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Utilization of Agro-Industrial Byproducts for Bacteriocin Production Using Newly Isolated *Enterococcus faecium* BS13

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Abstract**—**Microbial production of antimicrobials as biopreservatives is the major area of focus nowadays due to increased interest of consumers towards natural and safe preservation of ready to eat food products. The agro-industrial byproduct based medium and optimized process conditions can contribute in economical production of bacteriocins. Keeping this in view, the present investigation was carried out on agro-industrial byproducts utilization for the production of bacteriocin using *Enterococcus faecium* BS13 isolated from local fermented food. Different agro-industrial byproduct based carbon sources (whey, potato starch liquor, kinnow peel, deoiledrice bran and molasses), nitrogen sources (soya okra, pea pod and corn steep liquor), metal ions and surfactants were tested for optimal bacteriocin production. The effect of various process parameters such as pH, temperature, inoculum level, agitation and time were also tested on bacteriocin production. The optimized medium containing whey, supplemented with 4%corn steep liquor and polysorbate-80 displayed maximum bacteriocin activity with 2% inoculum, at pH 6.5, temperature 40^{o}C under shaking conditions (100 rpm).

Keywords**—**Bacteriocin, biopreservation, corn steep liquor, *Enterococcus faecium*, waste utilization, whey.

I. Introduction

DUE to the advent of awareness among consumers and harmful side effects of chemical preservatives, there has been increase in demand of biopreservatives such as bacteriocins [1]. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria with wide range of inhibition spectra against food pathogenic and spoilage microorganisms, thereby, enhancing the shelf life of food products. They are mostly heat stable, non-toxic, non-antigenic and can be degraded by proteolytic enzymes in the gastro-intestinal tract [2]. Because of their characteristics, these have become the prime focus area for researchers, industrialists and academicians in the field of biopreservation. Although bacteriocins can be produced by potential microbial sources (bacteriocin producing microorganisms) by fermentation but cost is the major factor associated affecting its use [3].

Methods like strain improvement/modification, optimization of various process parameters and cost effective downstream processing can be employed for over production of bacteriocin [4]. Another factor associated with cost is the fermentation media used for the production of bacteriocin. Almost 30% of the total production cost relies on synthetic media and nutritional supplements used for growth of micro-organisms [3]. Use of low cost media or agro-industrial byproducts as raw substrates (with least or no value) can make the fermentation process economically viable along with reduction in environmental pollution [5]. Agro-industrial byproducts contains appropriate and sufficient amount of balance nutrients required for the growth and product formation, thus, can serve as fermentation medium. Therefore keeping in view the increasing demand of consumers for cost effective natural food additives, studies on the utilization of agro-industrial byproducts has been carried out for the production of bacteriocin using *Enterococcus faecium* BS 13 isolate.

II. Material And Methods

A. Growth and Bacteriocin Production by Strain BS13

Enterococcus faecium BS13 was isolated in the Biotechnology Research Laboratory, SantLongowal Institute of Engineering and Technology, Longowal (Punjab, India) and was maintained on MRS medium [6], [7]. The inoculum was prepared by incubating the flasks containing MRS in a rotary shaker at 30^{o}C overnight at 100rpm. Bacteriocin production was carried out in 250 ml Erlenmeyer flasks each containing 100ml medium with 1% (vv^{-1}) inoculum at 37^{o}C and 100 rpm in different sets. After specific intervals of time, samples were taken out to determine bacteriocin activity and pH. For estimation of bacteriocin activity, the fermentation broth was centrifuged at 10,000 rpm for 10min at 4^{o}C. Supernatant was filtered through pre-sterilized 0.22µm filters (HiMedia, Mumbai).

B. Bacteriocin Assay

The supernatant obtained was used as crude bacteriocin. To eliminate the possibility of inhibition by hydrogen peroxide and lactic acid, dilutions were prepared from neutralized supernatant and 75µl of the supernatant was added into the wells to perform the well diffusion assay (in triplicate and

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mean values have been given) [8]. Antimicrobial activity against indicator strain *Lactobacillus brevis* MTCC 1750 was performed. Plates were incubated at 30°C for overnight and zone of inhibition were examined. Bacteriocin activity was calculated as reciprocal of dilutions indicating inhibition of indicator lawn and expressed in activity units per mL (AU mL^{-1}) i.e. AU mL^{-1} = Higher dilution producing a distinct zone of inhibition x 1000 uL vol $well^{-1}$

C. Optimization of Media Components and Process Parameters

1. Optimization of Carbon Source

Different agro-industrial byproducts like whey, deoiledrice bran (5% and 10 %, wv^{-1}) and molasses (5% and 10%, wv^{-1}) were utilized as carbon source for studying the effect on bacteriocin production.

2. Optimization of Nitrogen Source

Corn steep liquor (vv^{-1}) and soya okra (wv^{-1}) was further taken as nitrogen source at a concentration of 5% were used and pH of the medium was adjusted to 6.5. The nitrogen source showing better activity was also optimized for its concentration (2-8%).

3. Optimization of Metal Ion and Surfactants Concentration

To determine the effect of metal ions on the production of bacteriocin in the medium (whey and 4% (vv^{-1}) corn steep liquor), different metal ions (NaCl, $MnSO_4$, $MgSO_4$, $(NH_4)_2SO_4$, KCl and $CaCl_2$) at 0.5% (wv^{-1}) concentrations were added in the previously optimized medium. Various surfactants (polysorbate-20, polysorbate-60and polysorbate-80) were also studied for their effect on bacteriocin production.

4. Optimization of Process Parameters

Different process parameters were optimized by varying their respective values such as pH (5.5-7.0), temperature (25-45°C), inoculum volume (1-5%), agitation (50-150 rpm and stationary condition) for maximum bacteriocin production. Bacteriocin activity was measured at regular intervals as described previously.

III. Results And Discussion

A. Optimization of Medium Components

Production of bacteriocin is affected by different physical and chemical parameters including carbon and nitrogen sources, metal ions, temperature, pH, aeration etc. [1], [9]-[11]. To economize the process of microbial production/conversions, a number of low cost agro industrial by products or wastes have been used. Molasses, a byproduct of sugar industry or molasses based media has been reportedly used for various microbial processes including ethanol production and citric acid production [12]-[14].In the present study, bacteriocin production by *Enterococus faecium* B13 has been carried out using different byproducts including whey, molasses, potato starch liquor, kinnow peel and deoiledrice bran as carbon rich source (Fig. 1). Maximum production of 426AU mL^{-1} of bacteriocin was obtained in 18 h of incubation in whey. With molasses (5-10%), very less amount of bacteriocin production was observed which may be due to lack of specific nutrients required for bacteriocin production. However, using kinnow peel and potato starch liquor bacteriocin production of 213 AU mL^{-1} and 53 AU mL^{-1} respectively, observed. Deoiled rice bran, a rich source of nutrients having approximately 27% carbohydrates [15], resulted in very less amount of bacteriocin production. Unavailability of simple sugars for utilization by *E. faecium* BS 13 can be the possible reason for decreased bacteriocin production. In a study involving *Leuconostoc mesenteroides* in submerged shake flask culture using molasses, increase in bacteriocin production was observed with increase in molasses concentration from 2 to 3% [16]. Upto 200 AU mL^{-1} of bacteriocin was produced by *Lactobacillus plantarum* ST13BR, when grown on waste sugarcane molasses having total sugars of approximately 59% (ww^{-1})[13]. However, *Bacillus licheniformis* P40 produced maximum bacteriocin (3200 AU mL^{-1}) in cheese whey (70 gL^{-1}) at pH range 6.5-7.5 and temperature range 26-37°C in 15h of incubation as compared to other waste substrates[9].

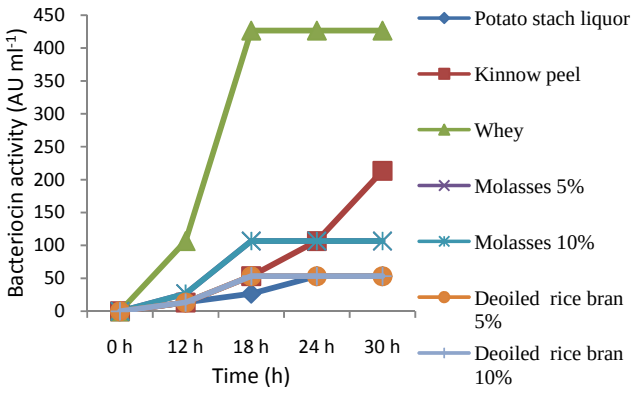

Fig. 1 Effect of different agro-industrial by-products as carbon source on bacteriocin production

The supplementation of whey with nitrogen sources like corn steep liquor, soy okra and pea pod brought drastic changes in pattern of bacteriocin production in present study. Effect of addition of industrial byproducts i.e. corn steep liquor or soy okra (5%) was observed, which led to increased bacteriocin production by 436907AU mL^{-1} and 1707 AU mL^{-1} fold respectively, as compared to whey alone (Table I). *E. facieum* BS13 was also grown in pea pod and soy okra supplemented media. As compared to soy okra supplemented medium, growth and production of bacteriocin was higher in pea pod supplemented medium as shown in Table I. An exponential increase in the bacteriocin production was observed after 18h of incubation which reached maximum after 24-30h of incubation. Corn steep liquor supplemented in MRS medium gave maximum growth of *Lactobacillus plantarum* 423 but with lower plantaricin 423 production as compared to other nitrogen sources [17]. Similarly, production of bacteriocin bacST4Sa (128000 AU mL^{-1}) was also reported

on corn steep liquor medium [18]. Further, corn steep liquor concentration was optimized (Table II) for production of bacteriocin. Maximum bacteriocin production was observed after 18h of incubation with 4% (vv^{-1}) corn steep liquor. Higher concentration of corn steep liquor did not show any positive effect on production of bacteriocin.

TABLE I
EFFECT OF DIFFERENT AGRO-INDUSTRIAL BYPRODUCTS AS NITROGEN SOURCES ON BACTERIOCIN PRODUCTION

Time	Bacteriocin Activity (AU ml^{-1})		
	Corn steep liquor	Soy okra	Pea pod powder
12 h	27306.667	426.67	426.67
18 h	436906.67	1706.67	27306.67
24 h	436906.67	1706.67	109226.67
30 h	436906.67	1706.67	109226.67

TABLE II
EFFECT OF DIFFERENT CONCENTRATION OF CORN STEEP LIQUOR ON BACTERIOCIN PRODUCTION

Time	Bacteriocin Activity (AU ml^{-1})			
	2%	4%	6%	8%
12 h	3413.34	27306.67	27306.67	27306.67
18 h	54613.34	436906.67	436906.67	436906.67
24 h	54613.34	436906.67	436906.67	436906.67
30 h	54613.34	436906.67	436906.67	436906.67

Metal ions are important components for any biological production process. Due to specific ionic and water binding capacity, these metal ions affect the bacterial metabolic activity [19]. In present study, inhibitory effect ofMg^{+2}, Mn^{+2}, NH_4^+ on the production of bacteriocin was observed. Further, addition of NaCl at 0.5% (wv^{-1}) led to decreased production of bacteriocin while K^+/Ca^{2+} has no effect on bacteriocin production in a mixture of whey and corn steep liquor (Table III). This may be due to presence of metal ions and salts in whey and corn steep liquor. However, low concentration of 0.014% $MnSO_4.H_2O$ found to stimulate growth and plantaricin production in *L. plantarum* 423 while $MgSO_4.7H_2O$ was not able to increase bacteriocin production [17], similar to results observed in present study. Eight fold increase in micrococcin GO5 was observed when concentration of K_2PO_4 was increased to 2-5% from 0.2% in growth medium [20]. In the same study, addition of $MgSO_4.7H_20$ enhanced the bacteriocin production upto 5-10%. Correlation of decrease bacteriocin with increased NaCl concentration is discussed by [19] while carrying out study on bacteriocin production by *Lactobacillus amylovorous* DCE 471.

TABLE III
EFFECT OF DIFFERENT METAL IONS ON BACTERIOCIN PRODUCTION

Time	Bacteriocin Activity (AU ml^{-1})					
	NaCl	$MnSO_4$	$MgSO_4$	$(NH_4)_2SO_4$	KCl	$CaCl_2$
12 h	13653.3	106.6	426.6	853.3	27306.6	27306.6
18 h	27306.6	426.6	1706.6	3413.3	436906.6	436906.7
24 h	27306.6	426.6	1706.6	3413.3	436906.6	436906.7
30 h	27306.6	426.6	1706.6	3413.3	436906.6	436906.7

Effect of different detergents on bacteriocin production was also observed. Addition of detergent polysorbate-80 in the whey based medium increased the bacteriocin production by approximately 4 times as compared to twice by polysorbate-60 (Fig. 2). Addition of polysorbate-20 does not affect the bacteriocin production in the present study. However, increased bacteriocin production on addition of detergent might be due to the stimulated protein secretion by changing the fluidity of membrane as observed by [21], whereas no curvaticin FS47 production was observed with polysorbate-80. Franz et al. [22] observed 50% increased bacteriocin production in a medium containing polysorbate-80. Similarly, polysorbate-80 (0.05-2%) increased bacteriocin production in a separate study [17].

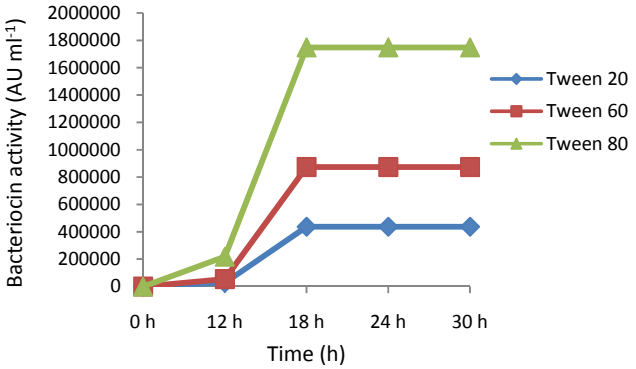

Fig. 2 Effect of various surfactants on bacteriocin production

B. Optimization of Process Parameters

Different process variables such pH, temperature, etc. also effect bacteriocin production. The effect of initial pH on the production of bacteriocin by *E. faecium* BS 13 was observed during the optimization process (Fig. 3). Maximum bacteriocin production (1.74×10^6 AU mL^{-1}) was obtained in the pH range of 6-6.5; however, further increase or decrease in the pH resulted in decreased bacteriocin production. Wide variation in optimized value of pH was observed by different groups working on bacteriocin studies from variety of microorganisms [23]-[25]. The change in bacteriocin production with pH might be due to change in growth behavior of microorganism, absorption/desorption from cell membrane, post-translational modification, secretion from the bacterial cells or modification in regulation of gene responsible for bacteriocin production [26], [27]. The maximum bacteriocin activity of 6400AU mL^{-1} was observed at pH 6-9 in *E. faecium* BFE 900 [22]. At pH 4-5 although growth was observed but no bacteriocin production was observed, however at pH 10 there is 50% reduction in bacteriocin production. In another similar study, maximum production (of 3600 AU mL^{-1}) was observed at initial pH of 5.8, while slight increase in pH to 6.2 decreased the activity to 2700 AU mL^{-1} proving that production of bacteriocin is very sensitive to change in pH [17]. Change in pH to lower or higher level (from pH 6.0) decreased the bacteriocin production by 1.3-2.5 times in *L. lactis* [28].

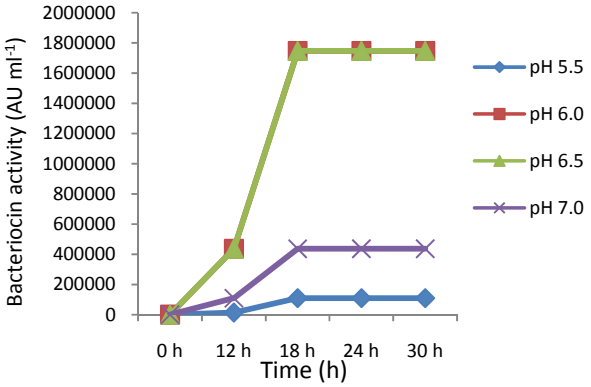

Fig. 3 Effect of pH of medium on bacteriocin production

Temperature is another critical factor which influences the rate of bacteriocin production (Fig. 4). With increase in temperature, the production of bacteriocin increased and maximum production was observed at 40°C (1.74×10^6 AU mL^{-1}). Beyond this temperature, bacteriocin production was inhibited and bacteriocin activity decreased by 50%. Wide variation in optimum temperature for growth and bacteriocin production was also reported by other researchers [23]. *Lactococcus lactis* A164 showed maximum bacteriocin production at 30°C but had optimum temperature for growth 37°C at which 50% reduction in bacteriocin production has been reported [28].

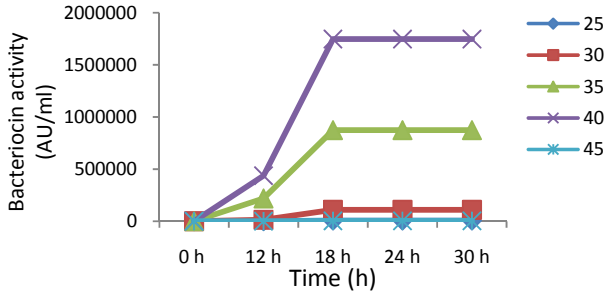

Fig. 4 Effect of incubation temperature (°C) on bacteriocin production

For the production of metabolites, volume of inoculums is very effective. Bacteriocin production was increased with the increase in inoculum level from 1% to 2% but no further increase was observed up to 5% inoculum level. As concentration of inoculum increased (2-5%), the production time of bacteriocin reduced from 24 h to 18 h of incubation (Table IV). A lower concentration of inoculum is represented by fewer numbers of viable cells thereby insufficient for high biomass and bacteriocin production [29]. However, high concentration of inoculum may result in competitive inhibition between large number of cells present [30]. Fast depletion of nutrient availability due to higher number of cells may also be reason for decreased production of bacteriocin by *Enterococcus facieum* BS13.

TABLE IV
EFFECT OF INOCULUM CONCENTRATION ON BACTERIOCIN PRODUCTION

Time	Bacteriocin Activity (AU ml^{-1})				
	1%	2%	3%	4%	5%
12 h	27306.6	436906.6	436906.6	436906.6	436906.6
18 h	436906.6	1747626.6	1747626.6	1747626.6	1747626.6
24 h	1747626.6	1747626.6	1747626.6	1747626.6	1747626.6
30 h	1747626.6	1747626.6	1747626.6	1747626.6	1747626.6

Under static conditions, 109227 AU mL^{-1} of activity was observed in 18 h of incubation. With increase in agitation from 50 to 100rpm, the bacteriocin production increased to 436907 AU mL^{-1} and 1747627 AU mL^{-1} respectively, with a similar incubation period. Lower agitation speed decreased the aeration and availability of dissolved oxygen during the growth. The nutrient distribution is also affected leading to less growth and production of bacteria [31]. Further increase in rpm to 150 decreased the bacteriocin production which might lead to lysis of cells or leading to drop in activity (Fig. 5). Higher agitation beyond optimal level also decreased the bacteriocin production which might be due to lysisof cells due to shearing effect [32]-[34].

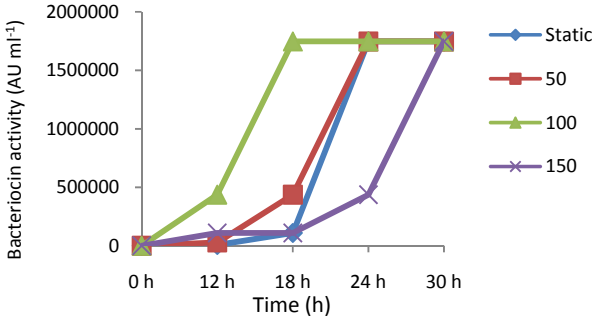

Fig. 5 Effect of agitation (rpm) on bacteriocin production

Further, the application of bacteriocin produced by *Enterococcus faecium* BS 13 was done in food system. Surface application of bacteriocin in unripened cheese/curd cheese (paneer) and khoya samples extended the shelf life up to 15 and 20 days, respectively as compared to control samples under refrigerated conditions [35].

IV. Conclusions

Various agro-industrial byproducts used as media components supported the production of bacteriocin by *E. faecium* BS13. Whey as carbon source resulted in the maximum bacteriocin production. The supplementation of corn steep liquor and soya okra increased the bacteriocin production but corn steep liquor (4%, wv^{-1}) resulted in maximum bacteriocin production. Addition of metal ions did not have any significant effect on the bacteriocin production. Among various surfactants, the supplementation of polysorbate-80 increased the production of bacteriocin in 18h of fermentation. Utilization of food industry and agro industrial waste for the production of biomolecules contributes towards economics of the process cost.

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