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RESEARCH ARTICLE

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Scheffersomyces stipitis ability to valorize different residual biomasses for vitamin B_9 production

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Abstract

Sugar beet pulp (SBP), sugar beet molasses (SBM) and unfermented grape marcs (UGM) represent important waste in the agro-food sector. If suitably pre-treated, hexose and pentose sugars can be released in high quantities and can subsequently be used by appropriate cell factories as growth media and for the production of (complex) biomolecules, accomplishing the growing demand for products obtained from sustainable resources. One example is vitamin B_9 or folate, a B-complex vitamin currently produced by chemical synthesis, almost exclusively in the oxidized form of folic acid (FA). It is therefore desirable to develop novel competitive strategies for replacing its current fossil-based production with a sustainable bio-based process. In this study, we assessed the production of natural folate by the yeast *Scheffersomyces stipitis*, investigating SBM, SBP and UGM as potential growth media. Pre-treatment of SBM and SBP had previously been optimized in our laboratory; thus, here we focused only on UGM pre-treatment and hydrolysis strategies for the release of fermentable sugars. Then, we optimized the growth of *S. stipitis* on the three media formulated from those biomasses, working on inoculum pre-adaptation, oxygen availability and supplementation of necessary nutrients to support the microorganism. Folate production, measured with a microbiological assay, reached 188.2 ± 24.86 μg/L on SBM, 130.6 ± 1.34 μg/L on SBP and 101.9 ± 6.62 μg/L on UGM. Here, we demonstrate the flexibility of *S. stipitis* in utilizing different residual biomasses as growth media. Moreover, we assessed the production of folate from waste, and to the best of our knowledge, we obtained the highest production of folate from residual biomasses ever reported, providing the first indications for the future development of this microbial production process.

INTRODUCTION

The continuous demand for nutritional security due to the fast world population increase is leading towards the finding of new solutions and technologies for waste valorization. 'The state of food security and nutrition in the world 2019' – a document by the Food and Agriculture Organization of the UN – reported that globally about 820 million people are still starving and/or suffering from food insufficiency. The 2030 agenda for sustainable food supply and the zero hunger challenge put forward the aim of producing 'quality food from waste' (Kapri et al., 2020; World Health Organization, 2019). Moreover, waste accumulation is a true problem for the ecosystem, since its high water and nutrients content promotes microbial decomposition leading to large methane and carbon dioxide emissions, causing problems for humans, animals and the environment (Helkar

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et al., 2016; Kumar et al., 2017). However, agricultural waste is increasingly appointed as a valuable source of nutraceuticals (or food additives), which are compounds that can provide additional health benefits in a regular diet (Lasrado & Rai, 2018). There are several examples in literature on the chemical extraction of diverse nutraceuticals from these residual biomasses, and in recent years, environment-friendly protocols are more and more applied (Ghafoor et al., 2022; Koraqi et al., 2022; Souza et al., 2022), following the principle of green chemistry. Nonetheless, the yield is generally low, and with the addition of economic considerations on the bioprocess, the problem of organic waste remains unsolved, unless alternative or complementary solutions are found. Among those, one of the most promising is to use these biomasses as feedstock in biorefineries. Lignocellulosic waste is composed of lignocellulose, a complex natural polymer consisting of a combination of lignin, hemicellulose and cellulose (Howard et al., 2003). The chemical structure of these three polymers makes them a substrate of enormous biotechnological value (Malherbe & Cloete, 2002), as they can be converted into products of interest through physical, chemical and biological processes. In particular, the latter comprises the use of microorganisms, such as bacteria, fungi or algae, defined as microbial cell factories. It is important to underline that the food and beverage industry produces a large amount of residues – among which lignocellulosic residues are also abundant, and not all of them are currently valorized in up-cycling processes. However, prior to being used as feedstock for microbial-based biotransformation, the raw biomass generally requires a pre-treatment step to open up the lignocellulosic structure to ease enzyme accessibility during hydrolysis, either in a separate enzymatic reaction (separate hydrolysis and fermentation, SHF) or combining the two steps (simultaneous saccharification and fermentation, SSF). Many pre-treatment strategies are available, and they can be exploited as is or in combination (mechanical, thermal, acid, base, oxidative and enzymatic) (Hendriks & Zeeman, 2009). Steam explosion is a common thermal pre-treatment strategy, in which the biomass is heated under pressure at around 240°C and then rapidly de-pressurized and cooled, making the water inside the biomass explode, opening up the lignocellulosic compact structure and releasing sugars oligomers and organic acids; the latter step further catalyses the hydrolysis of the released oligomers (Hendriks & Zeeman, 2009). Since this pre-treatment requires specific instruments, an autoclave pre-treatment is a strategy commonly used in a research laboratory setting to mimic the effect of a steam explosion, despite being less effective, especially with lignin-enriched biomasses. This pre-treatment is generally followed by a hydrolysis step, preferably enzymatic, since enzymes operate at conditions compatible with microbial growth (Katakojwala & Mohan, 2021) and produce cleaner waste when compared, for example, to chemical hydrolysis.

In this work, for the first time, unfermented grape marc (UGM) and other two important agricultural residues, sugar beet pulp (SBP) and sugar beet molasses (SBM), have been used for the production of vitamin B_9, exploiting the microbial cell factory *Scheffersomyces stipitis*.

Scheffersomyces stipitis is a Crabtree-negative yeast, naturally able to consume both hexose and pentose sugars (Jeffries & Van Vleet, 2009), making it an interesting cell factory for the valorization of diverse residual biomasses, such as those considered in this work. Nonetheless, the ability of this yeast to valorize residual biomasses into compounds of interest is almost unexplored, as it is mainly considered as a potential cell factory for bioethanol production (Biazi et al., 2022; Campos et al., 2022).

In this work, we initially tested different pre-treatment conditions to select the best on UGM for sugar monomers release: the results were compared to the theoretical maximum value previously determined by total acid hydrolysis. We selected a simple autoclave pre-treatment since it allowed the release of almost all the available sugars. Pre-treatment conditions for SBP were adapted from Martani and colleagues, while no pre-treatment was necessary for SBM (Martani et al., 2020).

Prior to use, the obtained supernatants were appropriately diluted, and growth supplements were added when necessary to prepare them as growth media. We then optimized the growth and sugar consumption of *S. stipitis* using the three different biomasses. In particular, we focused on inoculum pre-adaptation, oxygenation and the addition of key nutrients for supporting growth based on the experience from our previous study on defined media (Mastella et al., 2022).

Hence, folate production was assessed in shake flask fermentations where yeasts were grown in the optimized media derived from biomasses. Folate amounts and yield obtained from the different biomasses were calculated with an indirect microbiological assay to determine productions and yields. The results show that *S. stipitis* was able to produce comparable – if not competitive – amounts of folate, when compared to the data reported in literature with other cell factories. The highest production was achieved in SBM (188.2 ± 24.86 μg/L), followed by SBP (130.6 ± 1.34 μg/L) and UGM (101.9 ± 6.62 μg/L); the highest yield was obtained on SBP (9.54 μg/g ± 0.17) as a growth substrate.

These results confirm the potential of *S. stipitis* as a cell factory for the production of folates from residual biomasses and provide a solid starting point for further optimization at the bioreactor scale, where attention can be devoted to maximizing the production while fully exploiting all the nutritional elements present in the different residual biomasses.

MATERIALS AND METHODS

Chemicals and agricultural residual biomasses

All the chemicals used in this work, including glycerol ($C_3H_8O_3$), sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), sodium chloride (NaCl) and sodium citrate, were purchased from Sigma-Aldrich Company.

Sugar beet pulp (SBP) and sugar beet molasses (SBM) were provided by Cooperativa Produttori Bieticoli (CoProB), Minerbio (BO, Italy); UGM – obtained from the production of white wine – was collected from local farmers in the Lombardy region within the framework of the national funded project CREIAMO (https://creiamo-circulareconomy.com/). SBM was stored at 4°C, while SBP and UGM were stored at –20°C in order to better preserve their chemical and biological properties.

Yeast strain and growth conditions

Scheffersomyces stipitis (Culture Collection, CBS 6054) was grown in 100 ml, 250 ml or in 250 ml baffled-shake flasks at 30°C and 160 rpm, using different medium:flask volume (m/f) ratios. Hydrolysed SBP 3% and 5%, SBM 1:16 and UGM 5% were used as growth media (see next paragraph for details on the preparation). Cells were inoculated at an initial optical density (OD_{660}) of 0.05 in SBP and UGM, while in SBM, the inoculum was calculated to obtain an initial OD 0.0005 to be able to follow the exponential phase of growth. Growth was measured by monitoring the media absorbance at 660 nm (UV-1800; Shimadzu).

Folate detection

The extracellular or intracellular amounts of free folates (folate vitamers harbouring a maximum of three glutamyl units at) produced by the yeast strain were determined indirectly by a microbiological assay using *Lactobacillus rhamnosus* (NRRL culture collection, strain B-442) as the test microorganism. This bacterium is able to grow proportionally to the concentration of folic acid present in the medium: this allows to build a calibration curve that correlates the final OD reached by *L. rhamnosus* to the concentration of folates in the samples (Horne & Patterson, 1988).

The microbiological assay was performed in 96-well microtitre plates, following a protocol adapted from Sybesma and colleagues (Sybesma et al., 2003). The wells were filled by adding: 100 µl of twofold-concentrated Folic Acid Casei Medium (FACM, HiMedia, Mumbai, India), 100 µl of an unknown or reference sample in 0.1 M potassium phosphate buffer (pH 6.4) containing 1% (w/v) ascorbic acid (Sigma-Aldrich) and 20 µl of the *L. rhamnosus* inoculum. The plates were incubated at 37°C and the turbidity was measured after 18 h using a multiscan spectrophotometer set at 595 nm (VICTOR^{tm} X3, PerkinElmer). Control wells were inoculated without *L. rhamnosus* to check the absorbance of the FACM medium, later subtracted from the absorbance of the samples.

The analysis of the total folate concentration (including forms with long chains of polyglutamates) was performed in the same way as the free folate, but after enzymatic deconjugation of the folate samples with rat serum (Sigma-Aldrich) as a source for γ-glutamyl hydrolase activity. The purified rat serum was added to the folate samples at the final concentration of 20% (v/v) (Sybesma et al., 2003). After 3 h of incubation at 37°C, the enzyme was inactivated by heating for 5 min at 100°C. Samples were cooled down and after centrifugation at 14,000 rpm for 20 min at 4°C, the supernatant was collected and used for the microbiological assay, as described above.

Raw materials and preparation treatments

Sugar beet molasses was diluted 1:4 (vv^{-1}) with distilled water prior to autoclave sterilization. A pre-treatment step and enzymatic hydrolysis were required on SBP to release the sugars contained in the (hemi)cellulose fraction; a 10% stock was prepared as described by Martani and colleagues (Martani et al., 2020). A 10% stock of UGM was prepared as follows: 100 g of total solids (TS) of ground biomass was suspended in distilled water to a final volume of 1 L; after sterilization in an autoclave, the suspension was centrifuged in order to separate the solids from the liquid phase; the pH was adjusted to 5.5 with NaOH 15 M and stored at 4°C. When necessary and indicated, $(NH_4)_2SO_4$ (10 g/L) or urea (4.6 g/L), and/or KH_2PO_4 (6 g/L) and $MgSO_4 \cdot /L_2O$ (1 g/L) were added to the media.

The sugar content of all three biomasses was analysed by HPLC (as described below); nitrogen content (ammonia, urea and primary amino-nitrogen) in UGM 10% was determined with the Urea/Ammonia Assay Kit (Rapid) (K-URAMR) and the Primary Amino Nitrogen Assay Kit (PANOPA), both purchased by Megazyme.

In this work we also focused on the study of UGM, starting from characterization by acid hydrolysis, we then optimized the best conditions for its pre-treatment.

In order to identify the best condition in terms of time and released sugars, different enzymatic hydrolysis protocols were tested (no enzymes added, pH, temperature and enzymatic cocktail), either directly in the supernatant or in citrate buffer 50 mM (sterile sodium citrate tribasic 14.7 g/L at pH 5.5); the different conditions tested are listed in Table 1. Prior to enzymatic hydrolysis, a 3% w/v solution was autoclaved in order to both sterilize and pre-treat the biomass. Enzymatic

TABLE 1 Tested enzymatic hydrolysis conditions (S, supernatant; B, citrate buffer). Three independent experiments were performed for each condition.

Conditions	Biomass	Rpm	Temperature	pH	S/B	Cocktail
1a	Wet	130	30°C	6	S	–
1b	Wet	130	30°C	6	S	N S22119
2a	Wet	130	50°C	6	S	–
2b	Wet	130	50°C	6	S	N S22119
3a	Wet	130	30°C	5.5	B	–
3b	Wet	130	30°C	5.5	B	N S22119
4a	Wet	130	50°C	5.5	B	–
4b	Wet	130	50°C	5.5	B	N S22119
5a	Wet	130	30°C	6	S	–
5b	Wet	130	30°C	6	S	N S22201

hydrolyses were carried out by adding 100 μl/g_{TS} of the enzymatic cocktails NS22119 or NS22201 (which are the same cocktails used for SBP), kindly provided by Novozymes (Novozymes A/S). Enzymes were used in large excess to avoid limiting conditions and directly added to the supernatant. For the experiments in citrate buffer, the supernatant was pre-emptively substituted with an equal volume of citrate buffer. Sugar release was studied by taking 1 ml samples at 0, 3, 7 and 24 h from the addition of the enzymatic cocktail; samples were then analysed by HPLC.

The percentage of sugar released on total solids $\%(sug/TS)$was calculated as the ratio between the concentration of the released sugars (g/L) and the concentration of TS (3 g_{TS}/100 ml). The percentage of sugar released with respect to the total acid hydrolysis was evaluated with equation 1, where c_{enz} is the concentration of the sugar in the supernatant measured with the HPLC, $85mL$ is the volume of supernatant and $\%_{sug}(g_{acid}/g_{TS}) \bullet 3g_{TS}$ is the quantity of sugar released by the total acid hydrolysis.

$$\%\left(\frac{g_{enz}}{g_{acid}}\right)=\frac{\frac{g_{enz}}{L}\times 85mL\times\left(\frac{1L}{1000mL}\right)}{\%_{sug}\left(\frac{g_{acid}}{g_{TS}}\right)\times 3g_{TS}}\times 100 \quad (1)$$

In order to determine the initial concentration of raw materials being efficiently pre-treated by autoclaving, different UGM solutions (3%, 5% and 10% w/v) were used; after treatment and centrifugation, the different supernatants were neutralized with NaOH 15 M and a 1 ml sample was analysed by HPLC. Three independent experiments were performed.

Characterization of unfermented grape marc

UGM was stored at –20°C; before analysis and use, the frozen biomass was ground with a food processor to homogenize this heterogeneous biomass as much as possible. To determine the amount of total solids (%TS), 2 g of frozen ground UGM were lyophilized overnight; %TS was calculated as the ratio of the weight after and before lyophilization. Data refer to three independent experiments performed.

The total amount of glucose (Glc), arabinose (Ara) and insoluble fraction were determined with a modified version of the National Renewable Energy Laboratory (NREL) protocol for the determination of structural carbohydrates and lignin in biomass [https://www.nrel.gov/docs/gen/fy13/42618.pdf], to perform a complete acid hydrolysis. Briefly, 300 mg of TS were weighed in 50 ml bottles; 3 ml of 72% (v/v) H_2SO_4 were added and the solution was incubated at 30°C for 1 h, stirring every 10 min. The solution was diluted to 5.4% (v/v) by quickly adding 37 ml of distilled water, mixed by inversion and then autoclaved at 121°C for 20 min. The solution was filtered with a Büchner funnel, using pre-weighed filter paper. The retained solids were dried in a microwave until constant weight. The filtered liquid was transferred to a beaker and neutralized with NaOH 15 M until pH 5–6 was reached; the volume of the solution was measured, and the samples were analysed by HPLC (as described below). The total amount of sugars present in the biomass was calculated with equation (2):

$$[sugar]\,(g)=c_{HPLC}\times V_{filtered} \quad (2)$$

where c_{HPLC} is the concentration measured by HPLC, and $V_{filtered}$ the volume of the solution. Three independent experiments were performed.

To determine the total amount of fructose (Fru), 1.5 g of TS was added to 50 ml of distilled water to obtain a 3% (w/v) solution, autoclaved at 121°C for 20 min; samples were allowed to cool and H_2SO_4 was added until pH 2 was reached. Acid hydrolysis was carried out in a water bath set at 100°C, 100 rpm; 1 ml samples were taken after 1 h and 3 h, neutralized with NaOH 15 M and analysed by HPLC (as described below). The volume

of the solution was measured, and the total amount of fructose in grams was calculated with Equation (2). Three independent experiments were performed.

The presence of Glc and Fru was confirmed with the D-Fructose/D-Glucose Assay Kit (Megazyme).

HPLC analysis

Glucose, fructose, sucrose (Suc), arabinose, arabitol, acetic acid, galacturonic acid, lactic acid and ethanol (EtOH) concentrations were determined by HPLC (Agilent 1100/1200, Agilent Technologies, Inc.) using a Rezex^{tm} ROA-Organic Acid H^+ (8%) 300 × 7.8 mm column (Phenomenex), at 40°C; H_2SO_4 0.005 N was used as mobile phase, pumped at 0.5 ml/min; analysis time was set to 40 min. Separated components were detected by a refractive index detector and peaks were identified by comparison with known standards. Prior to analysis, all samples were centrifuged (14,000 rpm, 10′, 4°C) and filtered with 0.20 μm PTFE filters (AISIMÔ CORPORATION CO., LTD).

Statistical analysis

All statistical analyses where *p*-values are indicated were performed using a two-tailed, unpaired, heteroscedastic Student's *t*-test.

RESULTS AND DISCUSSION

Growth and folate production in sugar beet molasses (SBM)

SBM is a thick, dark brown liquid containing roughly 800 g/L sucrose and a minor quantity of fructose. Different dilutions (1:4, 1:8, 1:16, 1:20 and 1:32 v/v) were tested in shake flasks (m/f ratio of 2:5) as a growth medium, and the highest concentration that *S. stipitis* was able to withstand was the one of SBM 1:16; this dilution was thus selected for further investigations. In this condition, we observed only a partial consumption of the carbon source and the production of small quantities of ethanol (data not shown), suggesting insufficient aeration and the presence of one or more limiting nutrients, most probably in terms of the nitrogen source, of which SBM is poor (Martani et al., 2020).

To determine which nutrient(s) might be limiting, $(NH_4)_2SO_4$ (2, 5 or 10 g/L), or $MgSO_4$ (1 g/L) and KH_2PO_4 (6 g/L), or a combination of the three ($(NH_4)_2SO_4$ 10 g/L, KH_2PO_4 6 g/L and $MgSO_4$ 1 g/L) was added to SBM 1:16. These nutrients were specifically chosen since they are the main components of Verduyn medium (Verduyn et al., 1992), which is considered as a reference for the preparation of minimal medium for the yeast *Saccharomyces cerevisiae*. The results are shown in Figure S1. Interestingly, the addition of $(NH_4)_2SO_4$ as a nitrogen source had a negative effect on growth, leading to an OD_{660} lower than the one measured in the sole SBM 1:16; the addition of $MgSO_4$ and KH_2PO_4 (with or without $(NH_4)_2SO_4$) allowed to reach a biomass 2–3 times higher than in the control condition. Growth on SBM 1:16 + KH_2PO_4 (6 g/L) and $MgSO_4$ (1 g/L) was further investigated in 250 ml shake flasks (m/f ratio of 2:5) (Figure 1A): the cells reached an OD_{660} of 26, consuming less than 30% of the available sugars while showing two distinct growth phases ($\mu_1 = 0.08 \pm 0.014\,h^{-1}$, $\mu_2 = 0.02 \pm 0.003\,h^{-1}$) and the production of ethanol. To improve growth and sugar consumption, the kinetic was repeated by changing three different parameters (separately): nitrogen availability, salts concentration and aeration. We tested the addition of a lower concentration of $(NH_4)_2SO_4$ (2 g/L) to the medium optimized for salt content, but also in this case we saw a negative effect on growth rate (Figure S2A), confirming what was seen before; similarly, doubling the amount of $MgSO_4$ (2 g/L) and KH_2PO_4 (12 g/L) did not show any differences from the previous fermentation (Figure S2B). Differently, the reduction in the m/f ratio of 1:5 to improve aeration allowed to reach twice the amount of biomass (Figure 1B); however, the production of ethanol and the constant (not exponential) growth profile suggested that oxygen was still the limiting factor.

Thus, the growth kinetic was repeated in baffled flasks (m/f ratio of 1:5) on SBM 1:16 + $MgSO_4$ (1 g/L) and KH_2PO_4 (6 g/L): growth was indeed faster ($\mu = 0.42 \pm 0.022\,h^{-1}$) and more than 90% of the sucrose was consumed, ethanol was not detected and the culture reached a final OD_{660} of 70 (Figure 1C).

Here, it is interesting to discuss the assimilation of sucrose by *S. stipitis* to better understand glucose, fructose and sucrose consumption on SBM. Sucrose assimilation is well described in *Saccharomyces cerevisiae*: its genome contains seven loci-encoding invertase (*SUC*1, *SUC*2, *SUC*3, *SUC*4, *SUC*5, *SUC*7 and *SUC*8), but since the reference strains (S288C) only encodes *SUC*2, most studies have focused on that gene (Taussig & Carlson, 1983). In *S. cerevisiae*, *SUC*2 exists in two distinct forms that are localized in the cytoplasm as well as secreted, thus allowing invertase activity both intra- and extracellularly. BLAST alignment of *ScSUC2* on *S. stipitis* available genomes (taxid 4924 and CBS 6054) returned no results, suggesting that *S. stipitis* genome does not encode for an invertase homologue of *SUC*2. Research with the keyword 'sucrose' on *S. stipitis*' (CBS 6054) annotated genome on NCBI outputs four putative genes involved in sucrose metabolism: *SUC1.3*, *SUC1.4* and *SUC1.5* are identified as probable sucrose utilization proteins; *SNF8* (sucrose non-fermenting) is suggested to be involved in glucose de-repression. Local (Smith–Waterman algorithm) and

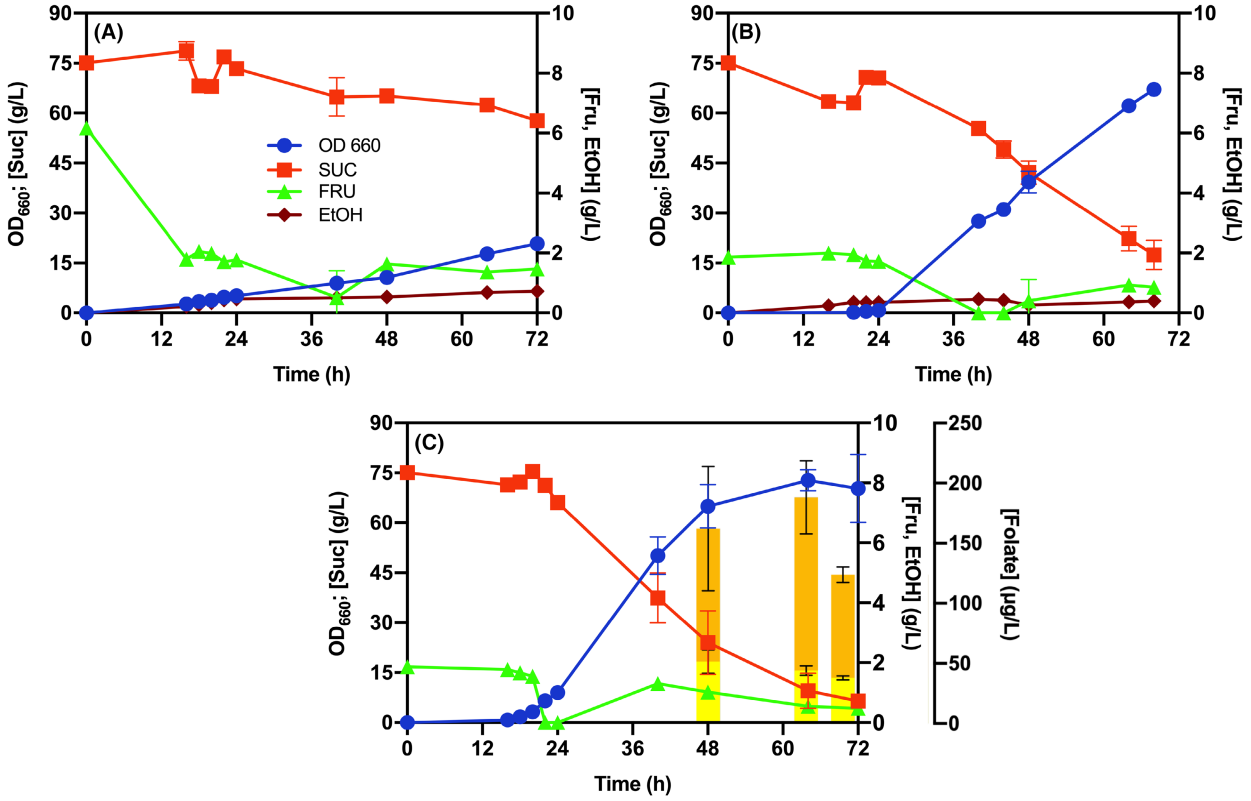


FIGURE 1 Growth and folate production profiles on SBM + $MgSO_4$ (1 g/L) + KH_2PO_4 (6 g/L). (A) Growth and sugar consumption with an m/f ratio of 2:5. (B) Growth and sugar consumption with an m/f ratio of 1:5. (C) Growth, sugar consumption and folate production in baffled flasks (m/f = 1:5). The left y-axis shows OD_{660} (blue, circles) and sucrose concentration (dark orange, squares); the right y-axis shows fructose (green, triangles) and ethanol (brown, diamonds) concentration; the secondary y-axis shows the concentration in bars of free (yellow) and total (orange) folate. Values are mean ± standard deviation of three independent experiments.

global (Needleman–Wunsch algorithm) alignment of *SUC1.3*, *SUC1.4* and *SUC1.5* showed identity around 50%, and similarity around 60–70% (BLOSUM62 matrix); no N-terminal secretion sequence was identified, but *SUC1.3* is characterized by an additional 45 amino acids at the C-terminal. Alignment of *SUC1.3*, *SUC1.4* and *SUC1.5* with *ScSUC2* showed no identity, in accordance with the results obtained with Blast. These results suggest that *S. stipitis* genome could encode for at least three similar invertases, non-homologues of *ScSUC2*, responsible for the ability of this yeast to consume sucrose; the lack of ER signal sequence suggests that these proteins are intracellular. Alignment of *SsSNF8* on Blast with *S. cerevisiae*'s genome suggests that *SsSNF8* is homologue to *ScSNF8* (36% identity), which is reported to be involved in glucose derepression in *S. cerevisiae*, in particular in carbon catabolite repression of polymerase II transcription by glucose (yeastgenome.org).

Indeed, when grown on a minimal medium with 20 g/L of sucrose as a carbon source (data not shown), no release of glucose or fructose in the medium was observed. These results are consistent with the work of Kobayashi and colleagues (Kobayashi et al., 2021), where no significant accumulation of monosaccharides derived from sucrose hydrolysis was observed in the culture supernatants when *S. stipitis* was grown on YPS. These results point to the existence of an intracellular invertase. In subsequent work, Kobayashi and colleagues (Kobayashi et al., 2022) investigated catabolite repression on sugars in sugarcane molasses, which has a relatively high glucose and fructose content when compared to SBM, where they only represent 0.3% of the dry mass each. Their results showed that sucrose consumption was clearly suppressed by the presence of glucose and fructose. This effect could also explain what we observed on SBM, where, however, the lower monosaccharides concentration and the high biomass titres probably alleviate the catabolite repression over sucrose. Moreover, it must be noted that precise HPLC quantification of small amounts of glucose and fructose in a complex matrix is challenging: the chromatograms were characterized by small shoulders corresponding to glucose and fructose peaks, but quantification was not possible (Figure S3). These results, in contrast with growth on defined media and the annotated genome, suggest the presence of an extracellular invertase. Taken together, these observations suggest that *S. stipitis* encodes both an intracellular and an extracellular invertase, yet further studies are needed to better characterize this aspect and to identify the putative extracellular invertase(s).

Folate production was evaluated in condition 1C at the end of the exponential phase and the onset of the stationary phase (Figure 1C, yellow bars). The highest production (188.2 ± 24.86 μg/L) was obtained at 64 h, with a yield on total sugars of 2.33 μg/g. The highest production on SBM found in literature is 150 μg/L, obtained with *S. cerevisiae* in a bioreactor, with a yield on total sugars of 2.21 μg/g (Hjortmo et al., 2008): these findings are comparable to our results obtained in shake flasks, suggesting the true potential of *S. stipitis* as a folate producer, especially when considering higher aeration and the controls available within a bioreactor.

Growth and folate production in sugar beet pulp (SBP)

It is known that SBP pre-treatment and hydrolysis allow the release of glucose and small amounts of arabinose, while also producing growth inhibitors, such as acetic, galacturonic and lactic acid; the latter is probably due to an initial contamination of the biomass from bacteria, as reported by Kühnel and colleagues (Kühnel et al., 2011); galacturonic acid is released from pectin, as described by Perpelea and co-authors (Perpelea et al., 2022). Thus, the use of SBP requires finding a compromise between a sufficiently high concentration of sugars and *S. stipitis*'s tolerance to inhibitors. Martani and colleagues have already characterized sugars and acids released during the pre-treatment process of SBP (Martani et al., 2020).

Given these information, SBP toxicity was assessed in a preliminary study by growing *S. stipitis* in different concentrations of SBP (3%, 4%, 5% and 6% w/v); SBP 3% allowed growth, while the growth on SBP 4% and 5% was characterized by a long lag phase; growth was not observed for higher concentrations (data not shown).

Initially, SBP 3% was selected for further investigations, and growth was monitored in shake flasks (m/f ratio of 2:5): as shown in Figure 2A, all the glucose is consumed, and arabinose starts being consumed at 40 h, probably due to glucose catabolite repression.

Since sugars concentration in SBP 3% is low (roughly 7 g/L of glucose, the most abundant), before characterizing the growth at higher aeration, *S. stipitis* was grown on SBP 5% (roughly 15 g/L Glc and 2 g/L Ara), but testing a pre-adaptation phase. Pre-cultures were grown in SBP 2.5% and 5%, and YPD was used as a control (Figure S4). The pre-adaptation on SBP 2.5% significantly reduced the duration of the lag phase and thus allowed efficient growth also on SBP 5% (Figure 2B): biomass reached 11 OD at 88 h, with still a minor but present residue of glucose, no consumption of arabinose, and the production of 2 g/L of EtOH. Since *S. stipitis* is a Crabtree-negative yeast (Jeffries & Van Vleet, 2009), to improve the aeration and thus avoid the production of EtOH (as with SBM), the m/f ratio was lowered to 1:5 and baffled shake flasks were used. Indeed, this condition allowed to double the amount of biomass with a faster consumption of glucose, completely depleted already at 48 h; arabinose was also completely consumed, and no ethanol production was observed (Figure 2C). Folate production (yellow and orange bars) was evaluated at the end of the exponential phase and reached 130.6 ± 1.34 μg/L at 48 h, with a yield on total sugars of 9.54 μg/g. These results are interesting because even if the production is lower than on SBM, the yield is four times higher. Furthermore, this residual biomass did not require any addition of specific salts or nutrients as in the case seen above. Here, only oxygenation and strain pre-adaptation were pivotal for improving the growth.

Growth and folate production in UGM

While SBM and SBP had been previously characterized in our laboratory on other non-*Saccharomyces* yeasts (Martani et al., 2020), UGM required a characterization study and the development of an efficient pre-treatment protocol for the subsequent use as a growth medium. Since the starting biomass is wet, %TS was determined for each batch.

Complete acid hydrolysis was carried out to determine the total amount of sugars in the biomass. The NREL protocol (see Methods), however, did not allow precise quantification of fructose, probably due to its degradation to 5-hydroxymethylfurfural, occurring at temperatures above 106°C and a pH lower than 2 (Bower et al., 2008). An alternative hydrolysis protocol that allowed sampling over time was developed, and the results obtained for the other sugars were in agreement with the NREL protocol. Our results show that more than 50% of the biomass is composed of sugars, namely Fru (26.56%), Glc (24.96%) and Ara (1.21%), expressed as a percentage on TS (g/gTS).

Development of a pre-treatment and hydrolysis protocol for UGM

To develop a protocol for releasing nutrients from UGM, we followed a strategy similar to the one we applied to SBP (Martani et al., 2020). The ground biomass is autoclaved to partially simulate a steam explosion, and then enzymatic hydrolysis is carried out. Since many factors can affect hydrolysis efficiency (Bertacchi et al., 2022), different enzymatic hydrolysis conditions were tested (Table 1, **par 2.6.2**) using two different enzymatic cocktails: NS22119 contains several carbohydrates to release both hexose and pentose sugars, while NS22201 contains cellulases for the degradation of cellulose and other β-glucans. Hydrolysis efficiency was determined as a percentage of released sugars with respect to the total acid hydrolysis, which was used as a baseline.

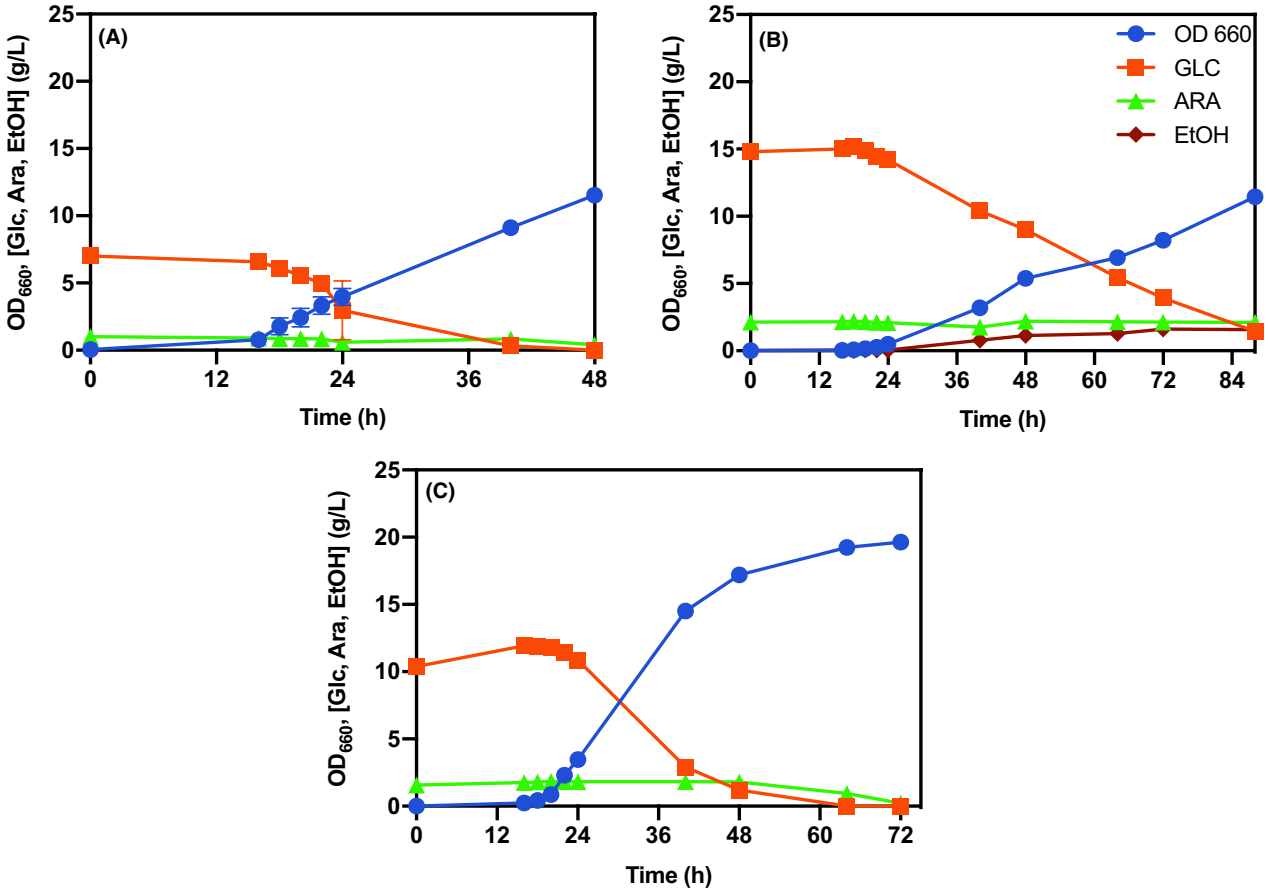
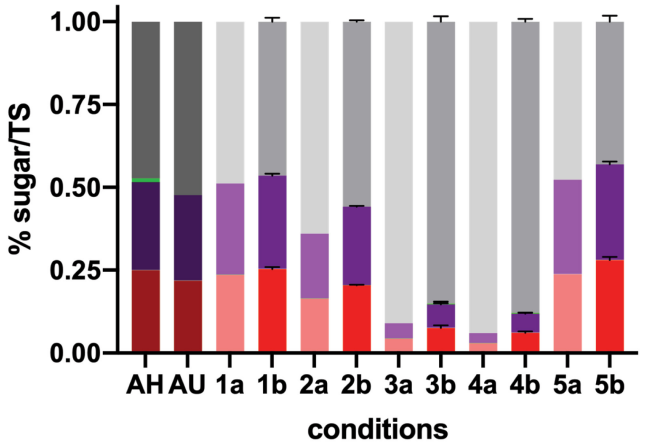


FIGURE 2 Growth and folate production profiles on SBP. (A) Growth and sugar consumption on SBP 3% with an m/f ratio of 2:5. (B) Growth and sugar consumption on SBP 5% with an m/f ratio of 2:5 with pre-inoculum adaptation in SBP 2.5%. (C) Growth and sugar consumption on SBP 5% with an m/f ratio of 1:5 in baffled flasks with pre-inoculum adaptation in SBP 2.5%. The left y-axis shows OD_{660} (blue, circles), glucose (red, squares), arabinose (green, triangles) and ethanol (brown, diamonds) concentration. The right y-axis shows the concentration in bars of free (yellow) and total (orange) folate. Values are mean ± standard deviation of three independent experiments.



Conditions	Biomass	rpm	Temperature	pH	S/B	Cocktail
1a	Wet	130	30°C	6	S	-
1b	Wet	130	30°C	6	S	N S22119
2a	Wet	130	50°C	6	S	-
2b	Wet	130	50°C	6	S	N S22119
3a	Wet	130	30°C	5.5	B	-
3b	Wet	130	30°C	5.5	B	N S22119
4a	Wet	130	50°C	5.5	B	-
4b	Wet	130	50°C	5.5	B	N S22119
5a	Wet	130	30°C	6	S	-
5b	Wet	130	30°C	6	S	N S22201

FIGURE 3 Pre-treatment results. The graph represents the percentage of released sugar on g_{TS} for each condition tested (listed in Table 1, par. 2.6.2). AH, acid hydrolysis; AU, autoclave pre-treatment. a, negative control (no enzymatic cocktail); b, enzymatic cocktail added. The results are the mean ± standard deviation of three independent experiments.

The histograms in Figure 3 show the percentage of sugar released on gTS for each condition tested, as well as the results obtained with the acid hydrolysis (AH) and the sole autoclave pre-treatment (AU). The autoclave pre-treatment process allowed to release amounts of glucose and fructose close to the results

obtained with the complete acid hydrolysis, representing 21.8% and 25.8% g/g_{TS} respectively (AU in Figure 3). To further improve the quantity of sugars released, different conditions for enzymatic hydrolysis were tested; preliminary studies allowed us to determine the best agitation parameters and showed no difference when the starting biomass was wet or dry (data not shown). The hydrolyses were tested at 30°C and 50°C, either by adding the enzymatic cocktail NS22119 directly in the supernatant of the autoclaved biomass or by replacing it with citrate buffer (1b, 2b, 3b, 4b, Figure 3, condition listed in Table 1, **par 2.6.2**). As highlighted in Figure 3, enzymatic hydrolysis conducted in the supernatant allowed recovery of around 75% of the total sugars present in the biomass, showing very little difference from the negative controls, regardless of the temperature or the enzymatic cocktail (conditions 1, 2 and 5, Table 1, **par 2.6.2**); hydrolyses in citrate buffer (conditions 3 and 4, Table 1, **par 2.6.2**) were far less efficient, and the same results were obtained by using the enzymatic cocktail NS22201 (**5b**, Figure 3), confirming that most of the sugars are released in the supernatant during the autoclaving step (condition AU). These results are similar to what Corbin and colleagues reported, who characterized the composition of Sauvignon Blanc white marc (Corbin et al., 2015): the main carbohydrates left after juicing are monosaccharides (Glc and Fru), oligosaccharides and water-soluble polysaccharides, which represent roughly 70% of the total carbohydrates and 38% of TS. In our case, the monosaccharides released after the pre-treatment represent more than 50% of the TS, and thus probably more than 70% of the total carbohydrates. Moreover, Corbin and colleagues (Corbin et al., 2015) reported that enzymatic hydrolysis with cellobiases from *Aspergillus niger* did not improve sugar release, which is consistent with our results. Since no significant improvements were observed – apart from the release of very small quantities of arabinose (<1 g/L) with the enzymatic cocktail NS22119 – in the logic of the development of an industrial process, the sole autoclave pre-treatment was selected to cut down both process time and reagents cost.

To prepare the medium for fermentation, different total solid concentrations were tested (3%, 5% and 10%). The amount of sugars released is proportional to the TS concentration (Glc: R^2 = 0.9971; Fru: R^2 = 0.9973), and no significant release of growth inhibitors, such as galacturonic acid, acetate, lactic acid or formic acid was observed. UGM 10% was, therefore, selected as the stock medium, containing on average of 19.1 g/L ± 2.14 g/L of Glc and 23.5 g/L ± 3.23 g/L of Fru. Ammonia, urea and primary amino-nitrogen (PAN) concentrations in UGM 10% were measured to assess the availability of nitrogen: ammonia concentration was 32.2 ± 1.38 mg/L, urea was not detected and the PAN value was 71.3 ± 1.94 mg_N/L, for a total amount of nitrogen lower than 100 mg_N/L. These quantities are one order of magnitude smaller than the concentrations of nitrogen generally used to cultivate *S. stipitis*.

Fermentation profile and folate detection in UGM

S. stipitis's fermentation profile was evaluated on different UGM dilutions (2%, 3%, 5% and 10% w/v); growth was observed in all conditions except for UGM 10%, which also caused problems when reading OD_{660} due to the turbidity of the medium (data not shown). Given the results obtained so far, growth kinetics on UGM were all carried out in shake flasks with an m/f ratio of 1:5, to allow sufficient aeration.

Growth profiles on UGM 3% (Figure 4A) and UGM 5% (Figure 4B) were further characterized, following the fermentation for 72 h after the inoculum: glucose is depleted in both conditions, but only 1.5 g/L of fructose are consumed; moreover, we noticed that growth caused an increase in the pH of the medium (from 5.5 to 6.5 at the end of the fermentation). Since there were no significant differences in the two conditions, UGM 5% was selected for further characterization, given its initial higher sugars concentration. Following the same strategy used for SBM, $MgSO_4$ (1 g/L) and KH_2PO_4 (6 g/L) and/or $(NH_4)_2SO_4$ (10 g/L) were added to UGM 5% to test for the presence of limiting nutrients (Figure S5). The addition of $MgSO_4$ and KH_2PO_4 did not significantly ameliorate the growth (compare with Figure 4B); the addition of $(NH_4)_2SO_4$ as nitrogen source – either alone or in combination with $MgSO_4$ and KH_2PO_4 – allowed to reach a higher biomass titre, with a faster and better consumption of sugars if compared with what observed on the sole UGM 5%; however, a very long lag phase (>72 h) was observed, and the pH at the end of the fermentation was low (pH 2.5–3). This could be explained by the mechanism of ammonium uptake, which requires the pumping of a proton in the medium (Cueto-Rojas et al., 2017; Mastella et al., 2022). These results suggest that nitrogen is a limiting factor, but that $(NH_4)_2SO_4$ is not the ideal source for *S. stipitis* on UGM 5%; moreover, the addition of $MgSO_4$ and KH_2PO_4 in combination with $(NH_4)_2SO_4$ allowed to reach a higher biomass titre and the complete depletion of both glucose and fructose, suggesting that these two salts are also a limiting factor.

Given these results, urea was tested as an alternative nitrogen source to $(NH_4)_2SO_4$; urea was used at a concentration of 4.6 g/L, which provides the same amount of nitrogen as 10 g/L $(NH_4)_2SO_4$. Growth in UGM 5% + 4.6 g/L urea +1 g/L $MgSO_4$ + 6 g/L KH_2PO_4 was assessed in shake flasks (Figure 4C): the cells reached 17 OD, doubling the value obtained in UGM 5%, and comparable to the biomass reached in kinetics with $(NH_4)_2SO_4$ and salts. Glucose and fructose are both depleted and, coherently to the proposed

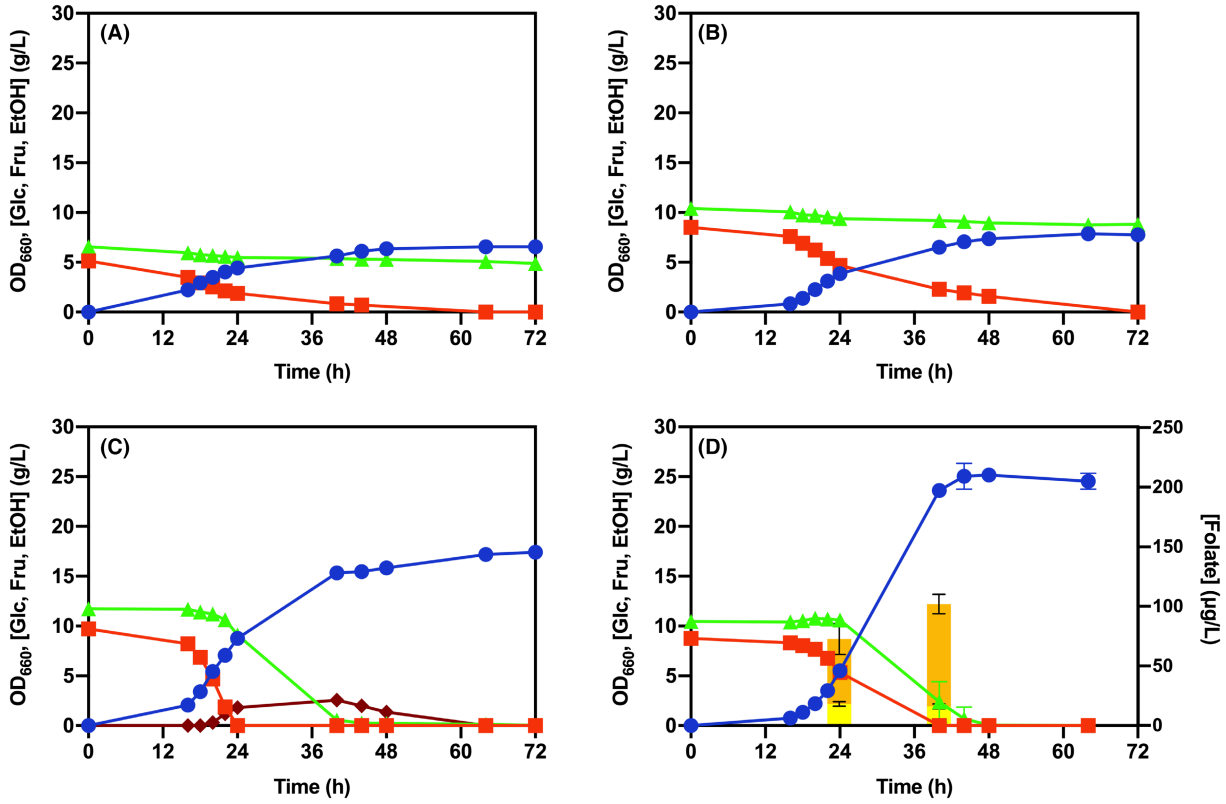


FIGURE 4 Growth and folate production profiles on UGM. (A) Growth and sugar consumption on UGM 3% with an m/f ratio of 1:5. (B) Growth and sugar consumption on UGM 5% with an m/f ratio of 1:5. (C) Growth and sugar consumption on UGM 5% + 4.6 g/L urea + 6 g/L KH_2PO_4 + 1 g/L $MgSO_4$ with an m/f ratio of 1:5. (D) Growth, sugar consumption and folate production on UGM 5% + 4.6 g/L urea + 6 g/L KH_2PO_4 + 1 g/L $MgSO_4$ in baffled flasks (m/f = 1:5). The left y-axis shows OD_{660} (blue, circles), glucose (squares), arabinose (green, triangles) and ethanol (brown, diamonds) concentration. The right y-axis shows the concentration in bars of free (yellow) and total (orange) folate. Values are mean ± standard deviation of three independent experiments.

mechanism of urea uptake (Mastella et al., 2022), an increase in pH at the end of the fermentation (from 5.5 to 7.5–8) is observed. Due to the faster growth, aeration becomes insufficient, as the production of 2.5 g/L ethanol is observed.

To avoid ethanol production, the growth was repeated in baffled flasks (m/f = 1:5) (Figure 4D). The higher aeration did avoid ethanol production and allowed cells to prolong the primary exponential phase, reaching 25 OD. Folate production was evaluated at the end of the exponential phase, with a peak concentration at 40 h of 101.9 ± 6.62 µg/L, and a yield of 5.30 µg/g on total sugars.

Folate production on UGM was the lowest obtained in this study, however, the yield was higher than in SBM.

CONCLUSION

S. stipitis was studied for the production of folate from different residual biomasses for the first time; while pre-treatment protocols were already available for SBM and SBP, UGM required a characterization step and the development of a pre-treatment and hydrolysis protocol. The NREL standard procedure was not efficient for fructose quantification, thus an alternative protocol was developed.

S. stipitis was able to grow on all three different media, formulated on the residual biomasses. Interestingly, SBM and SBP did not need the addition of nitrogen: these media contain enough yeast-available nitrogen (YAN) to sustain growth, even to high cell densities. This was not the case for UGM: the addition of $(NH_4)_2SO_4$ did improve growth but caused an extremely long lag phase, which was solved by using urea instead.

The highest production of folate (188.2 ± 24.86 µg/L) was obtained on SBM, showing, however, the lowest yield; the best yield was obtained on SBP (9.54 ± 0.17 µg/g). *S. stipitis* proved to be a versatile and robust yeast able to grow on different residual biomass, consuming all the sugars in the media (both hexose and pentose) with almost no additional nutrient requirements, making the overall process less costly. In terms of folate production on SBM, we were able to obtain a 33% higher titre than the best reported in the literature (obtained with *S. cerevisiae* in a bioreactor with SBM) (Hjortmo et al., 2008). To the best of our knowledge, this is the highest folate concentration obtained from residual biomasses ever reported. Future studies

should focus on scaling up the process in the bioreactor in order to better control and ameliorate the production while acquiring the data for a techno-economic analysis that will provide insights into the stage of technological development of this microbial production process.

AUTHOR CONTRIBUTIONS

Luca Mastella: Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Vittorio Giorgio Senatore:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); writing – original draft (equal); writing – review and editing (equal). **Tiziana Beltrani:** Writing – review and editing (supporting). **Paola Branduardi:** Conceptualization (lead); data curation (supporting); formal analysis (supporting); funding acquisition (lead); investigation (supporting); methodology (supporting); project administration (lead); resources (lead); supervision (lead); writing – original draft (supporting); writing – review and editing (supporting).

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article and its Supplementary Information file.

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SUPPORTING INFORMATION

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

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