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Bacteriocin production by *Enterococcus faecalis* VRE 1492 using different media at varying pH and temperatures

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ABSTRACT

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Bacteriocins are natural proteinaceous bactericidal substances produced by certain strains of bacteria and act against some other strains of the same or closely related species. This study was conducted to increase the level of bacteriocin production by *Enterococcus faecalis* VRE 1492 using selected growth media. Results showed that bacteriocin production by *Enterococcus faecalis* VRE 1492 was increased at different growth media, initial pH and fermentation time. Maximum production of bacteriocin was observed using De Man Rugosa and Sharpe (MRS) medium with glucose. The activity of bacteriocin was greatly increased in MRS medium with glucose at initial pH of 7.50 and 8.50 after 8 to 20 hours of fermentation at 30°C. Progressive increase in cell count from 0 to 24 hours of fermentation did not necessarily favor an increase in bacteriocin activity. After 20 hours of fermentation, the activity of bacteriocin decreased. The production of proteolytic enzymes by the bacterium was believed to inactivate the bacteriocin.

Keywords: bacteriocin. biopreservation. *Enterococcus faecalis* VRE 1492.

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INTRODUCTION

Bacteriocin purification and its subsequent use in foods has been recently reported as one of the modern and effective ways of food preservation. Its use on foods is a form of biopreservation technique conceived to replace the chemical preservatives that are generally hazardous to human health. Bacteriocins are natural proteinaceous bactericidal substances produced by certain strains of bacteria and act against some other strains of the same or closely related species (Nomura, 1967). They are known to inhibit the growth of spoilage and pathogenic microorganisms in foods but are not lethal to producer cells since these cells have specific immunity genes which are located near the producer genes (Lewus *et al.*, 1991; Okereke and Montville, 1991; Vaughan *et al.* 1992; Ray, 1996 and Franz, *et al.*, 1997).

The production of bacteriocin by microorganisms is dependent primarily on temperatures, pH and media composition. With increasing temperatures ranging from 4 to 30°C, the amount of bavaricin MN production increased (Lewus and Montville, 1992). In culture supernatant fluids, the bacteriocin levels rarely exceeded 5000 AU/ml and usually below 1000 AU/ml in the absence of pH-controlled fermentations (Joerger and Klaenhammer, 1986 and Vaughan *et al.*, 1992). In APT broth with 3.0 g per liter of beef extract under controlled pH of 6.0, growth rate and bacteriocin production of *Lactobacillus bavaricus* MN increased (Kaiser and Montville, 1993) An increase in the level of bacteriocin production by this microorganism was likewise reported with increasing fermentation temperatures from 4 to 30°C (Lewus and Montville, 1992).

A bacteriocin-producing microorganism identified as *Enterococcus faecalis* VRE 1492, was isolated from *agos-os*, a traditional fermented meat and sweetpotato mixture from the Philippines (Tan, 1998). This microorganism was reported to possess a wide range of activity against gram-positive and gram-negative bacteria. In order to purify this active compound, it is necessary that sufficient amount must be produced. This study therefore aimed to increase the level of bacteriocin production by *Enterococcus faecalis* VRE 1492 using selected growth media which are generally used in the cultivation and propagation of lactic acid bacteria. The effect of pH and fermentation time on the amount of bacteriocin production was also determined.

MATERIALS AND METHODS

The production of bacteriocin was optimized using some selected growth media which are generally used in the cultivation and propagation of lactic acid bacteria at different initial pH and fermentation time.

Growth media

Five different types of media with varying compositions were used in growing the bacteriocin-producing isolate. These media were as follows: GPY (Glucose Yeast Peptone), TSYE (Trypticase Soy Broth with 0.5% Yeast Extract) with and without glucose and MRS (De Man Rugosa and Sharpe) with and without glucose. Unadjusted and adjusted pH (7.5) were used in each medium. Ten ml each of the prepared media was dispensed into the test tube and sterilized at 15 psi for 15 minutes. One (1.0) percent each by volume of 18-hour fresh culture of the isolate was inoculated into each of the sterilized broth media in duplicate and incubated at 30°C for 18 hours.

After incubation, the fermented broth culture was centrifuged at 3,500 x g for 30 minutes. The cell-free supernatant was neutralized to pH 7.0 and filtered using cellulose acetate, (0.45 um pore). MRS agar in petridish, seeded with the indicator strain, *L. sake* 15521, was inoculated with sterilized 8 mm paper disc loaded with 80 ul of the neutralized supernatant and were incubated at 30°C for 24 hours. The diameters of the zones of inhibition around the paper disc were measured.

Initial pH

MRS with glucose, the medium that produced the highest amount of bacteriocin based on its widest zone of inhibition on the indicator lawn, was used in the determination of optimum pH for bacteriocin production. The broth medium was adjusted to pH 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 with sterilized 1 N HCl (for pH 3.5 to 5.5) and 1 N NaOH (for pH 6.5 to 9.5). Unadjusted pH (6.39) served as control. Each of the broth media which was adjusted to different pH as well as the unadjusted one was inoculated with 1.0%, v/v, of the freshly grown bacteriocin-

producing isolate. Incubation was done at 30°C for 18 hours. After incubation, the pH of the fermented culture broth was determined. The cell growth was monitored by measuring the absorbance at 660nm of the fermented broth culture.

Fermentation time

The pH of the medium was adjusted to 7.5 and was inoculated with bacteriocin-producing microorganism as above. Incubation was done at 30°C at different time intervals (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours). Sampling was done at the above fermentation time interval. The pH, cell growth and activity of bacteriocin were determined as above.

Bacteriocin assay

The activity of bacteriocin was measured by getting the arbitrary unit (AU). One Arbitrary Unit (AU) was defined as the reciprocal of the highest dilution yielding a definite and discernible zone of inhibition on the indicator lawn. The specific activity was determined by dividing the Arbitrary Unit (AU/ml). with the protein content (mg/ml). The protein content was determined using the Lowry method (Lowry *et al.*, 1951)

RESULTS AND DISCUSSION

Growth medium

The bacteriocin produced by the bacterium was affected by the kind of growth media and their initial pH. In general, wider zones of inhibition were observed in any growth media with an initial Ph of 7.5 compared with unadjusted pH (Table 1). The highest bacteriocin activity as reflected on its widest zone of inhibition was observed in MRS (with glucose) with an initial pH of 7.5. Results showed that the addition of glucose in MRS broth favored high activity of bacteriocin. However, in TSYE broth, the zone of inhibition was significantly the same in the presence or absence of glucose. Different types of bacteriocin producing bacteria seemed to respond differently under a given growth media.

Table 1. Effect of growth media on the production of bacteriocin.

Growth medium	Initial pH	Final pH	Zone of inhibition (mm[1])	Average zone of inhibition (mm[2])
GPY				6.05c
Unadjusted	6.80	4.32	5.50f	
Adjusted	7.50	4.34	6.60d-f	
TSYE (with glucose)				7.20b
Unadjusted	7.14	5.29	6.20e-f	
Adjusted	7.50	5.53	8.20b-c	
TSYE (without glucose)			7.60b	
Unadjusted	7.13	6.60	7.30b-e	
Adjusted	7.50	6.83	7.90b-d	
MRS (with glucose)				9.48a
Unadjusted	6.80	4.78	8.75a-b	
Adjusted	7.50	4.72	10.20a	
MRS (without glucose)				7.90b
Unadjusted	6.60	5.00	7.00c-f	
	Adjusted	7.50	4.95	8.80a-b

GPY – Glucose peptone yeast.
TSYE – Trypticase Soy with Yeast Extract.
MRS – De Man, Rugosa and Sharpe.
[1]Values are differences between the microbial growth zone and the clear zone around the microbial colony on the indicator lawn; LSD value = 1.553.
[2]Values are averages of the differences between the microbial growth zone and the clear zone around the microbial colony on the indicator lawn; LSD value = 1.098.

Large quantities of pediocin AcH was produced by Pediococcus acidilactici H in Trypticase broth with the addition of glucose, yeast extract, Tween 80, Mn^{2+} and Mg^{2+} (Biswas *et al.*, 1991). The zones of inhibition that were formed from GPY medium were found to be narrow suggesting that it was a poor medium for bacteriocin production by the isolate.

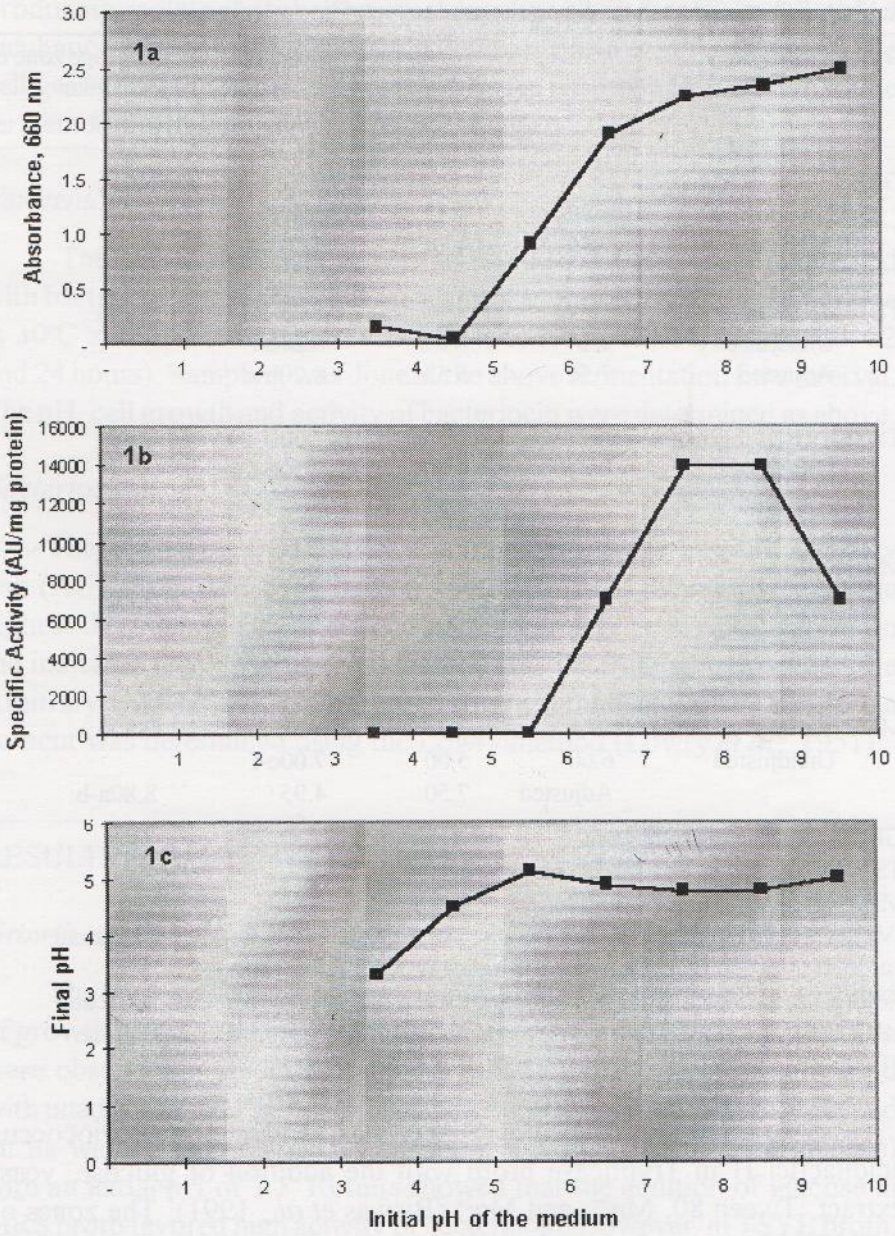

Figure 1. Growth and activity of bacteriocin-producing isolate at different initial pH of the medium after 18 hours of fermentation

pH

Highest bacteriocin activity was observed at 7.50 and 8.50 (Figure 1). Maximum cell growth appeared to be at about pH 9.50 as reflected by its high absorbance. However, the high level of cell growth at pH 9.50 did not seem to render maximum yield of bacteriocin. Similarly, Huot *et al.* (1996) reported parallel results regarding pH optima for nisin production. It was found that pH 6.0 was less favorable for nisin production but favorable for increased cell biomass. Results showed that there was a decrease in the activity of bacteriocin above pH 8.50. Ray (1996) reported that in general, bacteriocins can be destroyed at pH 9.0 or above.

At high pH, the inactivation of nisin was not attributed to simple denaturation since the products were composed largely of dimers and other multimers (Huot *et al.*, 1996). Nisin was found to be insoluble and unstable at neutral or alkaline pH (Lewus and Montville, 1992). Because of this, its activity decreased as pH increased.

Fermentation time

The production of bacteriocin by the bacterium was observed from 6 to 24 hours of fermentation (Figure 2). No bacteriocin activity was detected during the second and fourth hour of fermentation. The abrupt increase in bacteriocin activity from 6 to 8 hours corresponded to an abrupt increase in cell count as reflected by the abrupt increase in absorbance. The specific activity of bacteriocin remained at its maximum level from 8 to 20 hours of fermentation after which, it decreased after 20 hours of incubation.

It was clearly shown that the progressive increase in cells did not necessarily favor constant increase in the activity of bacteriocin. In nisin production, further increase in cell growth caused a decline in its activity due to proteolytic degradation (De Vuyst and Vandamme, 1992). Because of this, the activity of bacterioc in batch culture may decrease with extended incubation (Liao *et al.*, 1993). It is not advisable therefore to prolong the incubation time since the proteolytic enzymes that will be produced by the microorganisms may degrade the bacteriocin.

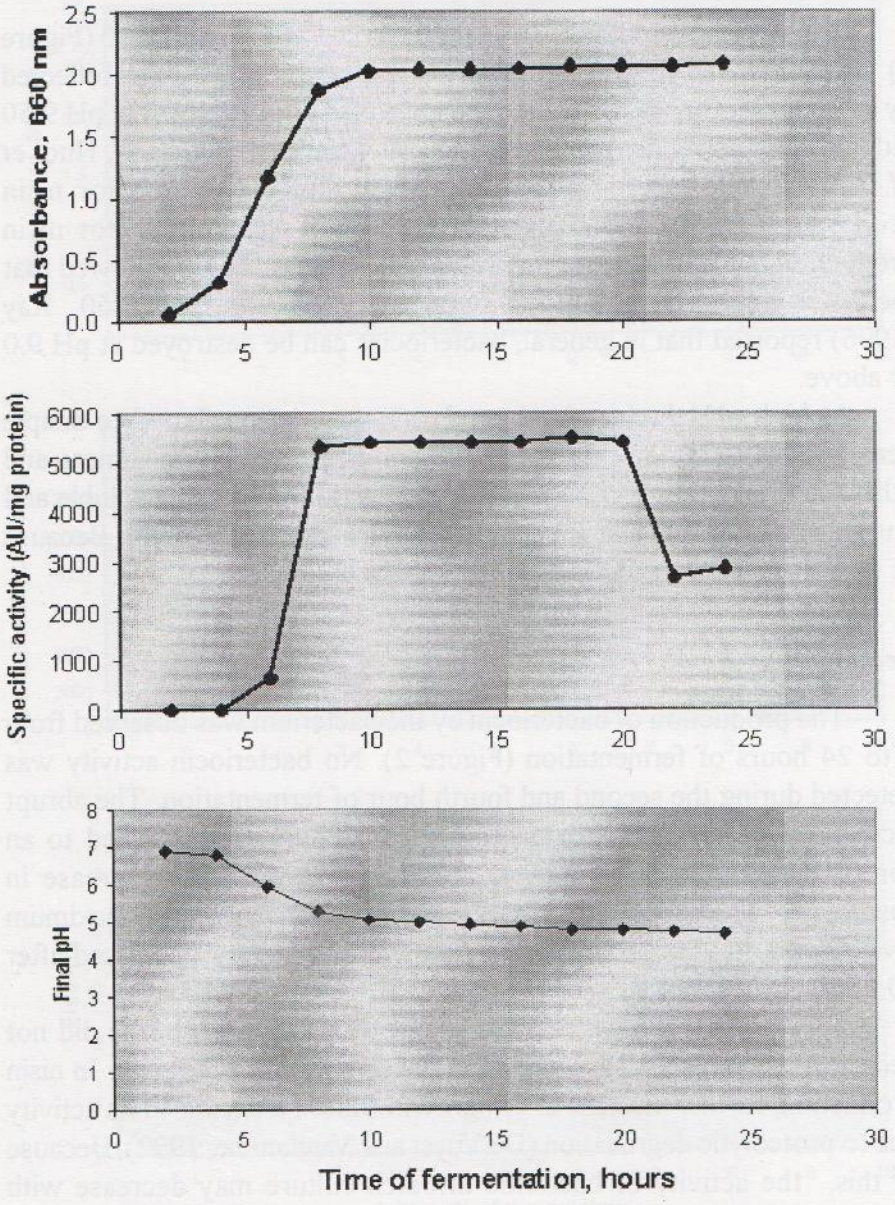

Figure 2. Effect of fermentation time on the growth and activity of bacteriocin-producing microorganism

The detection of bacteriocin activity was accompanied by the decrease in pH of the broth medium. Low pH at the end of fermentation may be needed for post-translational modification or the release of the active bacteriocin from the producing cells (Yang *et al.*, 1992 and Ray, 1996).

CONCLUSION AND RECOMMENDATION

Bacteriocin was produced by *Enterococcus faecalis* VRE 1492. A remarkable increase in the activity of bacteriocin was observed using MRS medium. At pH 7.5 and 8.5 in MRS medium with glucose, bacteriocin activity was likewise increased. Progressive increase in cells beyond 20 hours of fermentation resulted in the decline of bacteriocin activity. It is clearly shown that any further cell growth did not necessarily produce maximum bacteriocin activity in batch fermentation. It is believed that the bacteriocin-producing microorganism is capable of producing proteolytic enzymes which eventually inactivate the bacteriocin produced.

Different types of bacteriocin vary in their activity in varying media, pH and fermentation time. Previous studies have also shown that the activity of bacteriocin be tested at different temperatures at specified optimum pH level and time of fermentation.

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