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Growth tolerance of *Zygomycetes Mucor indicus* in orange peel hydrolysate without detoxification

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ABSTRACT

The capability of two zygomycetes strains, *Mucor indicus* and an isolate from tempeh (*Rhizopus* sp.), to grow on orange peel hydrolysate and their tolerance to its antimicrobial activity, was investigated. Both fungi, in particular *M. indicus*, tolerated up to 2% D-limonene in semi-synthetic media during cultivation in shake flasks, under aerobic as well as anaerobic conditions. The tolerance of *M. indicus* was also tested in a bioreactor, giving rise to varying results in the presence of 2% limonene. Furthermore, both strains were capable of consuming galacturonic acid, the main monomer of pectin, under aerobic conditions when no other carbon source was present. The orange peel hydrolysate was based on 12% (dry w/v) orange peels, containing D-limonene at a concentration of 0.6% (v/v), which no other microorganism has been reported to be able to ferment. However, the hydrolysate was utilised by *M. indicus* under aerobic conditions, resulting in production of 410 and 400 mg ethanol/g hexoses and 57 and 75 mg fungal biomass/g sugars from cultivations in shake flasks and a bioreactor, respectively. *Rhizopus* sp., however, was slow to germinate aerobically, and neither of the zygomycetes was able to consistently germinate in orange peel hydrolysate, under anaerobic conditions. The zygomycetes strains used in the present study demonstrated a relatively high resistance to the antimicrobial compounds present in orange peel hydrolysate, and they were capable of producing ethanol and biomass in the presence of limonene, particularly when cultivated with air supply.

1. Introduction

Vast quantities of citrus waste are generated each year in the citrus industry, during the production of juice and related products [1–3]. After juice extraction, about 50–60% of the fruit remain as citrus waste [4], mainly consisting of peels, seeds, and membranes, the dry weight being ca 0.2 g/g waste (w/w) [5]. Although not very cost-effective, citrus juice processors usually dry and sell the residues as raw material for pectin extraction or as animal feed [3,6]. As a consequence, a significant fraction of the industrial citrus waste is transported to waste disposal plants each year [7]. However, citrus peels are rich in pectin, cellulose, and hemicelluloses [8], which can be converted to fermentable substrates. Both orange and grapefruit peel waste have been successfully hydrolysed to glucose, galactose, fructose, arabinose, xylose, rhamnose, and galacturonic acid by enzymatic hydrolysis [1,6,9,10]. Several of these sugars can be used to produce ethanol and other chemicals [10,11].

If citrus waste could be used as an inexpensive feedstock for ethanol production, it would promote its application as a source for renewable fuels, simultaneously reducing its disposal problems [2,12]. However, the main obstacle to use citrus waste as raw material for ethanol production is the presence of peel oils, primarily limonene (95% D-enantiomer) [5,13]. D-Limonene, henceforth referred to as limonene, holds considerable antimicrobial activity, inhibiting yeast growth even at concentrations less than 0.05% [14]. Hence, to acquire a successful fermentation, the peel oils are usually removed from the peel hydrolysate [1,5]. Alternatively, using a microorganism less susceptible to limonene would reduce or even remove the need for a detoxification step.

Zygomycetes comprises a diverse group of fungi, found worldwide living as saprobes, parasites or as part of mycorrhizae [15]. Zygomycetes have been used by humans for centuries, mainly in Southeast Asia, for food production such as tempeh and tofu [16], while more modern uses include production of various enzymes, such as proteases, lipases and α-amylases [17]. Furthermore, *Mucor indicus* (formerly *M. rouxii*) is also excellent for ethanol production, in particular from lignocelluloses, since it is able to convert xylose

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into ethanol under aerobic conditions [18–20]. Previous reports on *Mucor* sp. revealed that they may be Crabtree-positive [21]. However, the NAD-dependent alcohol dehydrogenases of zygomycetes are more closely related to the bacterial alcohol dehydrogenases than to those of other fungi [22]. Some zygomycetes, on the other hand, lack the ability to produce ethanol [18]. The biomass produced in the process is also of interest, as it can be used for, e.g. fish feed [23], or be further refined into biodegradable superabsorbent polymers [24]. However, information on cultivation of zygomycetes on orange peel hydrolysate and its tolerance against limonene is lacking in the literature.

The aim of the present work was to study the feasibility of using zygomycetes for citrus waste recovery, investigating the growth of the zygomycete *M. indicus* on orange peel hydrolysate and on the resulting metabolite, along with fungal biomass production. For comparison, another zygomycete (*Rhizopus* sp.) isolated from tempeh was used. As part of the goal, the ability of these zygomycetes to tolerate limonene and to consume galacturonic acid, the main monomer of pectin, was also studied.

2. Materials and methods

2.1. Zygomycetes strains

M. indicus, CCUG 22424 (Culture Collection, University of Gothenburg) and *Rhizopus* sp., isolated from the Indonesian food tempeh (zygomycete IT [18]), were used for the experiments. The fungi were maintained on PDA slants containing 20 g/l glucose, 15 g/l agar, and 4 g/l potato extract, and were stored at 4 °C. The fungi were transferred to new agar slants every month and incubated aerobically at 28 °C for four to five days. Prior to the cultivations in liquid media, the strains were grown under identical conditions on PDA plates and then flooded with 20 ml sterile distilled water to release the fungal cells, mainly spores. Inoculations were carried out with 30 ml spore suspension/l medium, corresponding to $5.0 \pm 0.3 \times 10^5$ spores/l medium for *M. indicus*.

2.2. Orange peel and its hydrolysis

Orange peels from a mix of Mexican and Uruguayan oranges were provided by Brämhults Juice AB (Borås, Sweden). The peels were frozen, mixed with 20% dry ice, and milled with a laboratory mill (Retsch SM100, Retsch Germany) to obtain peels with a diameter less than 40 mm. The peels were stored frozen at −20 °C until used. The content of dry matter was established by drying the peels in quadruplicate at 105 °C for 24 h.

The milled peels were diluted to 20 l with water into a 12% (dry w/v) homogenate, after which enzymatic hydrolysis was carried out for 24 h as a single batch at 45 °C in a 35 l total volume bioreactor (Biostat C-20, B. Braun Biotech, Germany), with four impellers set to 500 rpm. Enzymes were added according to a previous study [6], including 0.24 FPU/g cellulase (Celluclast 1.5L, Novozyme, Denmark), 3.9 IU/g β-glucosidase (Novozyme 188, Novozyme, Denmark), and 1163 IU/g pectinase (Pectinex Ultra SP2, Novozyme, Denmark). The activity of cellulase was measured according to a standard method by NREL, based on the degradation of Whatman no. 1 filter paper [25], while the activity of β-glucosidase was measured according to a IUPAC standard method, based on the enzymatic hydrolysis of cellobiose into glucose [26], subsequently quantified by HPLC. The supplier provided data on activity of the pectinase (26,000 IU/ml).

During hydrolysis, the pH value was initially adjusted to 4.80 ± 0.07 with 10 M NaOH, and then kept constant by addition of 2 M NaOH. The size of the absolute majority of the peel units was reduced to micron size during the hydrolysis process, only a few being larger than 1 mm. After completed hydrolysis, the hydrolysate was centrifuged at $85{,}000 \times g$, using a continuous centrifuge (43 mm inner diameter, CEPA Laboratory Centrifuge LE, Carl Padberg Zentrifugenbau, Germany) with a flow rate of 5 l/h, to remove remaining sedimenting solids. The hydrolysate was then stored at −20 °C until used.

2.3. Cultivation on galacturonic acid

M. indicus and *Rhizopus* sp. were grown aerobically and anaerobically in a liquid semi-synthetic medium, containing 5 g/l yeast extract and 15 g/l galacturonic acid, supplemented with (in g/l): 7.5 $(NH_4)_2SO_4$, 3.5 KH_2PO_4, 1.0 $CaCl_2 \cdot 2H_2O$, 0.75 $MgSO_4 \cdot 7H_2O$ and 1 ml/l vitamin solution and 10 ml/l trace metal solution [19]. The pH value was initially adjusted to 5.5 with 2 M NaOH, and was maintained at this level (±1.0) during cultivation by addition of 2 M NaOH or 2 M HCl. The cultivation, a liquid volume of 150 ml in 250 ml baffled Erlenmeyer flasks, was carried out for two weeks in an agitated water bath at 32 °C and 125 rpm, with samples taken every 24 h. Aerobic cultures were provided with cotton plugs, while anaerobicity was maintained by a silicone plug with a water filled loop trap and a stainless steel capillary, through which samples were taken. The experiments were carried out in quadruplicate, and individual standard deviations were utilised for calculations of confidence intervals.

2.4. Effect of limonene

The antimicrobial effect of limonene was investigated by cultivating *M. indicus* and *Rhizopus* sp. in a semi-synthetic medium into which 0.25%, 0.5%, 1%, and 2% (v/v) limonene (Sigma–Aldrich Corporation, St. Louis, USA) had been added. The cultivations with 50 g/l glucose and 5 g/l yeast extract were carried out in a shaking water bath at 32 °C and 125 rpm, aerobically and anaerobically as described above (Section 2.3). The experiments were terminated after 84 h, at which point 12 samples had been taken.

The inhibitory effect of 1% and 2% limonene on *M. indicus* was investigated in a 2.5 l total volume bioreactor (Biostat A, B. Braun Biotech, Germany), with a working volume of 1.5 l, two impellers set to 300 rpm, and with 300 ± 2 ml/min aeration, regulated by a mass flow controller (Hi-Tech, Ruurlu, The Netherlands). The same medium, containing 0.13 ml/l silica antifoam (VWR International, West Chester, USA), was used, and pH was retained at 5.50 ± 0.07 by automatic addition of 2 M NaOH.

2.5. Cultivation on orange-peel hydrolysate

Rhizopus sp. and *M. indicus* were cultivated in 250 ml baffled Erlenmeyer flasks at 32 °C in an agitated water bath at 125 rpm, as described in Section 2.3, using 150 ml volumes of the hydrolysate, supplemented with salts, vitamins and trace metals.

Cultivation of *M. indicus* on supplemented orange peel hydrolysate was also carried out in a bioreactor, using similar conditions as described above. Anaerobic cultures were aerobically pre-cultured with 300 ± 2 ml/min airflow, using 750 ml of supplemented orange peel hydrolysate. After reaching maximal CO_2 production rate, the air was replaced by N_2 and another 750 ml portion of supplemented hydrolysate was added. Finally, after the second peak of CO_2 production, aerobic conditions were restored.

2.6. Analytical methods

Samples from cultivations were centrifuged for 5 min at $8000 \times g$ and then frozen at −20 °C, until analysed with HPLC (Waters, Milford, USA). For analyses of glucose, galacturonic acid, ethanol, glycerol, acetic acid, and pyruvic acid, an analytical ion-exchange column, based on hydrogen ions (Aminex HPX-87H, Bio-Rad, USA) and operating at 60 °C with 0.6 ml/min of 5 mM H_2SO_4 as eluent, was used. Galactose, cellobiose, fructose, xylose, and arabinose were analysed using a lead (II)-based column (HPX-87P, Bio-Rad) with two Micro-Guard Deashing (Bio-Rad) pre-columns operating at 85 °C, with 0.6 ml/min ultrapure water as eluent. A UV absorbance detector (Waters 2487), operating at the wavelength 210 nm, was used in series with a refractive index (RI) detector (Waters 2414 or 410).

Initial spore concentration was measured using a Buerker's counting chamber with a depth of 0.1 mm under light microscope. The spore solution was diluted ten times before the measurement, and the spores were counted in 50 E-squares, with a volume of 1/250 μl each.

Biomass concentration was determined at the end of each cultivation. The measurements of the cultivations in Erlenmeyer flasks were performed after harvesting all biomass by centrifugation for 5 min at $10{,}000 \times g$. For measurements of the bioreactor cultivations, 50 ml liquid samples were taken in triplicate. The resulting biomass was washed thrice with 50 ml distilled water, followed by drying at 105 °C for 24 h.

Limonene concentration of the orange peel hydrolysate was determined following extraction into n-heptane, by 40 ml n-heptane being added to 100 ml hydrolysate and thoroughly mixed, to promote the dissolution of limonene in the n-heptane. The n-heptane solution was separated from the water solution by centrifugation for 10 min at $10{,}000 \times g$, after which the top heptane fraction was removed and further diluted 100 times in n-heptane. The limonene concentration was determined by GC–MS (Hewlett Packard G1800C, Agilent, Palo Alto, CA), equipped with a 30 m HP-5MS column (Hewlett Packard). The injector and detector temperatures were 200 and 280 °C, respectively, and the initial column temperature was 50 °C, increased at a rate of 15 °C/min to 250 °C, at which point it was maintained for 3 min. Helium was used as carrier gas and was set with a flow rate of 1.0 ml/min. As internal standard, approximately 10 ppm 2-methylisohexanol was used and sample volume of injections was 1 μl. The experiment was carried out in octuplicate.

2.7. Statistical analyses

All experiments and analyses were carried out in duplicates, unless otherwise stated, and analysed using the software package MINITAB®. All error bars and intervals recounted, represent 95% confidence intervals, calculated from pooled standard deviations for that particular parameter of the experiment, unless otherwise noted. Results were analysed with ANOVA (analysis of variance) tables, using general linear models (multifactor) and one-way models, and factors were considered significant,