of media pasteurization on *D. hansenii*'s performance and the waste inherent microbial community

As a possible alternative to traditional sterilization, the pasteurization of both Arla by-products was studied. This strategy may contribute to *D. hansenii*'s advantage to outcompete the other naturally present microorganisms in the Arla effluents. Microscale fermentations were performed using the selected medium i (Table 1), pasteurized or non-pasteurized (Figure 2A,B). In addition, the growth rate of *D. hansenii* (μmax) and the YFP production (fluorescence) were calculated under these two conditions (Figure 2C). A control with only media (non-inoculated with *D. hansenii*) was also included in this study to analyse the evolution of the inherent by-product's microorganisms during the fermentation.

D. hansenii strains (both the WT and the YFP-transformant) always grew better than the media's inherent microorganisms, regardless of whether the pasteurization step was performed (Figure 2A,B). In both cases, in pasteurized and non-pasteurized media, the biomass of *D. hansenii* was more than two-fold higher than in control (with no *D. hansenii* cells) at the end of the fermentation. Moreover, the lag phase observed was shorter for the yeast (around 15 h) than the control, with a more accused difference detected when the by-products were pasteurized (approx. 30 h with non-pasteurized by-products and 45 h with pasteurized by-products) (Figure 2A,B). Therefore, at this scale (1.5 ml), and in both conditions, *D. hansenii* proliferates in the media better and faster than the natural species present in the dairy by-products.

Besides, the behaviour of the transformant *D. hansenii* strain was very similar to the WT strain, regardless of the pasteurization process. No statistically significant
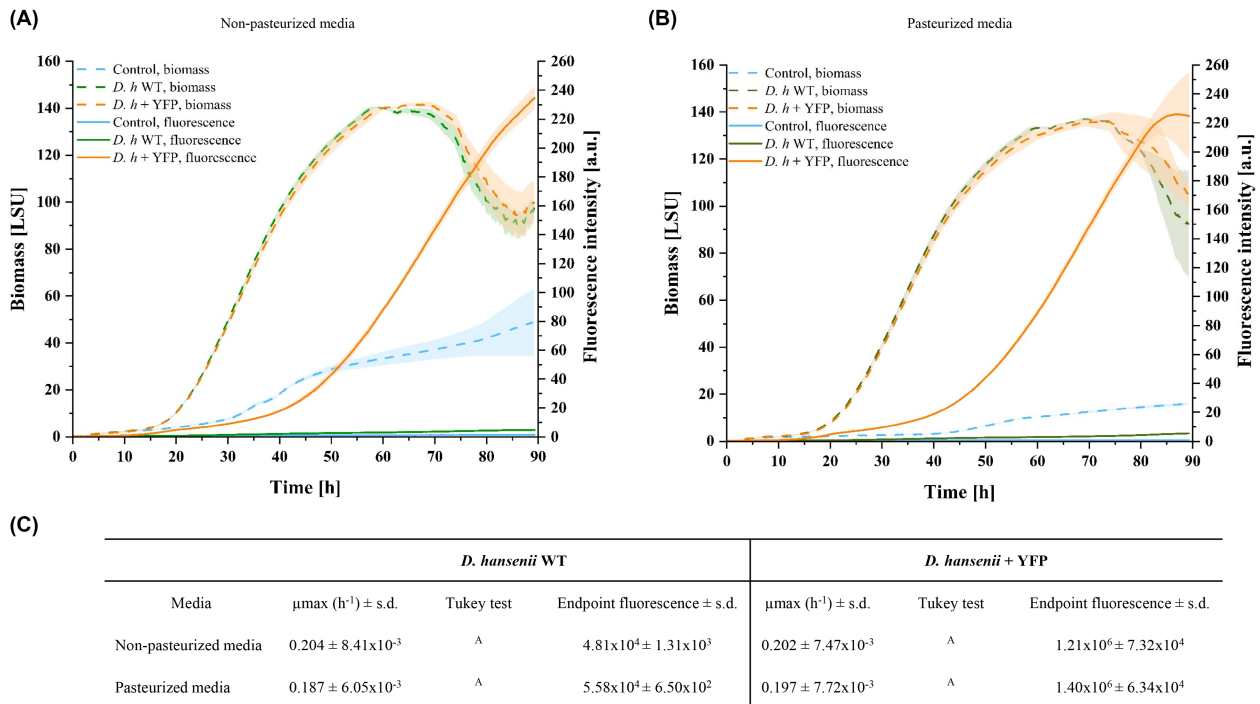
(A)

Non-pasteurized media

(B)

Pasteurized media

(C)

	D. hansenii WT			D. hansenii + YFP		
Media	μmax (h^{-1}) ± s.d.	Tukey test	Endpoint fluorescence ± s.d.	μmax (h^{-1}) ± s.d.	Tukey test	Endpoint fluorescence ± s.d.
Non-pasteurized media	0.204 ± 8.41x10^{-3}	A	4.81x10^4 ± 1.31x10^3	0.202 ± 7.47x10^{-3}	A	1.21x10^6 ± 7.32x10^4
Pasteurized media	0.187 ± 6.05x10^{-3}	A	5.58x10^4 ± 6.50x10^2	0.197 ± 7.72x10^{-3}	A	1.40x10^6 ± 6.34x10^4

FIGURE 2 Effect of media pasteurization on *Debaryomyces hansenii*'s performance and YFP production. Biomass and fluorescence profiles are represented in (A) non-pasteurized or (B) pasteurized Arla by-products. Experiments were run in biological triplicates, and each profile (biomass and fluorescence) represents the average with their corresponding standard deviation (in shadow). The control line in both cases represents media with no yeast inoculated. Note that the drop in the biomass levels or the higher standard deviations observed after 72 h of cultivation are due to BioLector limitations: at very high cell densities (usually after 72 h), the biomass filter loses resolution, as indicated by the manufacturer. (C) *D. hansenii*'s maximum specific growth rate values (μmax) obtained from biomass data, and fluorescence intensity emitted at the end of the cultivations (measured with CLARIOstar plus equipment) are specified in this table.

differences were observed between the μmax values calculated for both strains (Figure 2C), and their biomass profiles were almost identical (Figure 2A,B). Hence, producing YFP did not alter the growth performance of the transformant *D. hansenii* strain, although the YFP gene was randomly integrated into the genome of the yeast.

Regarding the fluorescence, none was detected in the YFP range for *D. hansenii* WT strain, neither for the control (non-inoculated with *D. hansenii*), and regardless of the media tested (Figure 2A,B). On the other hand, the fluorescence of the transformant *D. hansenii* increased over time, as it produced YFP throughout the entire fermentation. As mentioned before, the biomass and the fluorescence profiles followed the same trend, with a 10–15 h delay (Figure 2A,B).

Finally, minor differences were observed when the by-products were pasteurized compared with no pasteurization, even though, as mentioned before, the biomass and the fluorescence profiles were almost identical in both conditions. Figure 2C shows that slightly higher μmax values were obtained when the by-products were not pasteurized for both *D. hansenii* strains used. However, higher fluorescence values were detected at the end of the cultivation when the by-products were pasteurized. Some differences were also observed in the control between both conditions.

More specifically, when the by-products were pasteurized, the final natural biomass reached at the end of the fermentation was lower than when there was no pretreatment (Figure 2A,B). Therefore, we can assume that part of those microorganisms did not survive the pasteurization process, as was expected.

In order to give an extra advantage to *D. hansenii* to grow over the other microorganisms, pasteurizing the by-products was selected for the follow-up experiments performed at a larger scale in this work.

Shake flasks cultivations

Debaryomyces hansenii's growth on the Arla by-products and YFP production were also studied at a larger scale, first by performing 100 ml cultivations in shake flasks. A pasteurized mix of both by-products (DLP and SW, medium i) was used as a feedstock. The final biomass and the fluorescence profiles were compared with a control condition (without *D. hansenii*).

The results were similar to those observed on a smaller scale (Figure 2), although some differences were spotted. When comparing the biomass profiles, *D. hansenii* grew better and faster than the inherent microorganisms in the waste (control) (Figure 3). More specifically, after around 20 h of lag phase, *D. hansenii*

started to grow with a μmax of $0.048 \pm 1.08 \times 10^{-3}$ h^{-1}. On the contrary, about 55 h of lag phase was necessary for the inherent microorganisms on the Arla by-products to start growing (Figure 3). At the end of the fermentation, both control and *D. hansenii* cultivations reached closer final optical densities (OD$_{600}$ 99.2 vs. OD$_{600}$ 132.53, respectively), which was not observed on a lower scale (Figure 2B), where the microorganisms in control could not proliferate that much compared with when *D. hansenii* was inoculated.

Regarding the YFP production, the final fluorescence value measured by *D. hansenii* at the end of the fermentation was 8.17×10^5 (Figure 3), one order of magnitude less than in the smaller scale ($1.40 \times 10^6 \pm 6.34 \times 10^4$;
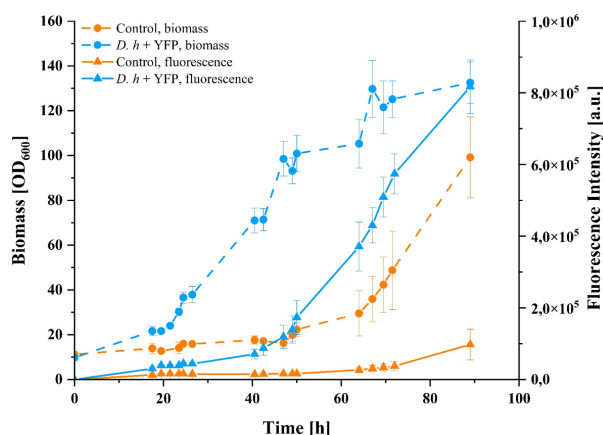
FIGURE 3 *Debaryomyces hansenii*'s biomass (OD$_{600}$) and YFP production (fluorescence) profiles observed throughout shake flask cultivations using a pasteurized mix of both Arla by-products as a feedstock. Experiments were run in biological triplicates, and each value corresponds to their calculated average. The standard deviation for each time point is also shown. The control line represents the waste with no yeast inoculated.
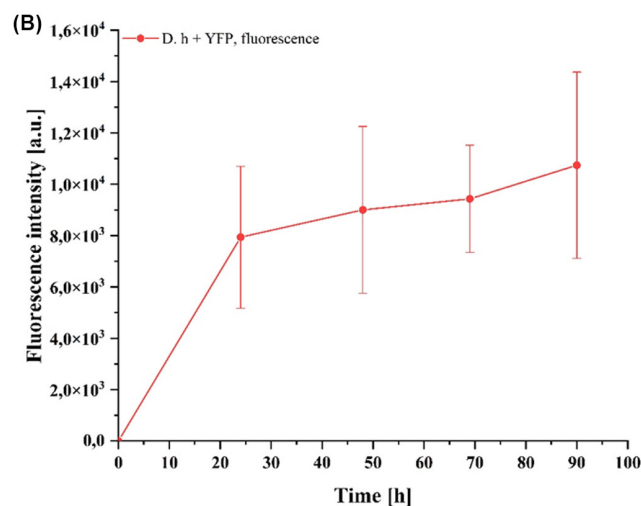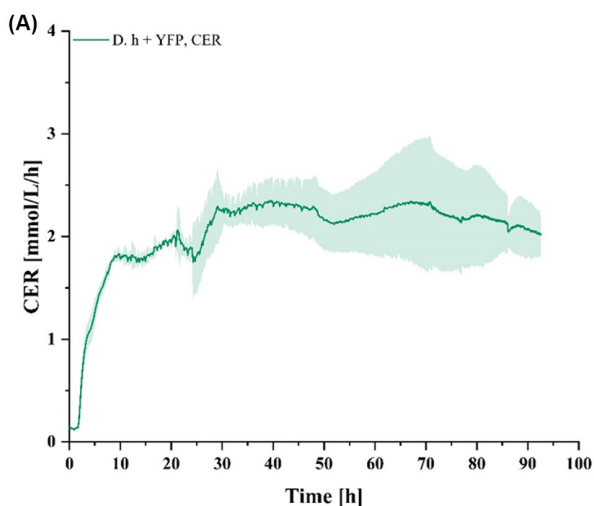
Figure 2C). However, in shake flasks, the fluorescence had not reached the stationary phase when the cultivations were stopped after 90 h. On the other hand, the fluorescence profile observed in the microfermentation experiments had already entered the plateau phase after around 70 h of cultivation (Figure 2B).

As observed before, the production of YFP was coupled with the growth of the yeast, following the same trend with a 20 h delay.

Bioreactor cultivations

The growth of *D. hansenii* in a combination of both pasteurized Arla by-products (medium i) was also studied in 1 L bioreactors (500 ml working volume). At this scale, it is possible to have controlled conditions, such as pH, temperature and the airflow, while monitoring additional parameters, such as the oxygen consumption or the CO_2 production during the cultivation, allowing a deeper insight into the process.

The Carbon Dioxide Evolution Rate (CER; used as an indirect measure of the yeast's metabolism) and the production of YFP were monitored throughout 90 h of cultivation.

As observed in the CER profiles, CO_2 was produced over the whole fermentation, indicating that *D. hansenii*'s metabolism was active and the yeast was proliferating (Figure 4A). Moreover, an increase in the fluorescence signal during the cultivation time was observed, confirming that *D. hansenii* over-performed the other microorganisms in the by-products and produced YFP (Figure 4B). However, the fluorescence levels reached at the end of the fermentation were lower than those observed on a smaller scale (microfermentations and shake flask experiments).

FIGURE 4 (A) Carbon dioxide Evolution Rate (CER) and (B) YFP production (fluorescence) profiles obtained from 1 L batch cultivations of *Debaryomyces hansenii* in both Arla by-products (pasteurized). Experiments were run in biological triplicates and each profile represents the average with their corresponding standard deviation represented in shadow (A) or vertical lines (B).

DISCUSSION

In this study, the halotolerant character of *D. hansenii* has finally been used to solve a rising challenge for the industry: waste management in a sustainable way. Our hypothesis that *D. hansenii* could be a suitable cell factory for industrial waste revalorization, especially for salt-rich effluents, has been confirmed. Not only *D. hansenii* demonstrates its ability to grow under complex and severe environmental conditions, but also to produce recombinant proteins (such as YFP) using dairy by-products as a feedstock.

Two different by-products from a dairy industry (Arla foods®/Arla Foods Ingredients P/S) were used as raw material for the growth of *D. hansenii*: DLP and SW (containing approx. 2 M NaCl). Media sterilization, which is expensive and requires a lot of energy (Yu et al., 2019), is avoided in this study. However, both by-products contain microorganisms from dairy production, which causes interferences with the optical density (OD) measurements traditionally used to study the growth of a microorganism in a media. Our results show that using a reporter fluorescent protein (in this case, the YFP) produced by the yeast works as an alternative to the OD to specifically monitor the growth of *D. hansenii* when other microorganisms are present. Fluorescent proteins (FP) have been extensively used before as reporter proteins for multiple purposes. For example, to study the activity of a promoter, to monitor gene expression, to follow the production of a target protein by tagging a FP in the N- or C- terminal, to study protein interactions, or for creating FRET-based sensors, among others (Chudakov et al., 2005; Van Roessel & Brand, 2002).

In this work, the YFP fluorescence emission observed in all the fermentation experiments performed (regardless of the work scale) followed the same trend as *D. hansenii*'s biomass profile, with 10–20 h delay (e.g., Figure 1). This means that the yeast produces the recombinant protein constitutively while cells are dividing, and the delay observed is probably due to the time required for the YFP gene to be transcribed and translated, for the chromophore to be formed, for the protein to fold completely, and for the equipment's sensor to detect a fluorescent signal, which also depends on the media and the temperature used (Miyawaki et al., 2005; Wiedenmann et al., 2009). Indeed, many authors have genetically modified the YFP to improve its maturation, reducing the folding time from hours to minutes (Aliye et al., 2015; Iizuka et al., 2011; Nagai et al., 1989). However, more studies would be needed to investigate the folding time of the YFP in the Arla by-products to draw a more accurate conclusion on the observed delay.

As expected, the fluorescence emission by the control (non-inoculated media) is minimal compared with the final fluorescence reached by *D. hansenii*. Thus, we can conclude that there are no interferences caused by the basal fluorescence of the dairy by-products on the fluorescence produced by our transformant yeast. Consequently, an increase in fluorescence is due to the growth and YFP production triggered by *D. hansenii*.

The role of each Arla by-product (DLP and SW) in *D. hansenii*'s performance was investigated separately by mixing them with commercial media (Table 2). One important conclusion from our work is that SW has a huge contribution to the growth of *D. hansenii*, as a shorter lag phase and higher µmax values are observed compared with when pure NaCl is used. One possible explanation for this finding is that, while commercial NaCl is pure, SW might contain additional nutrients, such as other salts (apart from NaCl) and minerals, enhancing the growth of the yeast even more.

Furthermore, pure lactose is probably more accessible for the yeast than the lactose from the DLP, part of which is trapped in aggregates from the by-products limiting its diffusion. Hence, when pure lactose is used as a carbon source, the metabolism of *D. hansenii* seems faster (higher µmax) than when using the DLP. Consequently, the yeast consumes the pure lactose rapidly until it is depleted, having less time to produce the recombinant protein. Thus, less fluorescence is detected at the end of the fermentation process. For this particular case, other commercial carbon sources (slower to be consumed) or other process configurations, such as a fed-batch, could be the most optimal approach (Minihane et al., 1986). As mentioned before, when DLP is used as a carbon source, the growth of *D. hansenii* is slower (lower µmax) than when using pure lactose. Hence, the yeast has more time to produce YFP, reaching higher fluorescence values at the end of the fermentation and improving protein production throughout the whole process.

Considering all of the above, a mix between both dairy by-products (DLP and SW) is the best feedstock for a better *D. hansenii* performance and YFP production. Moreover, some additional advantages of using this medium are: (i) the ability to revalue both dairy waste streams at the same time; (ii) the lack of use of pure water for the process, as the yeast grows directly in the media; and (iii) the absence of any commercial media needed, apart from an additional nitrogen source (YNB in our case). Hence, this was the medium selected for further experiments at a larger scale.

An interesting finding of this study is that *D. hansenii* is also able to grow using only the SW, and without any previous dilution (media iv). In this case, the initial concentration of NaCl is almost 2 M, which is toxic for most of the microorganisms (Yan et al., 2015) but not for *D. hansenii*, whose metabolism is slower but not inhibited (Navarrete, Frost, et al., 2021) unlike some authors had previously suggested (Capusoni et al., 2019). Nevertheless, further studies need to be performed to optimize the growth of the yeast in this particular

media, especially at a larger scale. In addition, our results open the possibility of growing *D. hansenii* using other alternative feedstock with similar salt concentrations, and using it as a protein production platform. For example, the alternative use of seawater could also be explored, avoiding the utilization of running water in the media. This approach could also reduce operational costs and increase production yields, as no sterilization of the media would be required.

On the other hand, avoiding the feedstock sterilization step brings another challenge: *D. hansenii* has to compete for the nutrients with the rest of the microorganisms present in the dairy effluents (mainly in the DLP) and that are already adapted to the media. In this work, the pasteurization of the media is presented as an easier and more competitive option than the classic sterilization method to give an advantage to *D. hansenii* to grow over the other microbial populations. Remarkably, even when the media is not pasteurized, *D. hansenii* has the capacity to outcompete the other species. In both cases, *D. hansenii* showed faster and higher growth than the control, with a slightly higher µmax observed when the feedstock was not pasteurized, but a lower YFP production was detected.

This can be explained by either the high salt concentration in the media, which impairs the growth of the non-halotolerant species, or the release of any kind of growth inhibitors by the yeast. The first case is clearly confirmed when we mix the DLP (where more microorganisms are present) with the SW, as the final concentration of salt in the media increases. Consequently, fewer microorganisms can stand the high osmotic pressure or need more time to adapt their metabolism to the high salt amounts, giving *D. hansenii* the advantage to grow. The second case has been already appointed by several authors who demonstrated that *D. hansenii* produces a killer toxin (mycocin) only in the presence of NaCl (Huang et al., 2021; Llorente et al., 1997; Marquina et al., 2001; Medina-Córdova et al., 2018). This toxin seems to be effective against different genres of yeast, fungi and bacteria. Therefore, we can expect that this toxic substance appears when the salt concentration in the media increases, although a more in-depth investigation is necessary to confirm this hypothesis. Interestingly, Liu and Tsao (2009) studied the biological control ability of *D. hansenii* in dairy products such as cheese and yogurt, and they found growth inhibition of several dairy moulds, like *Apergillus* sp., *Byssochlamys nivea*, *B. fulva*, *P. roqueforti* or *P. candidum*, among others.

Shake flask cultivations (100 ml) with pasteurized feedstock show similar results to microfermentation experiments, with comparable biomass levels and YFP production achieved by *D. hansenii*. In this case, the control could reach closer OD to *D. hansenii* than in smaller scale cultivations. However, the control lag phase was longer than *D. hansenii*'s lag phase,

indicating again that the microorganisms from the by-products do not suppose a 'competition' to the yeast, whose growth is faster. Therefore, in cultivations where *D. hansenii* is present, the yeast probably consumes all the nutrients until they are depleted before the other microorganisms start to grow.

Finally, the cultivations performed in controlled 1 L bioreactors (Figure 4A,B) show that the process is scalable at a larger scale (500 ml). However, less biomass and fluorescence are detected at the end of the cultivation compared with the lower scale tests (microfermentations and shake flasks). This could be due to some limitations during the fermentation process at a higher scale, such as insufficient oxygenation. In salt-rich environments, *D. hansenii*'s respirative metabolism is enhanced and the oxygen consumption is higher than when there is no salt in the media (Navarrete, Frost, et al., 2021). Therefore, proper aeration could be a bottleneck when producing recombinant proteins at a larger scale from the Arla by-products, where there is salt in the media and other microorganisms present that also consume oxygen to grow, increasing even more the oxygen demand. This limitation could be easily solved by increasing the aeration/agitation in the bioreactors. Follow-up experiments would be necessary to optimize specific production processes in 1 L bioreactors.

Overall, this study is a proof of concept of the intrinsic capacity of *D. hansenii* to revalorize dairy high-salt effluents, using them as a feedstock to grow and produce a desired recombinant protein. YFP has been chosen in this work and has been cloned into the yeast by random integration. Although CRISPR/Cas9 tools to genetically modify *D. hansenii* are now available (Strucko et al., 2021), it is still unknown which integration sites in the genome of this yeast are the most suitable to introduce a recombinant gene for optimal expression. Hence, random integration has been chosen as an alternative in this study. This approach has multiple advantages. For example, it allows inserting the gene into the genome, increasing the stability of the expression cassette over culture times (Löbs et al., 2017). This is not possible when the gene is expressed in an episomal plasmid, where it is necessary to constantly exert a selective pressure to avoid plasmid loss during cultivation (Bai Flagfeldt et al., 2009). Using random integration, many independent transformants are obtained with a high variability of gene expression between them (locus and number of copies). Thus, there are more chances to find a transformant strain with high protein expression. In this particular case, a transformant strain bright enough to provide sufficient signal above auto-fluorescence to be detected. However, this technology has some drawbacks. For example, it is difficult to ensure reproducibility among transformants. In addition, the insertion of the gene can activate or disrupt essential endogenous genes, which can cause an unstable

gene expression or an irregular yeast behaviour. For that reason, after the transformation, it is necessary to perform a laborious screening of the transformants to select the one with the desired phenotype and expression level (Kirchhoff et al., 2020). These limitations can be solved by integrating the gene into a target loci in the genome, which avoid disrupting essential genes and allows a consistent expression profile between transformants (Löbs et al., 2017). Hence, further investigation is needed to find suitable integration sites for recombinant gene expression in *D. hansenii*.

Finally, it has been proved that sterilizing the by-products is not required for an optimal performance of the yeast. This leads to the possibility of performing open fermentations where the yeast could be directly inoculated to the media and no pure water source would be required. However, finding a biological alternative to the addition of synthetic YNB as a supplementary nitrogen source would be necessary. This could make the process more affordable and boost a circular economy and a transition to a more sustainable industry in the near future.

AUTHOR CONTRIBUTIONS

Mònica Estrada: Formal analysis (lead); investigation (lead); writing – original draft (lead). **Clara Navarrete:** Conceptualization (supporting); formal analysis (equal); investigation (equal); writing – original draft (supporting); writing – review and editing (supporting). **Sønke Møller:** Resources (equal); validation (equal). **Alessandra Procentese:** Conceptualization (equal); investigation (supporting); supervision (supporting). **Jose L. Martinez:** Conceptualization (lead); funding acquisition (lead); investigation (supporting); project administration (lead); resources (lead); supervision (lead); writing – review and editing (lead).

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.