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Enhanced production of nisin by co-culture of *Lactococcus lactis* sub sp. *lactis* and *Yarrowia lipolytica* in molasses based medium

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ABSTRACT

Nisin is a safe, approved and commercial bacteriocin that is produced by *Lactococcus lactis* subsp. *lactis*. Since lactate accumulation in fermentation medium reduces *L. lactis* growth and nisin production, *Yarrowia lipolytica*, a lactate consuming yeast and *L. lactis* subsp. *lactis*, were simultaneously cultured in a molasses based medium. *Y. lipolytica* is not able to consume sucrose as carbon source, but rather consumes lactate and hence decrease lactic acid titer by 10% in the medium. Lactic acid consumption, 15% increased pH value and stimulated *L. lactis* growth. In the mixed culture, nisin production and *L. lactis* growth were 50% and 49% higher than that of pure culture, respectively. Also the results showed that specific growth rate of *L. lactis* increased 4 times more than that of the pure culture.

1. Introduction

Co-culture is defined as anaerobic or aerobic incubation of different specified microbial strains under aseptic conditions (Bader et al., 2015). These microorganisms metabolize the substrate(s) of the media together. Usually two microorganisms have been cultured together, but there are some examples of using more than two microbes in this system, too (Bader et al., 2010).

Co-culture systems have been used in various strategies, including: I) To overcome the limiting step(s) of a desired biosynthetic pathway: In this system, one microorganism provides the upstream section of the biosynthesis pathway and another microorganism completes the downstream section of the biosynthesis process (Zhang and Stephanopoulos, 2016). II) Prevention of biosynthetic enzymes from by-products biosynthesis: In this strategy, the second microorganism consumes undesired intermediate(s) that can be used for biosynthesis of undesired products (Chen et al., 2017). III) Increasing the recalcitrant substrate(s) availability for the main microorganism by extracellular enzymes of the auxiliary microorganism (Tesfaw and Assefa, 2014). IV) Inducing or increasing antibiotic production through co-culturing of producer strain and challenge organisms (Sung, 2016). V) Auxiliary microorganism consumes inhibiting metabolites produced by the main microorganism and improving the fermentation condition (Liu et al., 2006; Shimizu et al., 1999). This strategy is used in this research for nisin production.

Nisin is one of the most important members of bacteriocins and classified in bacteriocin group I or lantibiotics. This peptide consists of 34 amino acids including modified residues such as lanthionine and betamethyl lanthionine (Field et al., 2012). Its molecular weight is 3354 Da and is produced by *Lactococcus lactis* sub sp. *lactis* at industrial scale. Nisin has antibacterial effects against Gram positive bacteria and their spores, and is also used as a food preservative (Kim, 1997) which is approved in more than 50 countries as a food additive (Lv et al., 2004). It adjoins to lipid II in cytoplasmic membrane and resulting in cell wall synthesis inhibition as well as constructing pores in the membrane which in turn causes the loss of ions and disrupting of pH balances and death of microorganisms (González-Toledo et al., 2010).

There are various factors affecting on nisin production such as media ingredients, culture conditions and the metabolites produced by *L. lactis* during the fermentation (Liu et al., 2010). Nisin production in *L. lactis* is negatively affected by lactic acid in the fermentation process. By increasing lactic acid accumulation in the media, bacterial growth and consequently nisin production will be reduced (Wardani et al., 2007). There are different ways for decreasing the lactic acid titer in the medium such as extraction by solvents or alkaline neutralization. However, solvent extraction technique makes the final product to be far from food standards (Shimizu et al., 1999). Another approach for lactic acid removal from the fermentation broth is co-culture fermentation of microorganisms.

There are a few reports on using co-culture fermentation of *L. lactis* with *Saccharomyces* in whey (Liu et al., 2006) and *Kluyveromyces* in maltose-based media (Shimizu et al., 1999). These auxiliary

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microorganisms cannot use lactose and maltose, the main carbon source for nisin production by *L. lactis*, respectively. However, there is no report on using co-culture in sucrose based medium. Sucrose is one of the well-preferred carbon sources for nisin production by *L. lactis* (Parente and Ricciardi, 1999), auxiliary microorganisms should not use sucrose as a carbon source, and, they should be able to consume lactic acid. In this research, we tried to examine the applicability of *Yarrowia lipolytica* as an auxiliary microorganism for *L. lactis*, for the first time. *Y. lipolytica* is isolated naturally from cheese and dairy products (Nicaud, 2012) and therefore it seems a good candidate to be used for simultaneous culture with *L. lactis*. It shows no ability to use sucrose because of the lacking of sucrose cleaving enzymes. However, it is able to use fatty acids, alkanes and organic acids as carbon sources (Coelho et al., 2010). Ammonia production by amino acid degradation and lactic acid consumption by this yeast, caused to neutralization of pH (Mansour et al., 2008), that is useful for nisin production (Pongtharangkul and Demirci, 2006). Moreover, *Y. lipolytica* is able to grow in acidic pH (Coelho et al., 2010). Our hypothesis is that *L. lactis* and *Y. lipolytica* can grow together and lactic acid consumed by *Y. lipolytica* increases pH value in the fermentation medium resulting in enhancing nisin production by *L. lactis*.

2. Materials and methods

2.1. Microorganisms and media

Lactoccocus lactis sub sp. *lactis* (UTMC 106) and *Micrococcus luteus* (UTMC 1428) were used as nisin producing and nisin sensitive strains, respectively. These bacteria were obtained from University of Tehran Microorganisms Collection. *Y. lipolytica* (ATCC 18942) was used for co-culture fermentation with *L. lactis*.

The composition of different media was as follows: Seeding medium I, contained (g/l) sucrose 10, yeast extract 10, peptone 10, K_2HPO_4 10, NaCl 2, $MgSO_4$ 0.2, was used as seeding medium for *L. lactis* (Lv et al., 2004). Seeding medium II, contained (g/l) yeast extract 10, peptone 20, glycerol 30, was used for seed culture of *Y. lipolytica* (Bakkaiova et al., 2014). Molasses based fermentation medium, contained (g/l), sugar beet molasses 50–90, soybean meal 10–30, K_2HPO_4 10, NaCl 2, $MgSO_4$ 0.2, was used for co-culture fermentation of *L. lactis* and *Y. lipolytica*. Whey based fermentation medium, contained (g/l), whey 10–50, soybean meal 20, K_2HPO_4 10, NaCl 2, $MgSO_4$ 0.2, was used for comparison to molasses based medium as a control medium for *L. lactis* growth. BHI (Brain Heart Infusion) agar contained, (g/l) beef heart 5, calf brain 12.5, disodium hydrogen phosphate 2.5, D-glucose 2, peptone 10 with 1% tween 20 and 0.75 agar for using agar diffusion method. Plate count agar I, contained (g/l), maltose 5, yeast extract 5, peptone 5, cycloheximide 0.005, agar 15, that was used for measuring of CFU (Colony Forming Unit) numbers of *L. lactis* (Shimizu et al., 1999). Plate count agar II, contained (g/l), glucose 5, yeast extract 5, peptone 5, streptomycin 0.005, agar 15, used for CFU determination of *Y. lipolytica* (Shimizu et al., 1999).

2.2. Cultivation method

A loop full of 24 h culture of *L. lactis* and *Y. lipolytica* were inoculated into 100-mL Erlenmeyer flasks containing 20 ml I and II seeding media, respectively. The *L. lactis* containing flasks were incubated at 100 rpm, 30 °C for 12 h and the flasks containing *Y. lipolytica* were incubated at 160 rpm and 30 °C for 24 h. Equal volumes of seeding materials (8% v/v) were mixed and inoculated into 1000 ml Erlenmeyer flasks containing 200 ml fermentation medium. The fermentation flasks were incubated at 100 rpm, 30 °C for 24 h.

2.3. Optimization of molasses based medium culture

Three factors, including sugar beet molasses, soybean meal and the ratio of the volume of Erlenmeyer flask per volume of medium culture were studied to optimize the growth of *L. lactis* using response surface methodology (Box-Behnken design). It provides the experimental plan by the chosen condition, statistically designed experiments, estimation of coefficients in the mathematical model, accuracy of models and prediction of the optimum condition and the results are shown in plots. Each plots shows the effects of two variables within the considered range, while other variables fixed to the zero level. Each counter plot demonstrated the interactions between factors and when the shape of contours are elliptical indicating that the interaction effect between two factors is significant and when the shapes of contours are circular indicating that the interaction of two factors are non-significant (Guo et al., 2010).

2.4. Optimization of whey based medium

Whey has been used for nisin production by *L. lactis* and it was used as control medium in this research. By consideration of the source-dependent chemical composition of whey, as an agrochemical product, effect of whey concentrations (10–50 g/l) as carbon source on nisin production was studied using one factor at a time method. Other medium ingredients are the same for molasses based medium, without molasses that is replaced by whey.

3. Process analysis

3.1. Biomass measurement

Viable plate count was used to measure the biomass of *L. lactis* and *Y. lipolytica*. For growth measurement of *Y. lipolytica* and *L. lactis* in mixed culture, 5 mg/l streptomycin and cycloheximide were added to the isolation media, respectively (Shimizu et al., 1999). These antibiotics inhibited the growth of *L. lactis* and *Y. lipolytica*, respectively; and provided the appropriate conditions for measurement of the desired microorganism. Also, the specific growth rates of *L. lactis* as pure culture and mixed culture with *Y. lipolytica* in the molasses based medium were calculated by below formula.

$$\mu = \frac{lnN2 - lnN1}{t2 - t1}$$

N_2 and N_1 are the number of cells at the time of t_2 and t_1, respectively (Miao et al., 2012).

3.2. Lactic acid assay

Concentrations of lactic acid in fermentation broth were measured by titration with NaOH 0.1 M and phenolphthalein as the indicator after removing the biomass by centrifugation (Wasewar et al., 2002). The samples were provided each 3 h for 24 h in single and co-culture fermentation to calculate the amount of lactic acid.

3.3. Nisin bioassay

Antibacterial activity of the nisin was assayed by agar diffusion method (Pongtharangkul and Demirci, 2004) using BHI medium plus 0.75% agar and 1% Tween 20. *M. luteus* UTMC 1428 was cultured by pour plate method. The Petri dishes were filled with 25 ml of the inoculated bioassay medium. Then, 50 µl of each fermentation broth was poured into each well punched in the bioassay plates. The plates were incubated at 4 °C for 24 h, then incubated at 37 °C for 24 h and the inhibition zones were measured. Nisin (Handary Co. Belgium) at the concentration of 100–500 IU/mL was used as a standard.

3.4. Sucrose analysis

Total sugar in beet molasses was measured by phenol-sulfuric acid