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Optimisation of Enterocin A Production on a Whey-Based Substrate

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Abstract: This study demonstrates the optimisation of enterocin A production by *Enterococcus faecium* strains using a cheese whey substrate. To optimise enterocin A production, various factors such: temperature, oxygen, yeast extract and peptone were adjusted. Results have shown that the best condition for all strains were the same for temperature and oxygen factors, however the best condition for peptone and yeast extract were different for each strains. Also it was shown that aerobic condition is necessary to produce enterocin A in cheese whey by all studied strains.

Key words: Cheese whey · Enterocin A · Taguchi design

INTRODUCTION

Lactic acid bacteria are extensively used in food processing, such as in dairy and meat fermented products [1, 2] for their contribution to shelf life, texture, and organoleptic properties. These microorganisms have been used in food and feed preservation for centuries since they can produce a variety of antimicrobial agents, including organic acids, ethanol, carbon dioxide, diacetyl, and hydrogen peroxide [1]. Strains of lactic acid bacteria produce bacteriocins, which are peptides or proteins with an antibacterial activity against bacteria closely related to the producer strain [2, 3]. Among lactic acid bacteria, enterococci frequently occur in various traditional food systems, especially those from animal origin, such as dairy products. This genus can become an important part of the fermented dairy micro flora and its source seems to be not necessary related with fecal contamination. At present, these microorganisms are used in traditional food process and in probiotic therapy and their beneficial properties are widely recognized [4]. Most bacteriocins from enterococci are classified as class II bacteriocins; small, heat-stable, non-lantibiotics, such as enterocin A, B, As48 and ... [5]. Bacteriocins produced by enterococci have gained interest because bacteriocins producer strains can be isolated from a variety of fermented food or silage and because many are well active towards the food borne pathogens *Listeria* and *Clostridium* [4].

LAB are nutritionally fastidious and the production of bacteriocins are normally performed in complex growth media. Although these media promote exuberant growth and relatively high bacteriocin concentration levels, their high cost make them unsuitable for a large-scale production. On the other hand, some cheap raw materials such as whey, sugar molasses and mussel-processing wastes have also been reported as culture media for bacteriocin production [6]. Vessoni *et al.,* (2005) showed that skimmed milk in comparison to other synthetics media enhanced the expression of nisin from the cells into the medium [7]. For process cost reduction, Jozala *et al*. (2007) observed that diluted skimmed milk, at 25% of standard concentration, improved nisin production [8]. Whey is a byproduct of the dairy industry and contains rich nutrients such as lactose, soluble proteins and minerals salts. Unfortunately, whey and its associated nutritional qualities have traditionally been treated as waste and represent an important disposal and pollution issue because of its high biological and biochemical oxygen demand. Consequently, it is of interest to use this byproduct as a fermentation substrate for the production of value-added products [9, 10].

Flores and Alegre (2001), using supplemented whey during batch fermentation, obtained a maximum nisin activity of 5280 IU mL^{-1} after 9 h of processing (pH 4.9) [11]. Mondragon-Parada *et al.* (2006) verified that supplemented filtrated whey enhanced the biomass production of LAB [12]. Some researchers applied a mixed culture of *L. lactis* and *Saccharomyces cerevisiae* to whey-based medium to stimulate the production of nisin [13].

In this study investigated enterocin A production in cheese whey and evaluated the effect of four variables (temperature, oxygen, yeast extract and peptone) in enterocin A production by these strains in cheese whey.

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MATERIALS AND METHODS

Reagents, Media and Bacterial Cultures: Dried cheese whey powder was from Pegah Company (Isfahan, Iran). Brain heart infusion (BHI) was from Pronadisa (Farmacopea, Spain) and MRS was from Sharlua (Barcelona, Spain). Agar, gas pack and other reagents were from Merck (Darmstadt, Germany).The producer strains were isolated and characterized as *Enterococcus faecium* strains (a_2, a_5, a_{17}, a_{19}) [14, 15]. The indicator strain was *Listeria monocytogenes* PTCC19112.

Enterocin A Production: The inoculums were obtained in MRS broth at 30 °C. Then, 100 µl of culture (10^7 cell/mL) was transferred to 1 0 ml of freshen medium in tube at 30°C for 24 h. Culture supernatant was obtained by centrifugation at 12,000 rpm for 20 min and then was adjusted to pH 6.5 with 5 M NaOH and filtered through a 0.45-µm filter. Inhibition was tested by spotting 10 µL of the supernatant onto soft agar lawn (0.6%) seeded with 0.1 mL of an overnight grown *Listeria monocytogenes* PTCC19112 and incubated overnight. The enterocin A titer was determined by the serial two-fold dilution method. The reciprocal value of the highest dilution, where an inhibition zone was observed × 100 chooses as activity units mL^{-1}($AUmL^{-1}$) [14].

Table 1: Factors and level of factors used in this experiment.

Factors ≠ Levels	Level 1 (L1)	Level 2 (L2)	Level 3 (L3)
Peptone	0%	0.5%	1%
Yeast extract	0.5%	1%	1.5%
Temperature	30 C°	37 C°	-
Oxygen	Aerobic	Anaerobic	-

Table 2: Experimental design (Array type L-9) by Taguchi design

Experiment #				
Factor levels	Peptone	Yeast extract	Temperature	Oxygen
1	0%	0.5%	30 C°	Aerobic
2	0%	1%	37 C°	Anaerobic
3	0%	1.5%	30 C°	Aerobic
4	0.5%	0.5%	37 C°	Aerobic
5	0.5%	1%	30 C°	Aerobic
6	0.5%	1.5%	30 C°	Anaerobic
7	1%	0.5%	30 C°	Anaerobic
8	1%	1%	30 C°	Aerobic
9	1%	1.5%	37 C°	Aerobic
Total	18	18	12	12

Enterocin A Production in Cheese Whey: For selection of medium, cheese whey powder (70 g L^{-1}), cheese whey powder (70 g L^{-1}) supplemented with yeast extract (10 g L^{-1}), were tested individually. The pH was adjusted at 7.0 before autoclaving (121°C, 15 min) [10]. After inoculation with 100µL culture of every *Enterococcus faecium* strains(10^7 cell/mL) in 10 mL of these medium in tube, incubation was performed at 30 °C. enterocin A activity was determined after 24 h as described above.

Experimental Design: After selection of medium, the next step was to determine the optimal levels of two variables, temperature, O_2 and three variables nitrogen source (peptone and yeast extract) on enterocin A production (Table 1). For this purpose, Taguchi design (Qualitek-4(w32b) software) was used. A set of 9 experiments was carried out (Table 2) on based L_9 array [16, 17].

RESULTS

Enterocin A Production in Cheese Whey: Enterocin A produced by *E. faecium* strains (a_2, a_5, a_{17}, a_{19}) from the dairy products have been Characterised by Mirhosseini *et al*. In this work bacteriocin productin by these strains investigated in cheese whey, as the only source of carbon energy. Results were shown that all studied strains have grown in cheese whey with no bacteriocin production; however all of *E. faecium* strains produced maximum bacteriocin in cheese whey with supplemented of yeast extract (10 g l^{-1}). Thus yeast extract was useful supplemented in cheese whey for bacteriocin production.

Optimisation of Enterocin A Production by Taguchi Design: Results shown that quantity of enterocin A production was different in each strains and strain a_{17} had the most enterocin A production (32 × 100 $AUmL^{-1}$) among other strains (Figure 1). Statistical analysis of results with Qualitek-4 software with confidence level 90% and confidence interval +/-2.055 showed that the five variables have a significant effect on enterocin A production (Figure2). Interaction severity indexes (SI) between tow factors for each strain have shown in table 3. SI for variable factors in each strain was different. SI between tow factors for strain a_2 was yeast extract & temperature. In strain a_5 the best SI was interaction between yeast extract & oxygen and in a_{17} strain the most SI was peptone & yeast extract; however for strain a_{19} temperature & oxygen had main effect on enterocin A production. Results were shown that the most SI for each

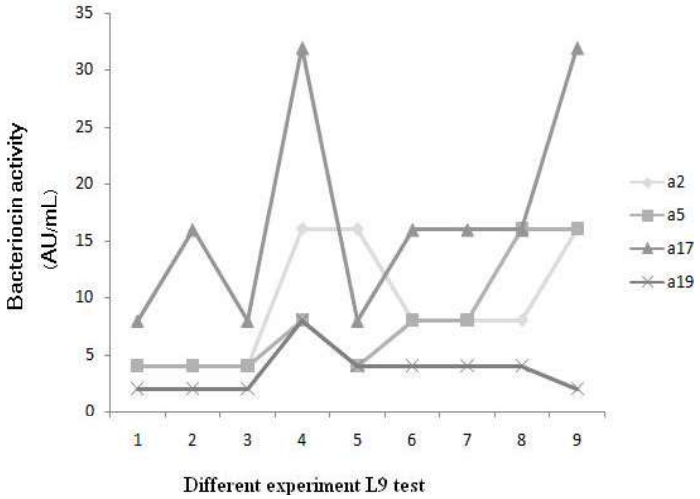

Fig. 1: Effect of different experiment L_9 test in *Enterococcus faecium* strains on enterocin A production. A: *Enterococcus faecium* a_2, B: *Enterococcus faecium* a_5, C: *Enterococcus faecium* a_{17}, D: *Enterococcus faecium* a_{19}

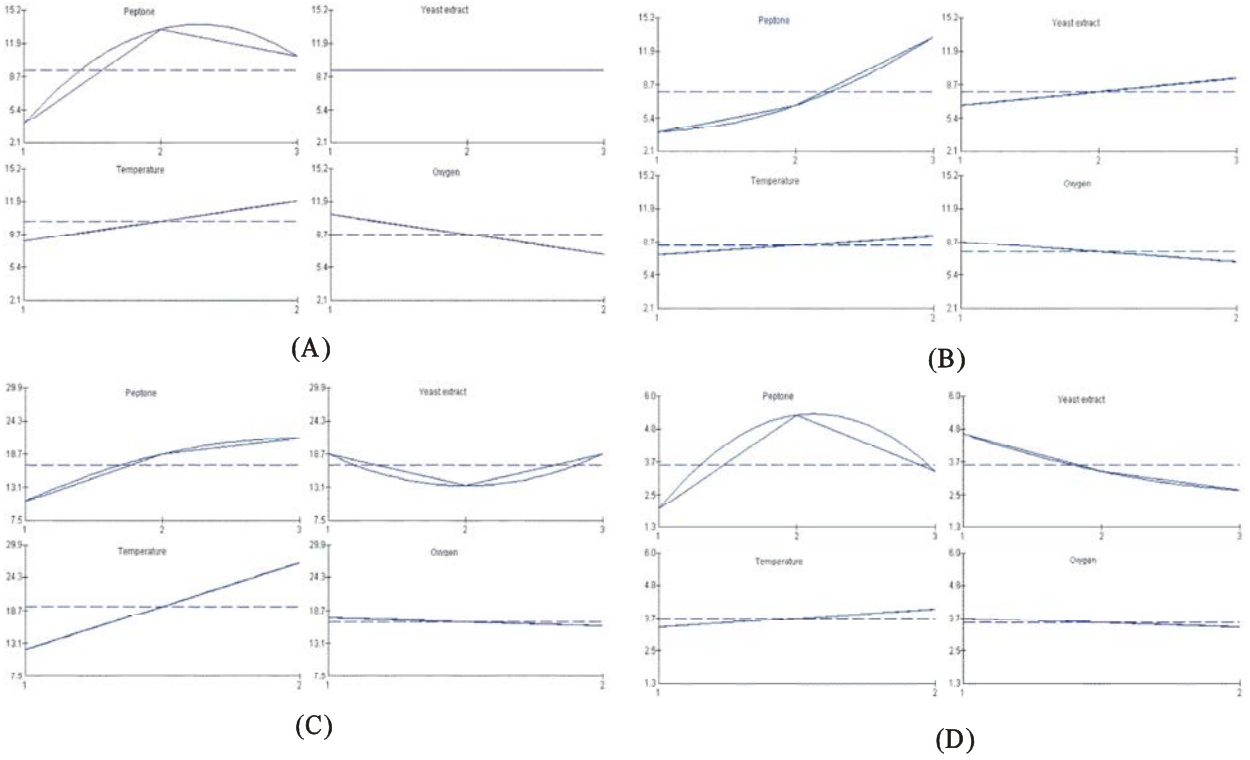

Fig. 2: Analysis of different condition in *Enterococcus faecium* strains on enterocin A production with Qualitek-4(w32b) software. A: *Enterococcus faecium* a_2, B: *Enterococcus faecium* a_5, C: *Enterococcus faecium* a_{17},D: *Enterococcus faecium* a_{19}

strain was dependent on factors which had low effect on enterocin A production. The effect of P value on different factors for 4 studied *E. faecium* are shown in table 4. As it is shown the percent of P value for peptone additive of strain a_2, a_5, a_{17} and a_{19} were 60.173, 69.696, 25.914 and 52.272 respectively. These data showed that addition of peptone had positive effect on all the isolated. However it has less effect on 25% on a_{17}. The low P value on this strain may be as the results of high P value for temperature (61.456) which compared to other stains and

Table 3: Interaction factors pairs (Order based on SI) for variable factors in *E. faecium* strains on enterocin A production. A: *E. faecium* a_2, B: *E. faecium* a_5, C: *E. faecium* a_{17},D: *E. faecium* a_{19} A: *E. faecium* a_2

Interacting Factor Pairs (Order based on SI)	Columns	SI(%)	Opt
Yeast extract × Temperature	2 × 3	75	L1, L2
Temperature × Oxygen	3× 4	50	L2, L1
Yeast extract × Oxygen	2 × 4	37.5	L2, L1
Peptone × Oxygen	1 × 4	33.33	L2, L1
Peptone × Temperature	1 × 3	16.66	L2, L2
Peptone × Yeast extract	1 × 2	0	L2, L1
B: *E. faecium* a_5			
Interacting Factor Pairs (Order based on SI)	Columns	SI(%)	Opt
Yeast extract × Oxygen	2 × 4	66.66	L2, L1
Temperature × Oxygen	3× 4	56.25	L2, L1
Yeast extract × Temperature	2 × 3	33.33	L3, L2
Peptone × Yeast extract	1 × 2	16.66	L3, L2
Peptone × Temperature	1 × 3	8.33	L3, L2
Peptone × Oxygen	1 × 4	8.33	L3, L1
C: *E. faecium* a_{17}			
Interacting Factor Pairs (Order based on SI)	Columns	SI(%)	Opt
Peptone × Yeast extract	1 × 2	66.66	L2, L1
Yeast extract × Oxygen	2× 4	50	L1, L1
Temperature × Oxygen	3 × 4	50	L2, L1
Yeast extract × Temperature	2 × 3	40	L1, L2
Peptone × Oxygen	1 × 4	37.5	L3, L1
Peptone × Temperature	1 × 3	25	L2, L2
D: *E. faecium* a_{19}			
Interacting Factor Pairs (Order based on SI)	Columns	SI(%)	Opt
Temperature × Oxygen	3 × 4	66.66	L2, L1
Yeast extract × Temperature	2× 3	58.33	L1, L2
Peptone × Yeast extract	1 × 2	33.33	L2, L1
Peptone × Temperature	1 × 3	33.33	L2, L2
Peptone × Oxygen	1 × 4	25	L2, L1
Yeast extract × Oxygen	2 × 4	16.66	L1, L1

SI-Interaction severity index (100% for 90 degrees angle between the line, 0% for parallel lines).

Opt-Indicates factor levels desirable for optimum condition (based strictly on the first 2 levels).

was high value for temperature factor. The data have shown that maybe strain a_{17} grow better in higher temperature. The addition of yeast extract and oxygen were low effect for all stains, however only peptone had high effect on enterocin A production and temperature and peptone were effective factors on enterocin A production by strain $a_{17.}$ Although peptone was positive effect but Taguchi design showed that best concentration of peptone was on level 2 (10%). But yeast extract with different concentration did not have any significant effect on enterocin A production (Figure 2). As it is shown in this figure only temperature have liner effect on a_{17} strain. In conclusion the optimum condition for *E. faecium* to produced enterocin A is shown in table 5. As it is shown, Peptone (5 grL^{-1}), yeast extract (5 grL^{-1}), temperature (37 $^{\circ}C$) and aerobic condition were best for strain a_2 and a_{19}. Peptone (10 grL^{-1}), yeast extract (15 grL^{-1}), temperature (37 $^{\circ}C$) and aerobic condition were best for strain $a_{5,}$ However peptone (10 grL^{-1}), yeast extract (5 grL^{-1}), temperature (37 $^{\circ}C$) and aerobic condition were best for strain a_{17}. Results have shown that best condition for all strains were the same in two factor temperature and oxygen and best condition for peptone and yeast extract were different in these strains. Also it was shown that aerobic condition is needed for all strains to produces enterocin A in cheese whey. Thus establishment anaerobic condition with CO_2 or gas pack is not necessary.

DISCUSSION

In this study was to evaluate the effect of four variables (temperature, oxygen, yeast extract and peptone) in enterocin A production by these strains in cheese whey. All these strains have grown in cheese whey but any strains have not produced enterocin A in cheese

Table 4: (analysis variance) ANOVA table for different factors in *E.faecium* strains on enterocin A production. A: *E. faecium* a_2, B: *E. faecium* a_5, C: *E. faecium* a_{17},D: *E. faecium* a_{19} A

Factors	DOF (f)	Variance (V)	F-Ratio (F)	Pure Sum (S)	Percent P(%)
1 Peptone	2	138.666	35.749	269.575	60.173
2 Yeast extract	2	-.001	-0.001	0	0
3 Temperature	1	64	16.499	60.121	13.419
4 Oxygen	1	64	16.499	60.121	13.419
Other/Errror	11	3.878			12.989
B					
Factors	DOF (f)	Variance (V)	F-Ratio(F)	Pure Sum (S)	Percent P(%)
1 Peptone	2	138.666	28.599	267.636	69.696
2 Yeast extract	2	10.666	2.199	11.636	3.03
3 Temperature	1	15.999	3.299	11.151	2.904
4 Oxygen	1	16	3.3	11.151	2.904
Other/Error	11	4.848			21.466

Table 4: Continued

Factors	DOF (f)	Variance (V)	F-Ratio (F)	Pure Sum (S)	Percent P(%)
C					
Factors	DOF (f)	Variance (V)	F-Ratio(F)	Pure Sum (S)	Percent P(%)
1 Peptone	2	184.888	47.666	362.02	25.974
2 Yeast extract	2	56.888	14.666	106.109	7.606
3 Temperature	1	860.444	221.832	856.565	61.456
4 Oxygen	1	7.111	1.833	3.232	0.231
Other/Error	11	3.878			4.733
D					
Factors	DOF (f)	Variance (V)	F-Ratio(F)	Pure Sum (S)	Percent P(%)
1 Peptone	2	16.888	15.481	31.595	52.272
2 Yeast extract	2	6.222	5.703	10.262	16.978
3 Temperature	1	1.777	1.629	0.686	1.136
4 Oxygen	1	0.444	0.407	0	0
Other/Error	11	1.09			29.614

Table 5: Optimum condition in *E. faecium* strains on enterocin A production. A: *E. faecium* a_2, B: *E. faecium* a_5, C: *E. faecium* a_{17},D: *E. faecium* a_{19} A

Factors	Level Description	level	Contribution
1 Peptone	0.5%	2	3.999
2 Yeast extract	0.5%	1	-.001
3 Temperature	37 C°	2	2.666
4 Oxygen	Aerobic	1	1.333
B			
Factors	Level Description	level	Contribution
1 Peptone	1%	3	5.333
2 Yeast extract	1.5%	3	1.333
3 Temperature	37 C°	2	1.333
4 Oxygen	Aerobic	1	0.666
C			
Factors	Level Description	level	Contribution
1 Peptone	1%	3	4.444
2 Yeast extract	0.5%	1	1.777
3 Temperature	37 C°	2	1.333
4 Oxygen	Aerobic	1	0.444
D			
Factors	Level Description	level	Contribution
1 Peptone	0.5%	2	1.777
2 Yeast extract	0.5%	1	1.111
3 Temperature	37 C°	2	0.444
4 Oxygen	Aerobic	1	0.111

whey. While all strains have enterocin A production in cheese whey supplemented with yeast extract. This indicated that nitrogen source had main effect on enterocin A production for all strains. In agreement with rsearch of Vazquez and *et al.,* 2006, they have shown that different peptones obtained from visceral and fish muscle residues, were tested too and promoted growth of lactic acid bacteria, demonstrating the favorable effects of fish peptones on the production of nisin and pediocin [18]. Cheese whey has been successfully used for bacteriocin production by several bacteria [19, 20]. However, cultivation on carbohydrate-rich byproducts like whey and grape waste resulted in undetectable production of bacteriocin by *Bacillus sp*. strain P349 [21]. Guerra and Pastrana (2001) observed that it is possible to convert the whey in suitable culture media to enhance nisin and pediocin production by means of supplementation with a complex nitrogen source and maintaining a low lactose concentration in the medium to prevent the catabolite repression effect [22]. Bacteriocin production was evaluated by growing *Bacillus licheniformis* on feather meal, hydrolysed feather meal, industrial fibrous soybean

residue, cheese whey, cheese whey supplemented with yeast extract and grape peel [10]. Among the media tested, maximum bacteriocin activity was observed during cultivation in cheese whey (3200 AU mL^{-1}), followed by industrial fibrous soybean residue (1600 AU mL^{-1}).

The best design experiment helps us to study many variables simultaneously and most economically. In this work the Taguchi design were employed and L_9 experiments were designed. In each set, 4 variables were considered, two factors in two level and others have 3 levels. Levels introduced quantitative amount of available in experiments. It is easy to study the results of design experiment with Qualitek-4(w32b) software to obtain the interaction of different factors. For example interaction between temperature & nitrogen source had main effect on enterocin A production in all stains, whereas interaction between peptone & temperature and peptone & oxygen were not significant. The effects of nitrogen source and temperature are very important for enterocin A production. The effect of temperature and pH on bacteriocin production has been reported for several bacteriocins produced by *Lactobacillus casei* [23] and *Leuconostoc mesenteroides* [24], among others. Using the Qualitek-4(w32b) software, were demonstrated that these parameters are also fundamental for enterocin A production by *Enterococcus faecium* strains. Best condition for oxygen factor on enterocin A producton was aerobic condition in all strains. This is indicated establishment of anaerobic conditions for enterocin A production in these strain are not necessary. Thus establishment anaerobic condition with CO_2 or gas pack is not necessary. This is economically. Qualitek-4 software proved to be a powerful tool in optimising bacteriocin production by other *Enterococcus* strains.

CONCLUSION

Optimisation of medium by the classical method involves changing one independent variable while fixing all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables and also may result in wrong conclusions. Taguchi design (Qualitek-4) is software for designing experiments, building models, evaluating the effects of factors, and searching optimum conditions of factors for desirable responses. Also results have shown that the cheese whey can be utilised as a substrate for bacteriocin production by *E. faecium*. This low-cost medium when used for microbial cultures has economic advantages and reduces environmental pollution which should stimulate research into its further use.

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