Effect of Bacterial Invasion on the Growth and Lipid Production of the Oleaginous Yeast *Rhodotorula glutinis*

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Abstract: Oleaginous microbes, grown heterotrophically on sugars derived from non-food crops or waste resources, are a renewable source of lipids. However, these cultures are prone to bacterial invasion. Ensuring optimal sterile conditions requires expensive pre-treatment techniques and has significant ramifications for the industrial-scale production of lipid derived biofuels, though, at present, it is unclear what effect a bacterial invasion would have on the organisms ability to accumulate lipid. In this investigation, the oleaginous yeast *Rhodotorula glutinis* (*R. glutinis*) was cultured under optimal conditions for lipid production (28 °C and pH 6.3) and the response to contamination by three common bacterial strains, *Escherichia coli (E. coli), Pseudomonas fluorescens (P. fluorescens)* and *Bacillus subtilis (B. subtilis*) was investigated. Bacterial strains were introduced to the yeast culture at 0, 4, 8, 12, 24 and 48 hours and their effect on the yeast growth and total lipid productivity was assessed. *R. glutinis* cultures that had been growing for less than 12 hours were unable to compete with any of the bacterial strains introduced. Lowering the temperature and pH allowed the yeast to compete more effectively, though it was found that these conditions were detrimental to the lipid productivity. The effect of invasion was also specific to the type of bacteria. *P. fluorescens* was found to be the most successful bacteria in competing with *R. glutinis,* while *B. subtilis* was found to be the least. Two common antibiotics, tetracycline and sodium metabisulfite were also investigated for their ability to limit the effect of a bacterial invasion.

Keywords: Biodiesel, lipids, yeast bacteria co-cultures, antibiotic, tetracycline.

INTRODUCTION

Diminishing supplies of fossil fuels, an increased awareness of their negative environmental impact and concerns over the security of their supply means that alternative liquid fuels for the transport sector are being increasingly sought. One such fuel is biodiesel, the Fatty Acid Methyl Esters (FAME) produced by the transesterification of triglyceride lipids. Biodiesel is predominantly produced from the transesterification of rapeseed, palm or soybean oils. However, there are environmental impacts associated with the production of fuels from such feedstocks as their production is land and water intensive, is widely viewed as unsustainable and can cause environmental degradation due to over farming. Palm oil production in Southeast Asia, for example, has been cited as a key source of deforestation, loss of biodiversity and social conflict [1]. In addition to the production of biofuels, lipids are also being increasingly used in the production of novel biopolymers and in personal care products [2].

One alternative to terrestrial oils is the production of lipids from single cell, micro-organisms such as microalgae, bacteria, fungi and yeasts. Species from all of these organisms have the potential to produce lipids, though phototrophic microalgae have attracted the most research interest to date [3]. An alternative option for lipid production is to harness heterotrophic growth as opposed to phototrophic, either in microalgae such as *Chlorella protothecoides* or in fast growing oleaginous yeasts such as *Rhodotorula glutinis, Yarrowia lipolytica* or *Lipomyces starkeyi* [4-8]. Such yeasts have been reported as containing over 20% of their dry weight in lipids, can be grown in dense colonies and on sugars derived from cellulosic materials, such as grasses, waste agricultural fodder or food waste residues [8-11]. While converting cellulosic materials to accessible inexpensive sugars is still a large technological challenge, cultivating yeast for energy has a number of advantages; it uses existing fermenting technology, it can potentially produce alternative co-products, it does not require large open spaces for cultivation and, unlike microalgae, neither sunlight nor land usage are limiting factors as yeast can grow in darkness allowing for round-the-clock production.

Oleaginous organisms such as *R. glutinis* do not accumulate high levels of triglycerides under balanced nutrient conditions, however, when nitrogen or phosphorous are limited in the system carbon is channelled into lipid production [12]. As such, yeast cultivation is a two-step process. Initially yeast is grown to stationary phase in a nutrient enriched environment; this stage is followed by a secondary phase where

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yeast is grown in a carbon enriched culture with no or little other nutrients, to allow for lipid accumulation.

One issue with the production of lipids from microbes in monoculture is the presence of invading species that can outcompete the target organism. In microalgae cultivation, this has been observed with alternative wind-borne algal species, or algal species contaminating waste feedstocks [13]. In the production of heterotrophic organisms, the largest threat of contamination is from bacteria. Most bacterial species have a higher growth rate in comparison to yeast species and also have the capacity to thrive in a diverse range of environments. Bacterial invasion is a specific problem when using sugars from a variety of sources coupled to waste streams. As such, to ensure the strict sterile conditions required for yeast production, severe pre-treatment of the feedstock, such as ozonation, microfiltration, UV treatment or autoclaving must first be undertaken. This significantly increases production costs and has significant ramifications for producing lipids on an industrial scale. In the industrial production of microbial fuels, strain selection is important and the risk of bacterial contamination can be reduced by choosing an organism that thrives at an extreme pH, temperature or salinity [14-15].

R. glutinis has attracted a large amount of research interest recently as a sustainable source of lipids [5, 16, 17]. *R. glutinis* is optimally cultured for lipid production at 28 °C, and tolerates a large pH range though slightly acidic is optimal [18]. Under optimal culture conditions it can produce between 30 – 70% dry weight of lipid after nutrient starvation, with a culture time of between 3-5 days. In this investigation the ability of *R. glutinis* to compete and produce lipids in the presence of one of three common bacterial strains, *E. coli, P. fluorescens* and *B. subtilis* was investigated.

MATERIAL AND METHODS

All chemicals were purchased from Sigma Aldrich, UK and were not further purified prior to use. All media, vessels and equipment were autoclaved at 121 °C for 20 minutes prior to use.

Culture Conditions

Stock solutions of *R. glutinis* (2439, purchased from the National Collection of Yeast Cultures, Norwich, UK) were maintained on sterile YMG (yeast extract 3 g L^{-1}, glucose 10 g L^{-1}, malt extract 3 g L^{-1} and peptone 5 g L^{-1}) plates with 1% (w/v) agar. These were used to inoculate 25 ml aliquots of sterile YMG media. These primary cultures were incubated for 48 hours at 28 °C in a shaking incubator, in accordance with previous studies (Yen *et al*. 2012), before inoculating 200 µL of this culture in 25 ml aliquots of sterile YMG in 50 ml sterile plastic centrifuge tubes. Stock cultures of *E. coli*, *P. fluorescens* and *B. subtilis* were streaked onto LB media agar (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl) and grown at 30 °C for 24 hours. The resulting bacterial colonies were used to inoculate the yeast-inoculated YMG in 50 ml plastic centrifuge tubes at the different time points. The bacteria were added to the cultures by picking up a bacterial colony from the agar plate with an inoculation loop, and dissolving it in the yeast culture. All bacterial contaminated yeast cultures, plus controls, were shaken at 23 °C or 28 °C at 180 rpm for the duration of the experiment. At 72 hours, cell cultures were centrifuged at 4,000 rpm for 5 minutes before the resulting YMG supernatant was removed and replaced with a sterile 20 g L^{-1} glucose solution. Repeats of each culture were carried out to calculate standard deviations.

Cultures with Antibiotics

The stock cultures of *R. glutinis, E. coli*, *P. fluorescens* and *B. subtilis* were used to inoculate 25 ml aliquots of sterile YMG media (pH 6.3), with additional tetracycline or sodium metabisulphite (0, 0.1, 0.5, 1, 2, 3, 4, 5 or 10 mg L^{-1}), in 50 ml sterile plastic centrifuge tubes. These cultures were incubated for 120 hours at 28 °C, 180 rpm, in a shaking incubator. Repetitive sampling was carried out to calculate standard deviations. The standard deviation was no higher than 8% for any sample investigated.

Analysis of the Cultures

1 ml aliquots of cell culture were pipetted into sterile 1 ml cuvettes before optical density measurements were read on a Cecil 1000 Series spectrophotometer at 600 nm. Sterile YMG was used as a control. For cell counts, cell-cultures were pipetted into 10-well FastRead microscope slides and examined under 40 X magnification. Total cell counts were taken from at least 6 different areas of the slides, the average and standard deviation were then calculated from these results.

Lipid Extraction and Analysis

The extraction of the lipids was carried out using an Anton Parr monowave 300 microwave reactor

equipped with a MAS 24 autosampler capable of loading 10 ml reaction vessels. Biomass was suspended in a 2:1 $CHCl_3$/MeOH mixture (6 mL) with a stir bar. The microwave was set on an automated cycle of three stages; heating to 80 °C (typically taking less than 1 minute) with 1000 rpm stirring, stirring at 600 rpm for 15 minutes to allow the reaction to take place and finally fast cooling using compressed N_2 (typically less than 2 minutes). The resulting oil was extracted into chloroform and washed with water three times to remove remaining water soluble components. The chloroform was removed under reduced pressure and the amount of lipid extracted calculated gravimetrically.

RESULTS

To establish the effect of bacterial invasion on a culture of *R. glutinis,* cultures of the yeast were grown for 120 hours. At 72 hours, the supernatant was replaced with a glucose solution to promote lipid production. The cultures were inoculated with *E. coli, P. fluorescens* and *B. subtilis* at 0, 4, 8, 12, 24 and 48 hour time intervals and sampling took place at 4, 8, 12, 24, 48, 72 and 120 hours for further analysis. Like the majority of yeasts, *R. glutinis* regulates the pH; the particular strain of *R. glutinis* used in this investigation was found to change the starting pH of the culture from 6.3 to 5 over a 60 hour time-frame (Figure **1**).

As most common bacteria grow preferentially at pH 6.5 or above, lowering the pH was investigated as a key method of reducing the impact of bacterial invasion. *R. glutinis* is optimally cultured at 28 °C for lipid production, though the yeast has also been shown to have reasonable growth rates at lower temperatures [19]. The effect of temperature on reducing the impact of bacterial invasion, by culturing at 23 °C, was therefore also examined (Figure **2**).

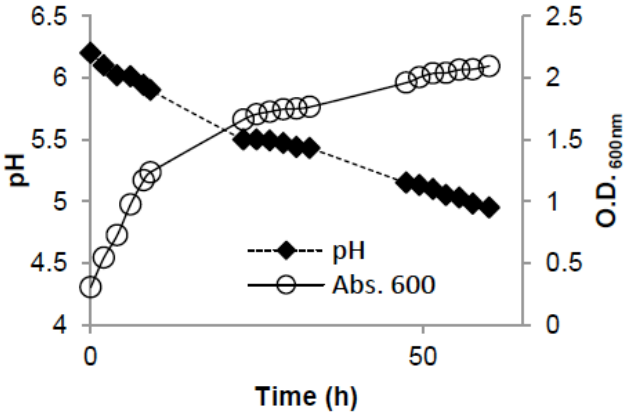

Figure 1: pH change in a pure culture of *R. glutinis* cultured at 28 °C.

Turbidity of the Cultures

To determine the growth of the cultures, turbidity was assessed by measuring the optical density at 600nm. Control experiments demonstrate that the yeast can survive at both lower pH (5 compared to the optimal 6.3) and a lower temperature (23 °C compared to 28 °C) with only a small reduction in the overall growth. By altering environmental conditions as a method of reducing the impact of bacterial invasion, the conditions are no longer optimal for yeast growth, though under these conditions the biomass yield is not greatly reduced (Figure **2**). As the optical density of a culture is related to the total biomass and is not species specific, the measurement gives an impression of the overall productivity of the co-culture. For all cultures containing *P. fluorescens* and *E. coli*, there is a significant amount of total biomass irrespective of the culture conditions and time of inoculation. The turbidity of the cultures inoculated with *B. subtilis* at 0, 4 or 8 hours are substantially lower than the other cultures except at 23 °C, pH 5. This is indicative of low levels of biomass, potentially due to *B. subtilis* having outcompeted the yeast though not growing optimally under the resulting conditions.

The Extent of Bacterial Contamination

To determine the effectiveness of the yeast to withstand bacterial contamination, cell counting was undertaken on the samples taken at 12, 24, 48, 72 and 120 hours. On the introduction of *P. fluorescens* at the optimal conditions used for *R. glutinis* (28 °C, pH 6.3) the yeast is outcompeted completely. This was even the case when the yeast had been growing for 24 hours prior to the introduction of the *P. fluorescens* (Figure **3**). It was only after the introduction of *P. fluorescens* at 48 hours that the yeast culture was well established and no *P. fluorescens* cells were observed in the final culture. Reducing the starting pH from 6.3 to 5 had little effect on the *P. fluorescens* which was still found to compete effectively with the yeast. This was even the case when the bacteria were introduced after 24 hours, although at the end of the experimental time-frame a small number of yeast cells were present in the culture. Reducing the temperature to 23 °C, in comparison to 28 °C, was more effective at controlling the bacterial growth. After inoculation with *P. fluorescens* at 24 hours, over 90 % of the cells are bacterial prior to the starvation stage; this then drops to near 70 % by the end of the culture. Similarly, the yeast is noticeably present in all of the final samples under these conditions. By reducing the original pH to 5, as

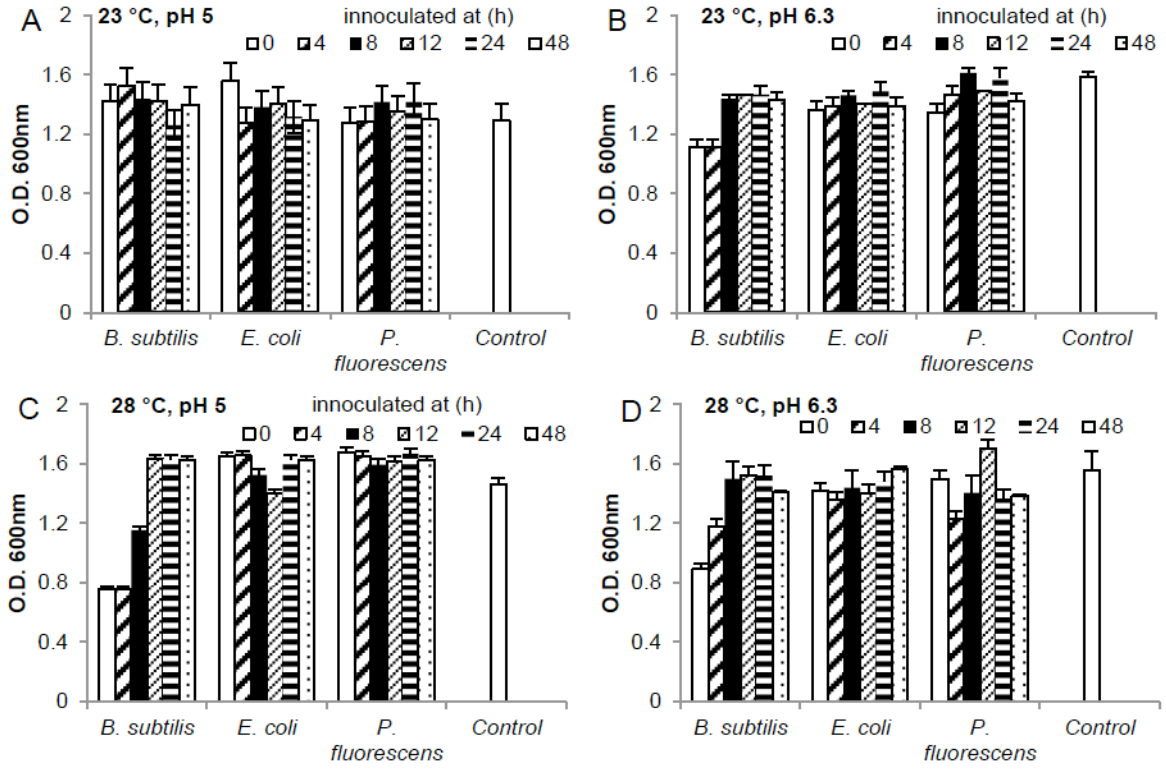

Figure 2: Optical density of the cultures of *R. glutinis* and bacteria after 120 hours of growth cultured at 23 °C or 28 °C at both pH 5 and 6.3. The separate data points indicate the time of inoculation of the bacteria.

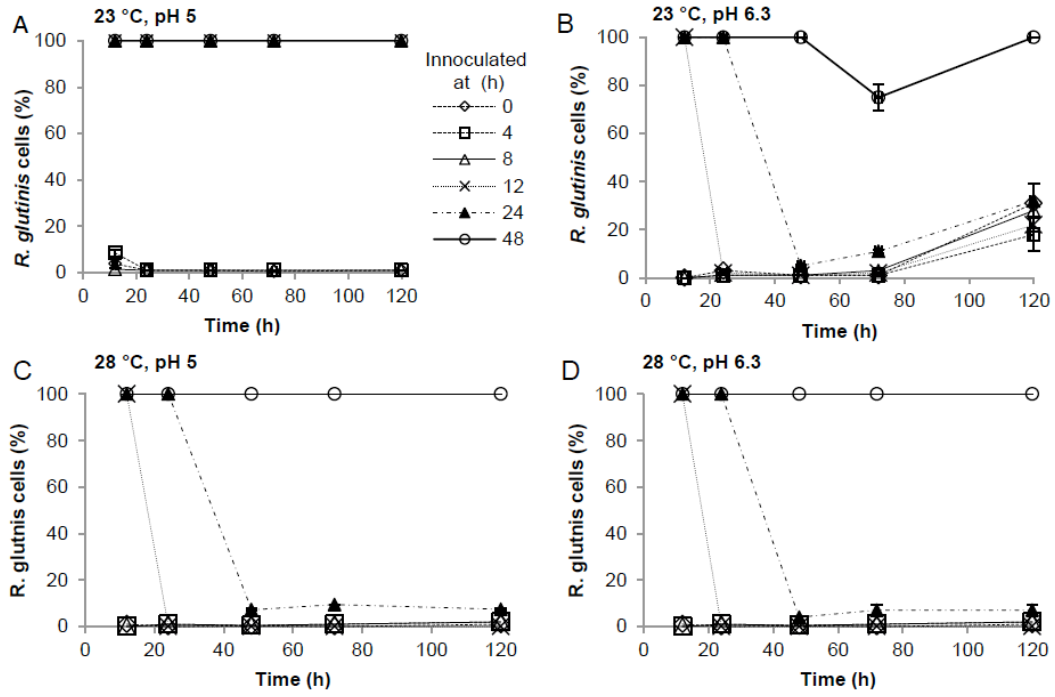

Figure 3: The percentage of yeast cells from the total in the cultures of *R. glutinis* inoculated with *P. fluorescens*, cultured at 23 °C, 28 °C, pH 5 and pH 6.3.

well as using the low temperature, a purely yeast culture was obtained from the samples inoculated at 12, 24 and 48 hours.

In comparison, when introduced to a *R. glutinis* culture at 23 °C, pH 5, *B. subtilis* was unable to compete, even when the bacteria was present from the

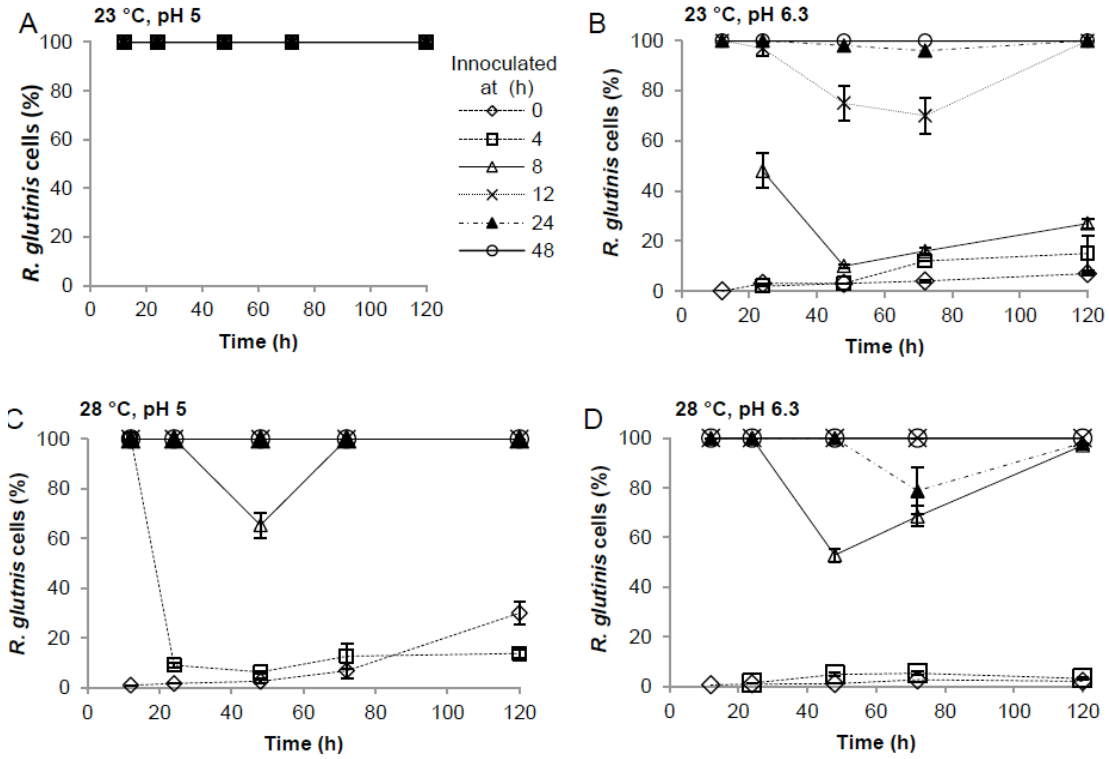

Figure 4: The percentage of yeast cells in the cultures of *R. glutinis* inoculated with *B. subtilis*, cultured at 23 °C or 28 °C at both pH 5 and 6.3.

start of the culture (Figure **4**). At temperatures of 28 °C and a pH of 5, *B. subtilis* introduced at 0 and 4 hours was found to outcompete the yeast. Under these conditions, however, the yeast only needs 8 hours of sterile growth before it is at a level at which is can completely dominate the culture by 120 hours. At 23 °C and pH 6.3 the *B. subtilis* competed far more successfully. On addition of *B. subtilis* at 0, 4 or 8 hours the bacterial cells compete effectively and were found to be dominant by the end of the culture. Even when *R. glutinis* was grown for 12 or 24 hours prior to the introduction of bacteria, *B. subtilis* is observed in the cultures in small percentages until the 72 hour starvation stage, after which only yeast cells were observed.

The majority of *E. coli* strains grow optimally between 30 - 37 °C and at a pH of between 6 and 8, though many strains have been reported to survive in far harsher conditions [20]. Unlike the other two bacteria detailed in this study, *E. coli* and *R. glutinis* were found to grow in co-culture together, irrespective of the conditions investigated or the time of inoculation (Figure **5**). At pH 6.3 and 28 °C, all of the cultures contained a significant percentage of *E. coli* cells. *E. coli* was found to compete effectively under these conditions and cultures were only found to be predominantly yeast if they were grown under sterile conditions for 24 hours or more. On reducing the starting pH to 5, while maintaining the higher temperature of 28 °C, *E. coli* growth was negatively impacted. However, by the end of the culture similar ratios of bacteria to yeast were observed.

Reducing the temperature from 28 to 23 °C, while retaining a starting pH of 6.3, had little effect on the total percentage of *E. coli* in the cultures. On reducing the pH and the temperature, however, *R. glutinis* was found to compete far more effectively. At the early stages of the culture, *E. coli* was found to be the dominant species, but over time the percentage of yeast increased until *R. glutinis* was the dominant microbe in all of the final stage cultures.

The Effect of the Bacteria on Lipid Production

None of the control *R. glutinis* cultures grown at 23 °C produced high levels of lipid, with 0.3 g L^{-1} recovered at pH 5 and 0.36 g L^{-1} observed at pH 6.3. The maximum lipid recovered, 0.85 g L^{-1}, was from the cultures grown at 28 °C with a starting pH of 6.3. Slightly less lipid, 0.78 g L^{-1}, was isolated from the cultures grown at the lower pH.

To estimate the level of yeast in the cultures the optical density was multiplied by the percentage of

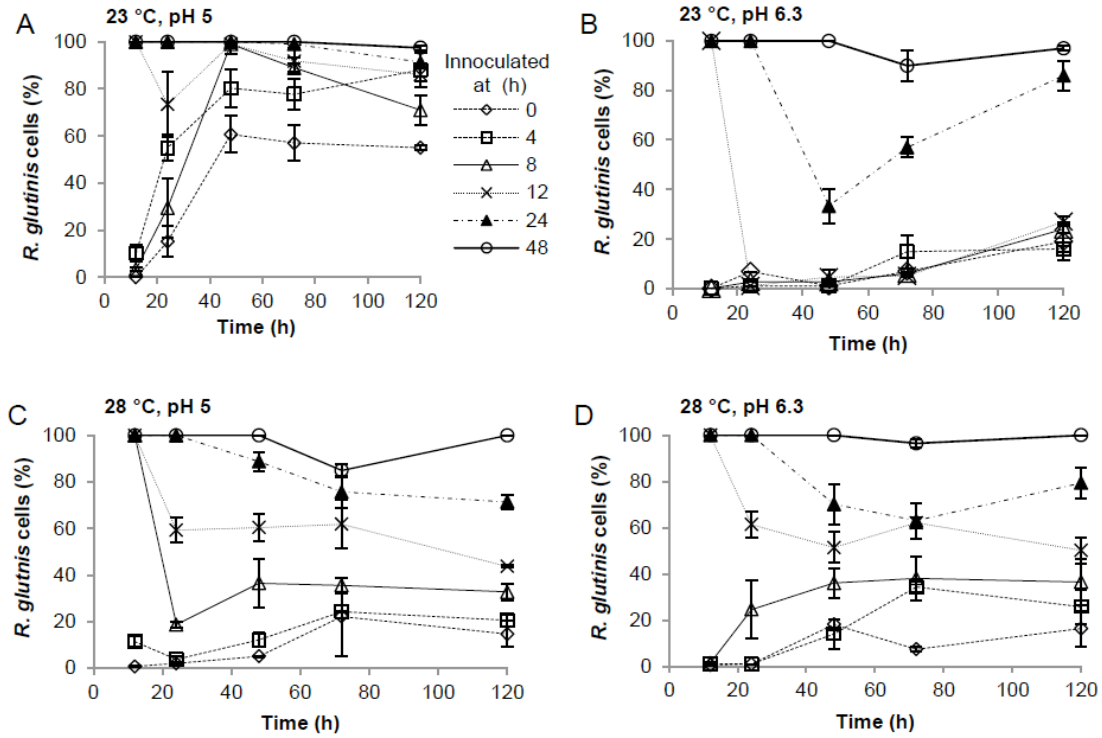

Figure 5: The percentage of yeast cells to bacterial cells in the cultures of *R. glutinis* inoculated with *E. coli*, cultured at 23 °C or 28 °C at both pH 5 and 6.3.

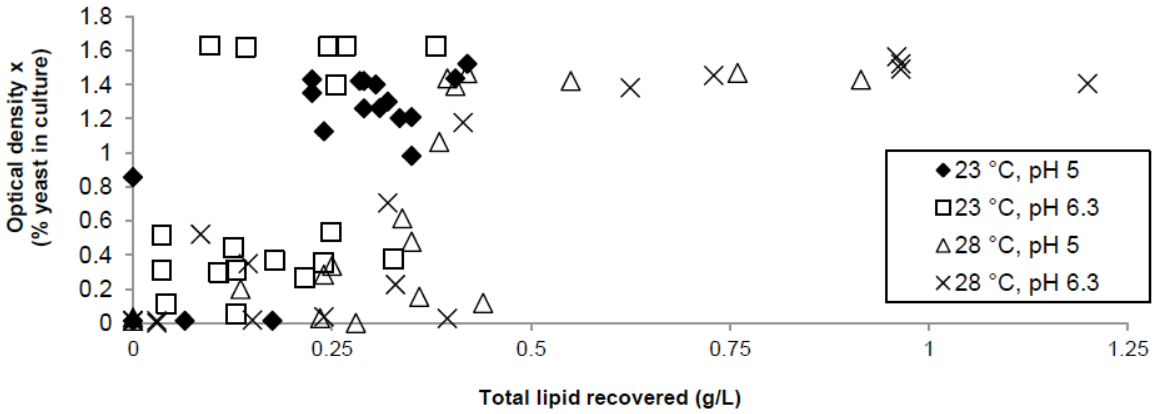

Figure 6: Composite analysis of the total lipid produced from the cultures inoculated with bacteria.

yeast cells in the cultures, this was then compared to the total lipid extracted (Figure **6**). This composite analysis gives an indication of the overall productivity of the cultures when bacteria are present. The lipid recovered for each individual experiment is given in the supporting information.

At 23 °C and pH 5, *R. glutinis* completely outcompeted *B. subtilis* irrespective of when the bacteria were inoculated into the cultures. Introducing *P. fluorescens* at 0, 4 or 8 hours resulted in predominantly bacterial cultures, on introduction of the bacteria at 12, 24 or 48 hours, the cultures were found to be predominantly yeast. Unlike *B.subtilis* or *P. fluorescens, E. coli* was observed in all the final cultures investigated, irrespective of the inoculation time or conditions. The maximum amount of lipid recovered from these cultures is only 0.4 g l^{-1}, though when high levels of yeast are present in the culture, irrespective of the identity of the bacteria, similar lipid levels to this were observed.

At 23 °C and pH 6.3, all of the bacteria competed more effectively with the yeast Under these conditions less lipid, in comparison to the control, was recovered from these systems, and most of the cultures produced less than 0.25 g l^{-1} of lipid. No matter what effect the temperature has on the bacteria in the cultures, 23 °C

is too low to produce suitable levels of lipid. Raising the temperature to 28 °C increased the total lipid in the control cultures. In the cultures inoculated with bacteria there is a clear correlation between the amount of yeast and lipid content. When the optical density due to the yeast is less than 0.8, low lipid levels were observed in all the cultures tested. However, when the yeast was present in large quantities 0.55 – 1.2 g l^{-1} of lipid were achieved.

The Effect of Antibiotics on the Microbial Cultures

While changing the environmental conditions has an effect on the bacterial composition, little lipid was isolated from the cultures grown at 23 °C. An alternative method to combat contamination by bacteria is the use of antibiotics. In an attempt to keep the cultures pure, two standard antibiotics were examined for their effect on *R. glutinis* growth. The first, tetracycline, is a broad range polyketide antibiotic which is commonly used to treat a variety of bacterial infections. The second type selected was sodium metabisulphite, a chemical agent commonly used to sterilise equipment for the production of beer and wine. Sodium metabisulphite has been shown to be particularly effective against bacteria which thrive in the conditions favoured by *Saccharomyces cerevisiae* [21].

Tetracycline is known to have little effect on the growth of yeasts, or specifically on *Rhodotorula* species [22, 23]. This was confirmed for the *R. glutinis* strain used in this study. Tetracycline was found to be extremely effective against the bacteria studied, however, with 2 mg l^{-1} being enough to inhibit the growth of the bacterial strains under investigation (see supporting information).

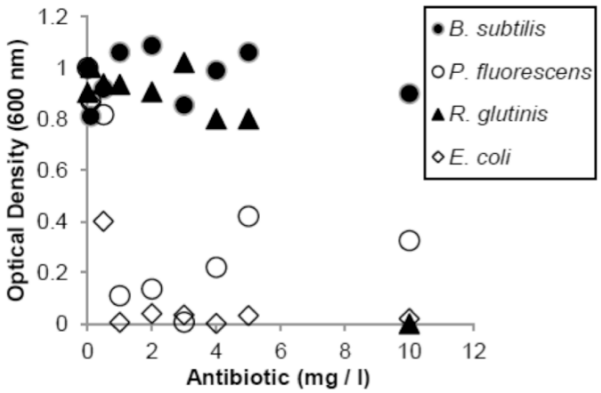

Figure 7: Normalised OD_{600nm} for cultures of *E. coli, P. fluorescens, B. subtilis* and *R. glutinis* which have been grown in the presence of sodium metabisulphite. Error bars have been excluded for clarity, but the standard deviation was found to be no higher than 8% for any sample.

Sodium metabisulphite was also extremely effective at inhibiting the growth of *E. coli* and *P. fluorescens* (Figure **7**). No growth of *R. glutinis* was observed when 10 mg l^{-1} sodium metabisulphite was present, though sodium metabisulphite had little effect on *B. subtilis,* even at this high concentration. To avoid bacterial invasions of *R. glutinis* in the laboratory, or potentially on a larger scale, then tetracycline seems to be the more effective antibiotic.

DISCUSSION

P. fluorescens has a wide temperature range that it can thrive in, and has been reported to grow at temperatures as low as 21 °C. The bacteria also typically prefer neutral or even alkaline conditions but also regulate the environment by producing basic extracellular components [24]. It is therefore unsurprising that the bacteria can compete so effectively with *R. glutinis* under the conditions examined. Alternatively, *B. subtillis* is reported to prefer alkaline conditions with optimal growth rates between pH 6.5 – 7 [25]. *B. subtilis* is also temperature sensitive and exhibits poor growth under 25 °C. Though somewhat surprisingly, at 28 °C and pH 6.3, *B. subtilis* did not outcompete the yeast unless it was inoculated at the start of the culture. When introduced at 8 hours, the bacteria was found to be present during the culture, though like the cultures at lower temperatures *R. glutinis* had completely outcompeted the *B. subtilis* after the starvation stage. This was possibly due to conditions being optimal for *R. glutinis* while the temperature was still too low and the pH too acidic for optimal *B. subtilis* growth.

E. coli is reported to thrive in a far wider range of conditions than either of the other two bacteria examined, so it was not surprising to find that it competed so effectively with the yeast under the specified test conditions. It is interesting that *E.coli* was found to be present in all the yeast cultures, yet was not found to fully outcompete the yeast or be outcompeted by the yeast as was the case for *B. subtillis* or *P. fluorescens*. It is possible that *E.coli* cells are not as affected by the yeast metabolites and potentially are utilising a product of yeast metabolism, allowing them to survive in these unfavourable conditions. *E.coli* have been reported to flourish under a wide range of environmental conditions and it could be that although conditions were adequate for survival, that they were not the optimal conditions required for sufficient growth to fully compete with the yeast.

For the cultures inoculated with *P. fluoresecens* or *E. coli*, irrespective of the growth conditions, the amount of lipid recovered was proportionate to the amount of yeast in the culture. The presence of these bacteria did not appear to impede lipid accumulation on removal of the nutrients. In some of the cultures containing *B. subtilis* there was a reduced level of lipid extracted, though this was potentially due to the *B. subtilis* interfering with the growth of the yeast rather than the lipid accumulation in the cells. Therefore, as long as a reasonable growth of yeast is achieved, through maintaining sterile conditions for the first stages of a culture, any subsequent bacterial invasion does not greatly affect the overall lipid productivity of the system.

While the use of antibiotics is practical on a laboratory scale, the cost is likely to be unfeasible in the industrial production of fuels. Additionally, extended use of common antibiotics at an industrial scale could soon produce resistant bacterial colonies [26]. While a low temperature offers some protection for *R. glutinis* from contamination, this also has a detrimental effect on the lipid productivity. The only feasible method found to reduce the impact of invasion was to increase yeast density prior to the introduction of the bacteria. This technique is currently used in the fermentation of sugars to bioethanol, however, culturing oleaginous species is a two-step process. If this method was applied to an industrial scale, a two-step semi continuous process would be needed. In the first stage it would be necessary to culture the yeast for 24-48 hours under rigorous sterile conditions. In the second, the yeast would be transferred to a less rigorously controlled system for the remaining growth stage and lipid accumulation stage.

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SUPPLEMENTAL MATERIALS

The supplemental materials can be downloaded from the journal website along with the article.

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