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preliminary communication

Effect of Cultivation Conditions on the Yeast-Induced Flocculation of *Escherichia coli*

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Summary

In humans, as well as in animals, Saccharomyces cerevisiae can be used to treat diarrhea caused by Escherichia coli. However, the antagonistic effect of the yeast on E. coli is still not fully understood. In order to elucidate the mechanism, the yeast-induced flocculation of both the nonfimbriated E. coli strain XL1 Blue and the fimbriated strain 4350, isolated from the faeces of a cow with diarrhea, was studied. Both strains flocculate in the supernatant of liquid cultures of a Sacch. cerevisiae strain that is used in antidiarrhea treatment. Presumably, this flocculation is induced by a glycoprotein released by the yeast in the culture medium during fermentation. This glycoprotein may link the E. coli cells together by a lectin type of binding.

The yeast-induced flocculation of the bacteria is dependent upon their cultivation conditions. For both E. coli strains, cells harvested at different cultivation stages showed a different flocculation response in the yeast supernatant. Stationary phase cells are less flocculent than cells from the logarithmic growth phase.

The flocculation assay, presented here, will be used to study the mechanism of the Sacch. cerevisiae – E. coli interaction in more detail.

Keywords: Escherichia coli, Saccharomyces cerevisiae, fimbriae, yeast-bacterium interaction, diarrhea

Introduction

The emergence of pathogens resistant to conventional anti-microbial agents makes it essential to intensify the search for new strategies for the prevention and treatment of infectious diseases. One approach is based on the knowledge that the adhesion of microorganisms to host cell surfaces is the first step in the development of a wide variety of infections. Interference with adhesion can prevent infection and may result in cure *(1)*.

Bifidobacterium and *Streptococcus thermophilus* strains have been used in infant formula to treat diarrhea, and showed a significant reduction in acute diarrhea from 30% to 7% in the group who received the supplement and also a reduction in rotavirus shedding, from 40% to 10%. The yeast *Saccharomyces boulardii* is another microorganism being studied for its antidiarrheal effects. When this yeast was administered along with antibiotics in several clinical trials, its use reduced the incidence of diarrhea by about half. In the chicken test, ovum-introduced yeast reduced colonization by *Salmonella* from 96% to 19% *(2)*.

Surface carbohydrates are indicated to be primarily responsible for cell recognition *(3)*. The simplest example is the role of carbohydrates in blood types which are differentiated by cell coat sugars. Bacteria have lectins (proteins or glycoproteins) on the cell surface which recognize specific cell receptors allowing them to attach. These receptors can be found on the surface of epithelial cells in animals. Binding of *Salmonella, Escherichia coli* and *Vibrio cholerae* has been shown to be mediated by a mannose-specific lectin-like substance on the bacterial cell surface *(4)*, and as for *Salmonella typhimurium* and *E. coli*, it has been demonstrated that the pili participate in the yeast-bacterium interaction *(5–9)*. Saturation of binding sites on the bacterial surface by mannan oligosaccharides prevents the attachment of these organisms to the epithelial membrane receptor, which contains mannose or a mannose-like structure. The mannan oligosaccharides have been compared to a raft passing through the gastrointestinal tract. During its passage, it binds to pathogens and then, these pathogens are washed out.

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Both soluble yeast mannan and intact yeast cells, which are known to contain mannans on their cell surface, agglutinated *E. coli* cells and thereby excluded *E. coli* cells from the binding surface *(10,11)*. In addition, the use of yeast cells as a probiotic additive in commercial animal feed products and additives has been claimed to have one or more beneficial health effects, such as:
I. colonization of the gastrointestinal and/or urogenital tract with potential antagonistic effects on pathogenic bacteria or improved recovery of intestinal disorders;
II. beneficial metabolic activities of the probiotic microorganism, *e.g.* production of vitamins, or bile salt hydrolyzing (BSH) activity; lowered blood cholesterol levels;
III. therapeutic effects, *e.g.* claimed stimulation of the immune response, possible protection against early events in carcinogenesis, and antidiarrheal effects *(12–14)*.

Our studies show that *E. coli* cells flocculate in a culture supernatant of *Sacch. cerevisiae*. This induced flocculation may be involved in the antagonistic effect of the yeast on *E. coli*.

Materials and Methods

Strains

The yeast strain *Sacch. cerevisiae* PRT was isolated from a commercial pharmaceutical preparation.

E. coli XL1-Blue MRF', JM101, C-3000 and HB101 were from the collection of our laboratory; *E. coli* strains 4350 and 26 were purchased from ATCC.

Preparation of E. coli cells

The *E. coli* strains were stored at –80 °C in TB broth with 40% glycerol, and were grown in TB broth at a temperature of 37 °C on a reciprocal shaker at 110 strokes · min^{-1} as described before *(15)*. TB broth contained, per liter of water, 12 g tryptone, 24 g yeast extract, 4 g glycerol, 2.314 g KH_2PO_4 and 12.54 g K_2HPO_4. Cells were harvested at different incubation times by centrifugation at 8000 g for 20 min. The cells were washed with distilled water and resuspended in phosphate buffer 1/15 M, pH = 5 to 6 (according to the strain) to obtain 130 mg/mL (wet weight) *(15)*.

Production of the yeast culture supernatant

The yeast strain was kept at –80 °C in SD medium with 15% glycerol. SD medium contained, per 100 mL of water, 0.67 g yeast nitrogen base (Difco) and 2 g glucose. Precultures were grown in 50 mL SD broth in 250 mL conical flasks, for 12 h at 28 °C on an orbital shaker, at 170 rpm. Fermentation was carried out in a 6.2 L BioFloIII fermentor (New Brunswick Scientific), containing 4 L of SD medium with 10% glucose. The fermentation was carried out at an agitation speed of 250 rev · min^{-1} and the temperature of 28 °C. Before pitching with 90 mL of preculture (of A equal to 0.8 at 600 nm), the medium was air saturated. Fermentation (with no further aeration) was stopped when the pH showed a clear increase. The yeast culture was centrifuged at 10,000 g for 10 min. To avoid microbial contamination during flocculation experiments, the final ethanol concentration was adjusted at 10% using 95% ethanol. The yeast supernatant could be stored at –20 °C until further use.

Flocculation assay

In the flocculation experiments, the formation of flocs of *E. coli* cells in the yeast supernatant was studied as described before *(15)*, modified from the method for studying the yeast-induced flocculation of *Pediococcus damnosus (16,17)*.

Flocculation experiments were set up using small bottles (12 mL) containing 200 μL of the *E. coli* cell suspension resuspended in 2 mL of buffer. Flocculation was initiated by adding 1.8 mL of yeast supernatant. The bottles were then shaken at 150 strokes · min^{-1} on a reciprocal shaker at 28 °C for 4 h, at which point the flocculation reached a steady state. To blank samples, 1.6 mL of buffer and 0.2 mL of 90% ethanol were added instead of yeast supernatant to make the 1.8 mL solution contain 10% ethanol.

After incubation, the suspensions were allowed to settle for 20 min. The upper 3.5 mL of the solution were removed and brought with water to a volume of 4 mL in a second tube (A). The precipitate was also diluted with water to 4 mL, and this suspension was designated as B. From the blank sample, 3.5 mL was also removed and the remaining 0.5 mL was diluted also to 4 mL. This was suspension C. All tubes were then vortexed to homogenize the suspensions, of which the cell densities (A_A, A_B and A_C) were measured spectrophotometrically using an Ultrospec IIE (LKB) instrument set at 600 nm.

The flocculation inducing activity is the percentage of the *E. coli* cells that flocculated in the yeast culture supernatant as follows *(15)*:

$$\text{Net flocculation}/\% = \frac{A\,(\text{flocculated } E.\ coli \text{ cells})}{A\,(\text{total } E.\ coli \text{ cells})} \times 100 = \frac{A_B - A_C}{A_A - A_B} \times 100$$

Results

Effect of E. coli growth phase on flocculation

Fig. 1 shows the flocculation of *E. coli* 26 in the yeast supernatant in function of the cultivation time. The maximal induction of flocculation was induced when the *E. coli* cells were harvested at the end of the exponential growth phase. When the cells were harvested in the stationary phase, the induction of the flocculation rapidly decreased. A similar behavior was also observed with all other *E. coli* strains.

Influence of pH on flocculation

To determine the influence of the pH on the flocculation inducing activity, the yeast supernatants were adjusted to pH values varying from 3.5 to 9. The corresponding buffer solutions to suspend the *E. coli* cells were also adjusted to the same values. 0.2 M of NaAc and 0.2 M of HAc were mixed to obtain the pH ranging from 3.5–4.5, while the phosphate buffer 1/15 M was used to obtain pH values from 5.0 to 9.0.

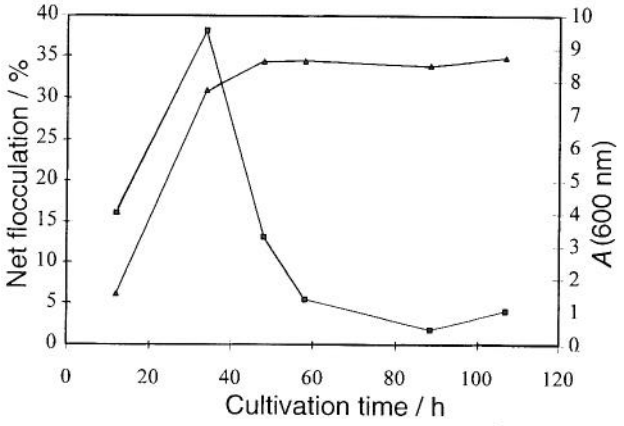

Fig. 1. Relation of *E. coli* 26 growth and flocculation in yeast supernatant. Each point represents the mean of three measurements. (Net flocculation -■-; *A* (600 nm) -▲-)

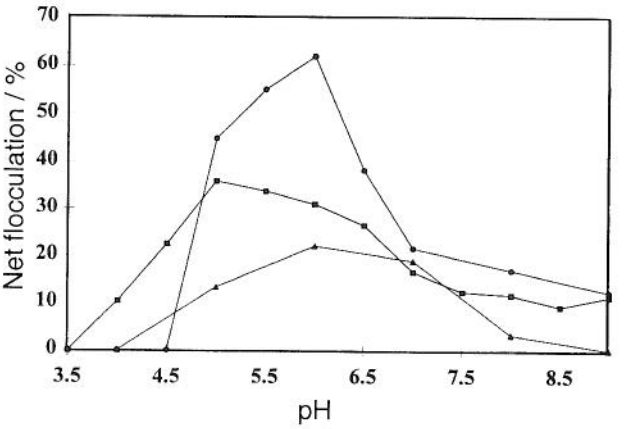

Fig. 2. Effect of pH on the flocculation of *E. coli* XL1 Blue MRF' in yeast supernatant. Each point represents the mean of three measurements.
(*E. coli* 26 -■-; *E. coli* 4350 -▲-; *E. coli* XL1-Blue -●-)

The induced flocculation of *E. coli* XL1 Blue MRF', 4350 and 26 as a function of the pH is shown in Fig. 2. Similar curves were obtained for the other *E. coli* strains. The pH optima are summarized in Fig. 3. Both figures show that pH values from 5 to 6.5 caused maximal flocculation. From pH = 3.5 to pH = 4.5, there was a high degree of flocculation in the blank. Between pH = 5 to pH = 9, flocculation in the blank was low and constant (data not shown). Therefore, the pH range from 5 to 6.5 was chosen to study the yeast-supernatant-induced flocculation.

Although all *E. coli* strains tested can flocculate in the culture supernatant of *Sacch. cerevisiae* (Fig. 3), the induction of the flocculation of the different *E. coli* strains varied largely (from 14% to 62% as tested in this study). *E. coli* XL1 Blue MRF' with a flocculation of 62% at pH = 6.0 was chosen for further study.

Influence of $CaCl_2$ on the flocculation

To determine the synergistic effects of calcium and yeast supernatant on flocculation activity, different volumes of 0.5 M calcium chloride in water were added to

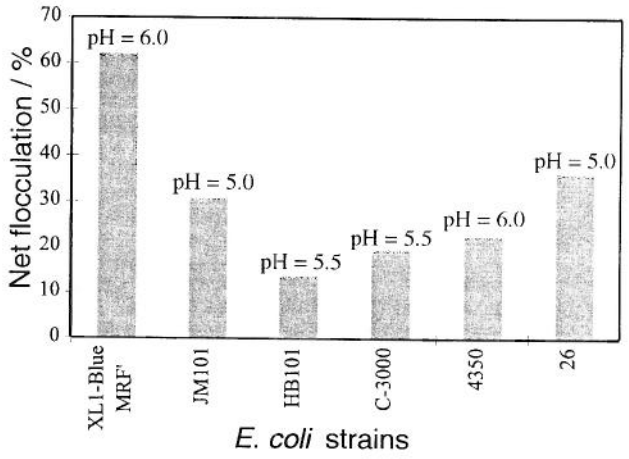

Fig. 3. Flocculation of different strains of *E. coli* cells at their optimum pH values. For *E. coli* XL1-Blue MRF' and 26, triplicate determinations from three batches of cultivated *E. coli* cells; as for the rest, triplicate determinations from one batch of cultivated *E. coli* cells.

the yeast supernatant to adjust the concentration of calcium in the mixed solution from 1 mM to 100 mM. Samples having the same $CaCl_2$ concentration but without yeast supernatant were used as blanks.

As shown in Fig. 4, $CaCl_2$ could induce flocculation in *E. coli* XL1 Blue MRF' cells even in the absence of yeast supernatant. The flocculation percentage increased with the increase of the $CaCl_2$ concentration. However, when the concentration of $CaCl_2$ reached 0.03 M, the flocculation was up to its maximum. More Ca^{2+} seemed to have no further effect on flocculation. The addition of $CaCl_2$ to the yeast supernatant only caused a small increase in flocculation.

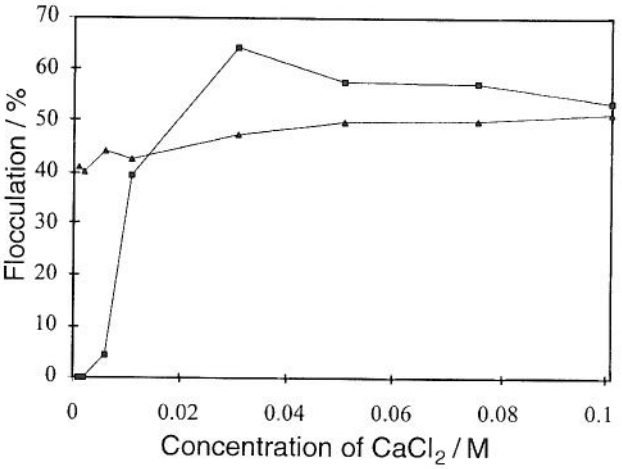

Fig. 4. Influence of $CaCl_2$ and synergistic effects of $CaCl_2$ and yeast supernatant on flocculation. Each point represents the mean of three measurements. (YS+ $CaCl_2$ -▲-; $CaCl_2$ -■-)

Influence of mannose and glucose on the flocculation

To measure the effect of mannose and glucose on the induction of flocculation, different concentrations of these sugars were added to the test bottles. Fig. 5 shows the inhibition patterns of the sugars on the flocculation

of *E. coli* XL1 Blue MRF' cells. Flocculation was found to be highly inhibited by mannose when the concentration reached 0.2 M. A small inhibitory effect was noticed with glucose at a very high concentration.

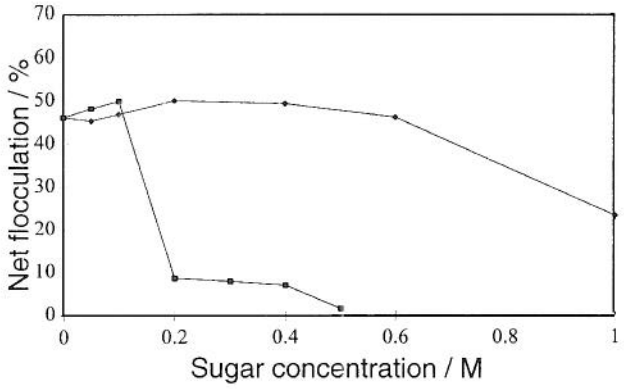

Fig. 5. Sugar inhibition of flocculation of *E. coli* XL1-Blue MRF' cells in yeast supernatant. Each point represents the mean of three measurements. (Glucose -◆-; mannose -■-)

EDTA inhibition on the flocculation

1 mM EDTA already reduced the flocculation of *E. coli* XL1 Blue MRF' cells in yeast supernatant, to about 10% of the control sample without EDTA (data not shown).

Discussion

Bacterial lectins, known as cell agglutinating and sugar binding proteins, frequently appear on the surface of the cell, or on specific organelles such as fimbriae and pili. Many bacterial strains can produce more than one type of fimbriae under different growth conditions, and therefore, may have different carbohydrate specificities *(18)*.

Earlier work on *E. coli* adhesion has been focused on cell-cell interactions. In the case of human epithelial cells and yeast cells, bacterial pili have been found to be the mediators of adhesion of *E. coli*. Mannose-specific lectin on the surface of *E. coli* was shown to be involved in the adhesion mechanism *(4–9)*.

In our laboratory, we have shown that yeast produces a secreted glycoprotein that can induce flocculation in *Pediococcus damnosus (16,17)*. In this study, we wanted to test if the same or a similar factor is able to induce flocculation in *E. coli* in order to identify the yeast glycoprotein(s) that participate in the yeast-*E. coli* interaction *(15)*. We could demonstrate that flocculation in *E. coli* cells was induced by a factor which was secreted into the medium during yeast fermentation, similar as for *P. damnosus*. This yeast-induced flocculation of the *E. coli* cells is dependent upon their cultivation conditions. Cells harvested at different cultivation stages showed a different flocculation response in the yeast supernatant. Stationary phase cells are less flocculent than cells from the logarithmic growth phase. We also found that all the strains of *E. coli* tested, regardless of whether they were fimbriated or non-fimbriated, flocculated in the yeast supernatant if harvested at the end of the exponential growth phase. Flocculation occurred over a broad pH range. Mannose and EDTA addition inhibited floc formation of *E. coli* cells. These inhibitions indicate a form of lectin-mediated flocculation *(19,20)*.

In previous reports on intrastrain flocculation of yeast *(21–25)*, calcium was shown to play a very important role as inducer of flocculation in brewing yeast strains. Mill *(24)* claimed that flocculated cells are linked by 'salt bridges', *i.e.* calcium ions joining carboxyl groups on the surface of two different cells. He proposed that both ionic and hydrogen bonds would form a stable intercellular chelate structure. Another model proposed by Miki *et al. (19)* suggested that flocculent cells had a recognition factor which attached to α-mannan sites on other cells. Here we found that both calcium and yeast supernatant could induce flocculation of *E. coli* cells, and the flocculation was found to be dependent on calcium concentration. There seemed to be no large effect on flocculation with an addition of calcium to the yeast supernatant. This observation may be explained by following hypotheses: 1. calcium ions are directly involved in bridging the carboxyl or other anionic groups on the surface of *E. coli* cells, and the binding sites are saturated at a certain calcium concentration; 2. specific receptors on *E. coli* cell surface bind the factor secreted by yeast during fermentation, these receptors may or may not be the same as binding with the calcium; 3. in the presence of both calcium and yeast factor, *E. coli* cells can either bind calcium or the factor, however, their bindings are competitive in case that they bind to the same site on *E. coli* cell surface, and saturation of binding sites on *E. coli* cells by the factor prevents the attachment of the calcium to the *E. coli* cells; 4. in case that the receptors on *E. coli* are different for calcium and factor (which has higher molecular weight), binding of the factor to the receptors on *E. coli* prevents the formation of calcium-bridges. Therefore, no obvious flocculation increase is caused by addition of calcium to the yeast supernatant.

Conclusion

We have shown in this study that yeast supernatant of *Sacch. cerevisiae* PRT can induce flocculation in *E. coli* strains. This induced flocculation is found to be pH dependent and a lectin type of binding. Future work shall be carried out on isolation of the factor in the yeast supernatant which is responsible for the flocculation.

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Utjecaj uvjeta uzgoja na kvascem induciranu flokulaciju bakterije *Escherichia coli*

Sažetak

Sacharomyces cerevisiae može se upotrijebiti za liječenje proljeva uzrokovanih bakterijom E. coli u ljudi i u životinja. Međutim, antagonistički utjecaj kvasca na E. coli još nije potpuno objašnjen. Da bi se to objasnilo, proučavana je kvascem inducirana flokulacija E. coli soja XL1 plavo bez fimbrija i soja 4350 s fimbrijima, izoliranog iz fecesa krava oboljelih od proljeva. Oba soja flokuliraju u supernatantu tekućih kultura soja Sacch. cerevisiae koji se koristio u liječenju dijareje. Ta je flokulacija vjerojatno inducirana glikoproteinom koji se oslobađa iz kvasca tijekom fermentacije. Oslobođeni glikoprotein vjerojatno povezuje stanice E. coli lektinskim tipom vezanja. Kvascem inducirana flokulacija bakterije ovisi o uvjetima uzgoja. Oba soja E. coli, izolirana u raznim stadijima uzgoja, pokazivala su različiti stupanj flokulacije u supernatantu kvasca. Stanice iz stacionarne faze manje flokuliraju nego stanice iz logaritamske faze rasta. Pokusi flokulacije koji su ovdje prikazani koriste se za iscrpnije proučavanje mehanizma interakcije Sacch. cerevisiae – E. coli.