PERFORMANCE OF AQUEOUS IMPREGNATED RESIN SYSTEM FOR PURIFICATION OF BACTERIOCIN FROM *Lactobacillus bulgaricus* FTDC 1211

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by

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Nur Fazrin Husna binti Abdul Aziz

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LIST OF SYMBOLS

%(v/v)	Volume percentage
%(w/w)	Weight percentage
&	And
~	Approximate
<	Less than
=	Equals
>	More than
±	Plus minus
µ	Micro
^{o}C	Degree Celcius
α	Alpha
β	Beta

LIST OF ABBREVIATIONS

AIRS	Aqueous Impregnated Resin System
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
ATPS	Aqueous Two Phase System
BLIS	Bacteriocin-like Inhibitory Substance
BSA	Bovine Serum Albumin
CCD	Central Composite Design
CFU	Colony Forming Unit
Da	Dalton
FDA	Food and Drug Administration
GF	Gel Filtration
GRAS	Generally Recognized as Safe
LAB	Lactic Acid Bacteria
MS	Mass Spectrometry
NaCl	Sodium chloride
PEG	Polyethylene glycol
RPM	Rotation per minute
RSM	Response surface methodology
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TAPPIR	Tunable Aqueous Polymer Phase Impregnated Resin
TCA	Trichloroacetic
TEMED	Tetramethylethylenediamine
TRIS	Trisaminomethane
UV	Ultraviolet

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PRESTASI SISTEM RESIN TERIMPREGNAT AKUEUS UNTUK PENULENAN BAKTERIOSIN DARIPADA *Lactobacillus bulgaricus* FTDC 1211

ABSTRAK

Kaedah penulenan bakteriosin konvensional sering memerlukan rangkaian prosedur yang sukar, mengakibatkan kos yang tinggi dan penghasilan yang kurang. Langkah penulenan tunggal baru yang menggunakan sistem dua fasa akueus (ATPS) mempunyai tempoh keredaan yang panjang, tegangan antara muka dan perbezaan ketumpatan yang rendah. Untuk mengatasi masalah ini, sistem resin terimpregnat akueus (AIRS) dicadang dan dibangunkan dalam penyelidikan ini untuk menulenkan bahan perencat serupa bakteriosin yang dihasilkan oleh *Lactobacillus bulgaricus* FTDC 1211 yang menunjukkan aktiviti antimikrob menentang *Staphylococcus aureus*. Dalam AIRS, satu fasa akueus yang hidrofobik diimpregnatkan dalam resin sementara satu fasa akueus yang hidrofilik bertindak sebagai larutan pengekstrakan untuk mengasingkan bakteriosin dalam supernatan mentah bebas sel. Bakteriosin akan diekstrak ke dalam resin yang diserapi polimer. Sebelum menjalankan eksperimen penulenan bakteriosin, prestasi AIRS dalam mengekstrak produk biologi dikaji dengan menggunakan albumin serum bovin (BSA) sebagai model protein. Kestabilan memperimpregnasi polimer hidrofobik dalam tiga jenis resin (Amberlite XAD4, pelet kaca VitraPor 4.0 mm dan 8.0 mm) telah dikaji. Resin Amberlite XAD4 menunjukkan penyerapan PEG yang paling stabil dengan kadar larut-lesap tidak melebihi 1%. Permukaan yang licin dapat diperhatikan pada Amberlite XAD4 yang terimpregnat polimer di bawah mikroskopi elektron penskanan. Parameter penulenan yang dikaji dalam eksperimen pengasingan BSA adalah kesan kepekatan dan jisim

molekul polietilen glikol, pH dan kepekatan akueus natrium sitrat dan jenis bahan resin. Hasil pemulihan sebanyak 50.55% BSA diperoleh dengan AIRS yang mengandungi 40%(w/w) polietilen glikol 2000 terimpregnat pada pellet kaca VitraPor berdiameter 8.0mm dan 10% (w/w) natrium sitrat pada pH 7.0. Dalam eksperimen penulenan bakteriosin, pengoptimuman faktor-faktor penting yang mempengaruhi penulenan bakteriosin dalam AIRS telah dikaji dengan menggunakan kaedah gerak balas permukaan. Faktor tersebut merangkumi jisim molekul dan kepekatan polimer, pH dan kepekatan akueus garam, jenis resin dan kepekatan NaCl. Bakteriosin tersebut tertulen separa dengan hasil pemulihan sebanyak 82.96% dan fakor penulenan sebanyak 3.2 dengan sistem yang mengandungi 11.44% (w/w) PEG 4000 yang terimpregnat pada Amberlite XAD4 dan 1.98 %(w/w) natrium sitrat pada pH 6.06 dan 4%(w/w) NaCl. Satu jalur dengan saiz ~14kDa diperoleh dalam SDS-PAGE membuktikan bahawa sistem resin terimpregnat akueus boleh digunakan sebagai kaedah alternatif untuk penulenan bakteriosin.

PERFORMANCE OF AQUEOUS IMPREGNATED RESIN SYSTEM FOR PURIFICATION OF BACTERIOCIN FROM *Lactobacillus bulgaricus* FTDC 1211

ABSTRACT

Conventional purification method of bacteriocins often requires laborious series of complicated procedures, high cost and low yield. Recent single purification step using aqueous two-phase system has long settling time, low interfacial tension and small density difference. To overcome this problem, in our study, the aqueous impregnated resins system (AIRS) was proposed and developed to purify bacteriocin-like inhibitory substances produced by *Lactobacillus bulgaricus* FTDC 1211 which exhibited antimicrobial activity against *Staphylococcus aureus* in our study. In AIRS, hydrophobic polymer phase was impregnated into resins and hydrophilic aqueous phase acts as extraction solution to separate the bacteriocin in cell-free crude supernatant. The bacteriocin was then extracted in the polymer impregnated resins. Before conducting bacteriocin purification experiment, the performance of AIRS in extracting biological product was investigated using bovine serum albumin (BSA) as protein model. The impregnation stability of the hydrophobic polymer in three different types of resin (Amberlite XAD4, VitraPor glass pellets 4.0 mm and 8.0 mm) was investigated. Amberlite XAD4 resins showed the most stable impregnation of PEG with less than 1% (w/w) of leaching factor. A smooth surface on PEG impregnated Amberlite XAD4 was observed under scanning electron microscopy. The purification parameters investigated in BSA partitioning experiment are the effects of different polyethylene glycol (PEG) molecular weights and concentrations, pH and concentration of sodium citrate aqueous solution and

type of resins. It showed that 50.55% of BSA recovery yield was obtained by AIR System with 40% (w/w) of PEG 2000 g/mol impregnated in VitraPor glass beads with 8.0 mm diameter and 10% (w/w) of sodium citrate at pH 7.0 extracting aqueous solution. In bacteriocin purification experiment, optimization of crucial factors affecting purification of bacteriocin in AIRS were studied using response surface methodology. The factors included the molecular weight and concentration of PEG, pH and concentration of salt, type of resins, and NaCl concentration. The bacteriocin was partially purified at recovery yield of 82.96 % and purification factor of 3.2 with the system composed of 11.44% (w/w) of PEG 4000 g/mol impregnated in Amberlite XAD4 and 1.98% (w/w) of extraction solution of sodium citrate at pH 6.06 and 4%(w/w) of NaCl. A band with size ~14kD was obtained in SDS-PAGE that proves AIRS can be used an alternative method in the purification of bacteriocin.

CHAPTER 1

INTRODUCTION

1.1 Research Background

In bioprocessing area, the upstream process for the biological molecules production is growing steadily for the past three decades. The downstream processing, however, have always become the production footprint. There are four major parts in downstream processing of biomolecules which are recovery, isolation, purification and polishing. Among these four steps, purification is said to be the major contributor of the total production costs due to the usage of high technology equipment (Raja Murty et al., 2011). Over the past decades, researchers have shown interest on a single step purification technique known as Aqueous Two-Phase System (ATPS). ATPS have gained interest in purification because it is said to be the alternate method to reduce the costs in purification step. Nevertheless, the physicochemical properties of the two-phase cause long settling time and requires additional equipment. These footprints led to a development of cutting-edge technology in downstream processing.

Thus, in this study, a novel approach purification method known as Aqueous Impregnated Resin System (AIRS) was proposed and developed to purify bacteriocin from *Lactobacillus bulgaricus* FTDC 1211. AIRS imply the concept of hydrophobicity of polymer and the hydrophilicity and electrostatic charge of sodium citrate as salt aqueous solution in purifying the target biomolecule. The hydrophobic polymer will be impregnated into resins while the contaminant from the culture broth will be separated by the salt solution. The bacteriocin is chosen as target biomolecules in this research because it can exhibit antibacterial effect toward

Staphylococcus aureus, strains that are potential risk to humans since it may cause wide range of clinical infections (Tong et al., 2015).

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins which have the ability to either kill or inhibit the growth of bacteria to its closely related species. Bacteriocins are being produced by Lactic Acid Bacteria (LAB) that Generally Recognized as Safe (GRAS) microorganism. Its non-toxic nature, heat-stable, highly selective makes it promising characteristics for the bacteriocin to be used as antimicrobial agents or preservatives in pharmaceutical and food industry. It has been known widely that the bacteriocin is proven to inhibit many pathogenic bacteria such as *Staphylococcus* sp., *Lysteria* sp., *Eschericia* sp., *Vibrio* sp., *Shigella* sp., *Pseudomonas* sp., *Aeromonas* sp. *et cetera* (Karpinski and Szkaradkiewicz, 2013). This characteristic makes bacteriocin as huge potential antimicrobial agent in pharmaceutical industry. For instances, De Vust and Leroy (2007) states that the contribution of bacteriocin in gut health is it plays a role during *in vivo* interactions occurring in the human gastrointestinal tract. Bastos et al. (2010) reported that Lysostaphin, a Class III bacteriocin has the potential to kill human and animal staphylococcal pathogens. Lancaster et al. (2007) stated that bacteriocins are being used for the treatments of malignant cancers.

While the upstream processing of bacteriocin production was grown rapidly, the downstream processing of bacteriocin production, conversely, have become one of the major problems in the marketplace. This is because the downstream processing of bacteriocin involves the separation of the bacteriocins from a complex crude feedstock (Bali et al., 2014; Deraz et al., 2007; Jozala et al., 2008; Jozala et al., 2015). Over the years, there are many works related to purification and production of bacteriocin. The most common purification method of bacteriocin is by multistep

operation which commonly involved ammonium sulphate precipitation and chromatography. Although this conventional method is known to give high purity of product, however, the disadvantages associates with this conventional method is that it requires a multistage operation that are expensive, time consuming, produce low yield and are not efficient for industrial scale requirements (Parante et al., 1999 and Jamaludin et al., 2017). Besides that, secondary metabolites that have low molecular weight of biomolecules such as bacteriocin can be easily degraded in high toxic organic solvents in traditional extraction and separation techniques. Therefore, an alternative purification method is needed.

One of the alternate methods of bacteriocin purification is as described by Abbasiliasi et al. (2014). In their research, they carried out an experiment to purify bacteriocin-like inhibitory substances (BLIS) using ATPS. In the experiment, the BLIS was successfully purified with the final product achieved purification factor and recovery yield of 8.43 and 81.18% respectively. This outcome indicates that ATPS is a reliable method in bacteriocin purification. Nevertheless, low density differences in ATPS that leads to the long separation time between the two phases have become the bottleneck of this system (Bernhard Burghoff, 2013). Furthermore, Grilo et al. (2014) states that it is difficult to optimize and sorting out the mechanism of the biomolecules partitioning in ATPS due to the poor understanding on how the mechanism works.

Thus, in this study, a novel approach method known as AIRS was introduced. AIRS have potential to purify biomolecules by maintaining the advantages of ATPS and eliminates the drawbacks of ATPS mentioned previously. In AIRS, instead of having two aqueous phases in a system, one of the aqueous phases is impregnated into the resins, while the other aqueous phase acts as bulk phase surrounding the

impregnated resins. By this way, the long settling time of ATPS can be overcome while the purification of biomolecules is carried out in non-toxic and biocompatible system.

1.2 Research Scope and Objectives

In this experiment, the principle of a new developed purification method known as AIRS in purifying bacteriocin from *L. bulgaricus* FTDC 1211 was studied. Since this method is new, the preliminary study on the functionality and mechanisms of this method was studied using bovine serum albumin (BSA) as an exemplary protein. Few factors that may give effects to the BSA partitioning and bacteriocin purification experiment using AIRS were examined and the final recovery yield was evaluated.

The objectives of this research were:

1. To investigate the impregnation stability and the effect of purification parameters on the recovery of bovine serum albumin (BSA) using AIRS.
2. To optimize the effect of parameters on the purification of bacteriocin produced by *Lactobacillus bulgaricus* FTDC 1211 using AIRS.

CHAPTER 2

LITERATURE REVIEW

2.1 Purification of Biological Product in Bioprocessing

In bioprocessing, the process is divided into two major parts which are upstream processing and downstream processing. Upstream processing of biomolecules commonly depends on the biological limits such as the media compositions. The downstream processing, conversely, is the major contribution on total production costs especially in pharmaceuticals. The separation and purification of pharmaceutical ingredients can be up to 70-90% of total production costs (Gronemeyer, Ditz and Struber, 2014; Molla et al., 2018)

The reason of high cost in purification of pharmaceutical ingredients is because of multistep operation and the usage of expensive equipment like chromatography. These multistep operations normally involve ammonium precipitation and a series of chromatography technique, which further lead to low yield recovered and significant loss of biomolecule activities. Besides, it is not economical friendly to use this conventional method for large production scale (Rosa et al., 2011; Asenjo and Andrews, 2012)

Since the purification of biomolecules are important in pharmaceutical industry, there are alternative methods reported which is believed to reduce the purification costs and produce acceptable biomolecule purity. For the past years, a single purification system known as ATPS was introduced in purification of biomolecules. ATPS is a single purification step which use the hydrophobicity of polymer to interact with the target biomolecule and the second aqueous phase, normally the salts will precipitate the contaminants from the culture broth. The advantages of ATPS include the simplicity of the process, gentle environmental

conditions (70% to 90% of water), rapid and selective separation with little denaturation, eco-friendly and not involving toxic solvents (Raja et al., 2012).

Nevertheless, the density difference between the two phases and the interfacial tensions are low, causing long phase separating times. Besides, these two aqueous phases also very viscous due to the high concentration of both phases as well as the nature of polymer, which contributes to the footprint of this purification system (van Winssen et al., 2014). Although ATPS have many promising results in downstream processing, there are still room of improvement for this method.

In this study, a novel purification method system known as AIRS is developed to overcome all the limitations of ATPS but maintaining the advantages of it. The application of this new developed method is tested by targeting bacteriocin as the target biomolecule.

2.2 Bacteriocins: A Valuable Biomolecule in Bioprocessing Area

The target biomolecule to undergo purification in this research is bacteriocin. Bacteriocins are small, ribosomally-synthesized peptide produced by Lactic Acid Bacteria comprises the heterogenous group of physicochemically diverse peptides or protein that show narrow or broad antimicrobial activity against pathogenic bacteria but is not effective against the producer cells even at low concentration (Cintas et al., 2001; De Vuyst and Lerroy, 2007).

Even though bacteriocins have the capability to inhibit the growth or kill microorganisms, they are not categorized as antibiotics. The major difference between them is that bacteriocins have narrower and specific activity than antibiotics. Their activity is restricted to strains of species which is related to their producing strain and particularly to the strain of same species. In contrast, antibiotics

have wider activity spectrum and even though it has specific activity, it does not show any preferential effect on closely related strains (Zacharof and Lovitt, 2012).

For the past years, resistance to antibiotics issues have risen that the international organization have ruled global action plans aimed to ensure that infectious diseases being treated and prevented with safe and effective medicines (WHO, 2015). At this point, the bacteriocins which act as an alternative to antibiotics have gained interest in research area.

The reasons that bacteriocins have gained interest in research area is because their characteristics that have fast acting mechanism. Besides, due to their proteinaceous nature, the bacteriocins is easily degraded by proteolytic enzyme. United States Foods and Drug Administration (FDA) have classified LAB and its by-products as GRAS ~~as~~ in human food ingredients. Furthermore, due to high specificity of some bacteriocins against clinical pathogens, bacteriocins have shown its potential to become a viable alternative towards antibiotics (Perez et al., 2014).

In this study, the antibacterial effect of bacteriocin is tested against *Staphylococcus aureus,* a pathogenic bacterium belong to the Gram-positive Micrococcaceae family which can normally be found in our body. The carriage of *S. aureus* which is the anterior nares can be found in 20 to 30% of all humans. It can produce several toxins such as staphylococcal enterotoxins which may cause several illnesses. These pathogenic bacteria are found to be the most common cause for the skin and soft tissue infections (Bouvet et al., 2017). Skin and soft tissue infections caused by *S. aureus* usually start with minor boils or abscesses. However, if it is not treated properly, it may lead to severe infections involving muscle or bone to which may disseminate to the lung and heart valves (McCaig et al., 2006). This makes *S.*

aureus a threatening factor for health and the need for treatment is demanding. Because of this reason, researchers have found out that the antibacterial effect of bacteriocin have the potential to inhibit the growth of Staphylococcus sp. (Jiang et al., 2017).

2.2.1 Inhibitory Effect of Bacteriocins towards S*taphylococcus aureus*

There are few reports that shows positive results of inhibitory effect by bacteriocin toward *Staphylococcus aureus*. For example, in research carried out by Varella Coelho et al. (2007), it is seen that aureocin A53 and aureocin A70 (bacteriocins produced by *Staphylococcal* sp.) show positive inhibitory effect towards 87 and 30 Brazillian *Staphylococcal* strains respectively. By combining both aureocin A53 and aureocin A70, 107 Brazillian *Staphylococcal* strains were inhibited, including 20 *Staphylococcal* strains that were not inhibited by either aureocin A53 or aureocin A70 alone.

Besides that, a novel bacteriocin known as pentocin JL-1, produced by *Lactobacillus pentosus* that was isolated from the intestinal tract of *Chiloscyllium punctatum* shows inhibitory effect toward multidrug-resistant *Staphylococcus aureus*. By using concentration in range 50ng/mL to 15 µg/mL of pentocin JL-1, an inhibition zone with diameter 22 to 24 mm was observed on agar plate inoculated with 2×10^6 CFU/mL of multidrug-resistant *S. aureus* (Jiang et al., 2017).

Apart from that, bacteriocin (BAC-1B17) produced by *Bacillus subtilis* KIBGE-IB17 found to be effective against the methicillin resistant *Staphylococcus aureus*. The purified BAC-1B17 shows an arbitrary unit of 80 AU/mL and minimal inhibitory concentration of 50µg/mL against methicillin resistant *S. aureus* (Ansari et al., 2018).

In addition, Okuda et al. (2013) carried out a research to study the effect of bacteriocins on methicillin-resistant *S. aureus* biofilm. In their study, the antibacterial effect by three types of bacteriocins (nisin A, lacticin Q and nukacin ISK-1) were studied against *S. aureus* MR23 planktonic calls and *S. aureus* MR23 biofilm cells. It shows that, after 1 hour incubation as for nisin A and 4 hours incubation as for lacticin Q, the *S. aureus* MR23 planktonic cells (with concentration of $1x10^8$ CFU/mL) have completely being killed, in a way that no colony formed can be observed on agar after the incubation time. As for *S. aureus* MR23 biofilms, nisin A shows about 10x higher bactericidal activity than lacticin Q. Nukacin ISK-1 on the other hand only show bactericidal effect towards *S. aureus* MR23 planktonic cells but no bactericidal effect was observed on *S. aureus* MR23 biofilm.

2.3 Current Purification Method of Bacteriocins

Over the past decades, bacteriocins purification have been done by various purification methods. The conventional method which normally involves precipitation and chromatography have been carried out as early as 1990s.

Nevertheless, there are few disadvantages associated with conventional methods. Firstly, as these steps usually involve multistep operations (precipitation and chromatography), the major problems with this method is high costs, time consuming and may cause significant loss of the bacteriocins activity. Besides, even though chromatography can give high purity, the percentage of yield obtain using this method is low. As shown in Table 2.1, where Lactocin S, bacteriocin extracted from *Enterococcus faecalis* KT2W2G and *Weissella paramesenteroides* DFR-8 have below 5% of recovery yield (Mørtvedt et al., 1991; Pal and Ramana, 2010 and H-Kittikun et al., 2015).

On another hand, precipitation by ammonium sulfate is not suitable for low molecular weight bacteriocins especially for Class I and Class II of bacteriocin as the bacteriocin is poorly precipitated using this method. Besides, proteins may be eliminated either fully or partially when passing through the dialysis sac. Furthermore, since the culture medium is complex, the result obtained is unsatisfactory. The protein pellet will be floating even after centrifugation, causing the separation process is difficult (Guyonnet et al., 2010; Borzenkov et al., 2014). In Table 2.1 adopted from Jamaluddin et al. (2018) below shows the conventional purification method for bacteriocin.

Table 2.1: Summary of conventional methods for purification of bacteriocins (adopted from Jamaluddin et al., 2018)

No.	Methods/Purification scheme	Bacteriocins	Microbial strain	Activity yield (%)[a]	Purification fold[b]
1	Ammonium sulfate or acid (pH 2.5) precipitation, hydrophobic interaction chromatography, gel filtration, RP-HPLC	Leucocin AUAL 187	*Leuconostoc gelidum* UAL 187	58	4500
2	Ammonium sulfate precipitation, ion exchange, hydrophobic interaction, RP-HPLC, gel filtration	Lactocin S	*Lactobacillus sake* L45	3	40000
3	Ammonium sulfate precipitation, 1-butanol extraction, ion-exchange chromatography	Cerein 8A	*B. cereus* 8A	6.7	54.2
4	Ammonium sulfate precipitation, cation exchange chromatography	Pediocin PD-1	*Pediococcus damnosus* NCFB1832	34	1700
5	Ammonium sulfate precipitation, RP-HPLC	Salivacirin CRL 1328	*Lactobacillus salivarius* CRL 1328	7.3	-
6	Ammonium sulfate precipitation, gel exclusion column chromatography	Not specified	*Bacillus subtilis* R75	22.10	22.30
7	Ammonium sulfate precipitation at isoelectric point, cation exchange chromatography, RP-HPLC	Pediocin PA-1	*Pediococcus pentosaceous* NCDC 273	134.4	320
8	Ammonium sulfate precipitation, cation exchange chromatography, hydrophobic interaction chromatography	Not specified	*L. mirunus* AU06	28.92	4.74

Table 2.1 (cont.)

No.	Methods/Purification scheme	Bacteriocins	Microbial strain	Activity yield (%)[a]	Purification fold[b]
9	Ammonium sulfate precipitation, reverse phase cartridge, cation exchange chromatography, RP-HPLC	Not specified	*Enterococcus faecalis* KT2W2G	4	48.10
10	pH-mediated cell adsorption/desorption	Sakacin A Pediocin AcH Nisin Leuconocin Lcml	*Lactobacillus sake* *Pediococcus Acidilactici* *Lactococcus Lactis* *Leuconostoc comasum*	44.3 106.7 93.3 96.2	- - - -
11	Adsorption—desorption of the bifidin I onto /from silicic acid, Cation exchange chromatography, RP-HPLC	Bifidin I	*B. infantis* BCRC 14602	25.6	1390
12	pH-mediated cell adsorption—desorption method, gel permeation chromatography, RP-HPLC	Not specified	*Weissella paramesenteroides* DFR-8	4.42	177.94
13	Adsorption-desorption of the bacteriocin from culture supernatant onto Micro-Cel (diatomite calcium silicate), cation exchange chromatography	Mesentericin W3	*Leuconostoc mesenteroides*	64	-
14	pH mediated adsorption and desorption, SP-Sepharose Fast flow cation exchange column, GF Sephadex G10, RP-HPLC	Bifidocin A	*Bifidobacterium animals*	7	115

Table 2.1 (cont.)

No.	Methods/Purification scheme	Bacteriocins	Microbial strain	Activity yield (%)[a]	Purification fold[b]
15	Cation-exchange chromatography, hydrophobic interaction chromatography, HPLC	Sakacin A Sakacin P Enterocin A Pediocin PA-1	*L. sakei* 2675 *L. sakei* 2525 *E. faecalis* 336 *P. acidilactici* 1521	10 50 66 25	- - - -
16	Cation exchange chromatography—pressure reverse column	Pediocin PA-1	*Pediococcus acidilactici* LMG2351	80	-
17	Cation exchange chromatography, reverse phase column R1, RPHPLC	Enterocins A5-11A Enterocins A5-11B	*Enterococcus durans*	16 64	1650 2250
18	Hydrophobic interaction chromatography, elution of nisin with water or PBS	Nisin	*Lactococcus Lactis*	Water: 284.88 PBS:152.02	Water:724 PBS: 384
19	Immunoaffinity chromatography	Nisin A	*Lactococcus lactis* BB24	72.7	10
20	Immunoaffinity chromatography	Enterocin B	*Enterococcus faecium* BFE 900	25	10.7
21	Immunoaffinity chromatography	Enterocin P	*Enterococcus faecium* P13	1.1-5.9	-
22	Ultrafiltration	Lactacin F	*Lactobacillus acidophilus*	100	-
23	Microfiltration, diafiltration, nanofiltration	Pediocin PA-1	*Pediococcus acidilactici* 003	71.6	4.5

[a]Activity yield: The percentage of the activity remaining on the recovered bacteriocin compared with the initial bacteriocin activity.
[b]Purification factor: Calculation of how many times the specific activity of bacteriocin increased after each purification step as compared with the initial bacteriocin specifc activity.

Although the conventional methods are well known for their satisfactory result, alternate method is in high demand considering the disadvantages caused by the conventional method as mentioned previously. For the past few years, researchers show high interest on a more simple and convenient purification step. This includes the simplicity of the process, less equipment used, cost and time saving with an acceptable range of purification factor and yield. One of the ways to achieve this target is by eliminating the complex chromatography technique.

Few alternative purification methods include expanded bed adsorption, a macroporous monolith, aqueous two-phase extraction, and an aqueous micellar two-phase system. These alternate methods prove that even though the purification system is easy to operate and less equipments needed, these purification systems can still give high purity and recovery yield of bacteriocin. In Table 2.2 below, Jamaluddin et al. (2018) summarize the alternate purification method for bacteriocin that have been reported.

Table 2.2: The alternate method of bacteriocin purification (adapted from Jamaluddin et al., 2018).

No.	Methods/Purification scheme	Bacteriocins	Microbial strain	Activity yield (%)	Purification fold
1	Cation exchanger - based EBA EBA system (Streamline SPTM)	Amylovorin L471 Pediocin Enterocin A	*Lactobacillus amylovoros* DCE 471 *Pediococcus acidilactici* ATCC 8042 *Enterococcus faecium* CTC 492	30 26 15	- - -
2	Phenyl—pAAm monolith	Sakacin P	*Lactobacillus sakei* CCUG 42687	80	150-160
3	ATPS by PEG, ammonium sulfate ATPS by PEG, ammonium sulfate 1M NaCl	Cerein 8A	*B. cereus* 8A	81.7 65.3	0.96 0.81
4	Aqueous two-phase system (ATPS) consisting of PEG with sodium citrate	Bacteriocin-like inhibitory substance (BLIS)	*Pediococcus acidilactici* Kp10	81.18	8.43

Among the alternative methods reported, bacteriocin-like inhibitory substances purified by ATPS shows a promising result. With 80% of recovery yield and 8.43 purification fold, purification of bacteriocins by ATPS shows a great purification strategy. In ATPS, the incompatibility of the two aqueous solutions causes the separation between them. The two aqueous solutions can either be two polymers or a polymer and salt at ionic strength (Asenjo and Andrews, 2011).

To overcome the problems associated with the conventional ATPS, instead of having a mixture of 2 aqueous phases that needs time to settling down, one of the phases (normally the PEG which acts as the hydrophobic aqueous solution) is being impregnated into resins. This PEG impregnated resins will be added into salt aqueous phase containing NaCl and crude bacteriocin. By controlling the salting-out effect of the salt, the bacteriocin may attached to the PEG-impregnated resins due to hydrophobic interaction (the same mechanisms observed in conventional ATPS). To recover the bacteriocins from the PEG-impregnated resins, the resins will be suspended into salt aqueous solution without NaCl for desorption (Figure 2.1).

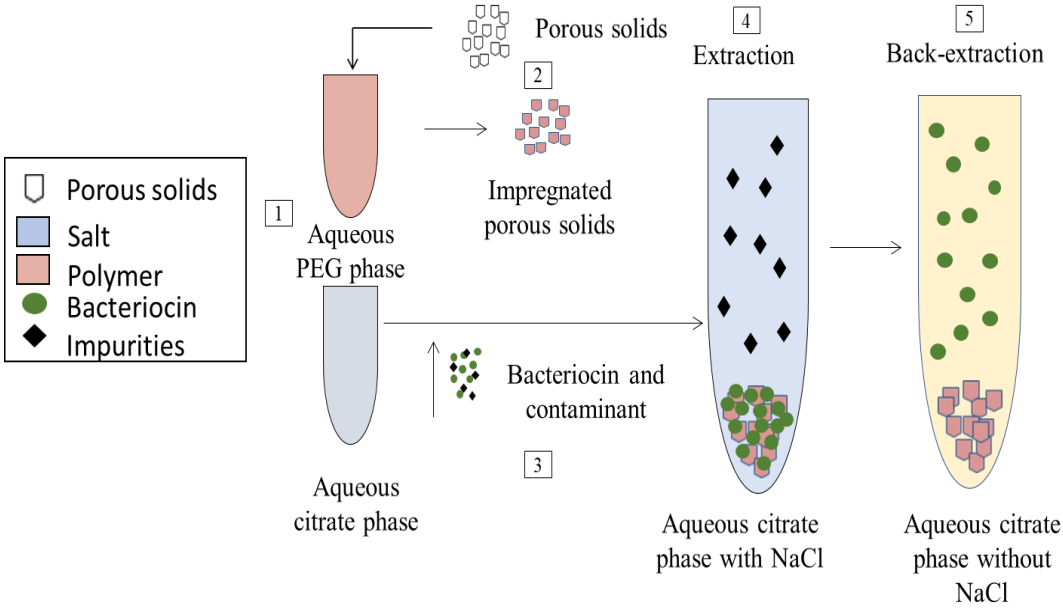

Figure 2.1: Illustration of the AIRS. 1) The preparation of aqueous PEG phase and aqueous citrate phase according to desired concentration. 2) The impregnation of porous solid into the aqueous PEG phase via dry impregnation method. 3) Crude bacteriocin containing contaminants was mixed into the aqueous citrate phase containing NaCl. 4) Extraction of bacteriocin into the PEG-impregnated resins. 5)Back-extraction of bacteriocin using aqueous citrate phase without NaCl.

An almost similar purification technique using AIRS mechanisms has been reported, known as Tunable Aqueous Polymer Phase Impregnated Resins (TAPPIR) carried out by van Winssen et al. (2014). However, in TAPPIR, the technology is still relying on ATPS mechanism, at which the impregnation of one phase into the resins occur after equilibration of the two phases. By this way, the technology still requires the long separation time for the mixture to form two phases. In addition, this limits the concentration range for the two phases since the total weight of the system still depends on each other to get the formation of two phases. In contrast, AIRS which do not depend between the two phases may have wider concentration range as the two phases can have their own concentration, provided the impregnated polymer will not leached.

2.3.1 Impregnation Stability of PEG in Resin

The stability of the impregnated PEG in the resins is very crucial in AIRS. This is because, the leaching of PEG will reduce the efficacy of bacteriocin partitioning in AIRS. Kaplanow et al. (2018) carried out a research on the impregnation stability of polymer in TAPPIR. In their research, the effect of temperature on impregnation stability was studied. Three temperatures were set (293.15, 298.15 and 303.15K), and the volumetric leaching factor were calculated. It is shown that not more that 2% of leached PEG volume reported, regardless the temperature. This indicates that the temperature does not affect the impregnation stability of PEG in the resins.

On other hand, pH value of salt may affect the impregnation stability at which the PEG tends to leach at higher pH (pH above 8.0). according to Kaplanow et al. (2018), high pH will increase the miscibility gap between the two phases and change the protonation state of the salt. Increasing the salt ions will increase free water molecule that solubilizes the PEG, which further led to PEG-rich phase got too hydrophobic and salt phase was displaced.

2.3.2 Factors Affecting the Purification of Biological Product in AIRS

Since the development of AIRS is still new and scarce, the factors affecting the purification of biological product in AIRS is not fully understood yet. Tan et al. (2018) have reported the purification of lipase from *Escherichia coli* BL21 by using AIRS and discussed about the parameters affecting lipase purification by AIRS. the parameters are discussed in the following sections.

2.3.2(a) Effect of Polymer Molecular Weight and Concentration in AIRS

In AIRS, the purification of target biomolecule can occur due to the hydrophobic interaction between the impregnated PEG and the hydrophobic area of

protein. By varying the molecular weight and concentration of polymer, the number of polymer-protein interaction can be altered and the optimum polymer molecular weight and concentration for the bacteriocin purification can be achieved (Asenjo and Andrews, 2011).

2.3.2(b) Effect of Ionic Strength of Salt in AIRS

The ionic strength in salt influences the purification of target biomolecule in AIRS in a way that it affects the protein-ligand interaction as well as the protein retention. As the salt concentration increases, the water molecules will recede from protein's surface and thehydrophobic surfaces of protein will be exposed to hydrophobic ligands. However, too high salt concentration will destabilize the protein structure by twisting the capsid protein. The capsid protein is the shell of viruses which enclose its genetic materials. Changes in ionic strength in high salt concentration may induce the conformational changes in capsid protein and sestabilize them (Tan et al., 2018).

2.3.2(c) Effect of pH of Salt in AIRS

To promote selective separation in AIRS, the pH of salt can be manipulated. This is because pH of the system may alter the charge and surface properties of the solute. Increasing the pH value above the isoelectric point of target biomolecule may induce additional affinity towards the polymer stationary phase (Glyk et al., 2017).

2.3.2(d) Effect of Resins' Characteristics in AIRS

In AIRS, the resins chosen is important as it will impregnate the polymer for extraction process. The characteristics of resins that may give different purification behavior of target biomolecules in AIRS include the size and surface area of the

resins, the structure of the resins as it will influence the interaction with the target molecule (Shuang et al., 2015) and the pore size of the resins as it may control the impregnation stability of the polymer. All these characteristics need to take into consideration as it may control the purification performance of the target biomolecule in AIRS.

2.3.2(e) Effect of NaCl Concentration in AIRS

As NaCl is used in AIRS to induce salting-out effect, the effect of NaCl in AIRS should be studied. The importance of NaCl in AIRS is to control the salting-out effect in salt extraction solution so the target biomolecules can be bind to the stationary PEG-rich phase. When the same concentration of extraction of salt is used during the desorption, the target biomolecule is move to the salt-rich phase, indicating that NaCl plays a major role in inducing salting-out effect to control the extraction and desorption phenomenon.

2.3.3 Adsorption Kinetic of Protein

In AIRS, adsorption kinetic analysis is also an important analysis besides all of the parameters aforementioned to know more details about its performance and mechanism. By analyzing the adsorption kinetics, information on adsorption capacity, kinetic performance of adsorbent and the solute uptake rate may be established (Qiu et al., 2009). In general, the modeled adsorption isotherm is an invaluable non-linear curve describing the adsorption phenomenon at a constant temperature (Foo and Hameed, 2010). There are many adsorption isotherm theories have been developed and studied. Among these theories, Langmuir and Freundlich isotherm are commonly known and used in adsorption kinetic studies.

Langmuir adsorption isotherm was primarily designed to quantify and contrast the adsorptive capacity of various adsorbents. According to Langmuir’s monolayer-

based theory, the adsorption phenomenon was understood such that the density of a fluid at an interface decreases gradually toward the bulk. It accounts for the adsorbent surface coverage by balancing the relative rates of adsorption and desorption on adsorbent. The general equation of Langmuir model can be written as:

$$Q_{eq} = bQ.C_{eq}/(1 + bC_{eq})$$

Where C_{eq} is the concentration of material in solution at equilibrium (mg/L), Q_{eq} is the amount of material adsorbed per unit weight of sorbent at equilibrium (mg/g), Q is the maximum sorbate uptake under the given conditions (mg/g) and b is the coefficient related to affinity between sorbent and sorbate (L/mg) (Ayawei et al., 2017).

Besides Langmuir adsorption isotherm, Freundlich adsorption isotherm is also commonly used to evaluate the adsorption phenomenon. The Freundlich isotherm describes the non-ideal and reversible adsorption. It can be applied to multilayer adsorption. The non-linear Freundlich isotherm model can be written as:

$$Q_e = K_f C_e^{1/n}$$

where K_f is the adsorption capacity and n is the adsorption capacity constant.

For the determination of the best-fitting adsorption isotherm to the experimental data, the correlation coefficient (R^2) was used. The equation for R^2 is as follows:

$$R^2 = \frac{\sum(q_m - q_{e(ave)})^2}{\sum(q_m - q_{e(ave)})^2 + \sum(q_m - q_e)^2}$$

where q_m is the constant obtained from the isotherm model, q_e is the equilibrium capacity obtained from experimental data and $q_{e(ave)}$ is the average of q_e (Chen, 2015).

The adsorption phenomenon is said to be unfavourable according to Langmuir adsorption isotherm when the R^2 value is more than 1, linear when R^2 value equals to 1 and favourable when R^2 value is in between 0 to 1. The closer the R^2 value to 1, the more favourable for the adsorption isotherm to be applied.

CHAPTER 3

SALT-INDUCED EXTRACTION OF POLYETHYLENE GLYCOL IMPREGNATED RESIN SYSTEM FOR RECOVERY OF A BIOLOGICAL PRODUCT, BOVINE SERUM ALBUMIN.

3.1 Introduction

Biological products such as proteins, nucleic acids, microorganisms, animals and plant cells have extensively been widely used in food, pharmaceutical and other different industries. While the upstream processing in producing these biological products are developing widely, the downstream processing for the productions of these biological products always lead to the production bottleneck. There are four stages in the downstream processing of biomolecules such as recovery, isolation, purification and polishing (Raja Murty et al., 2011). Researchers have developed numerous purification strategies in order to obtain high yield of products. However, the production costs for biological products can be up to 80% (Rosa et al., 2009). Alternatively, these problems can be overcome by introducing a cost-effective purification strategy known as aqueous two-phase system (ATPS**)** which implies the concept of hydrophobicity in the first aqueous phase and hydrophilicity of the salt aqueous solution**.**

ATPS is a promising purification method which is easy to control, because low toxicity of phase forming chemicals and biocompatibility making it suitable to be used with ionic environment sensitive solutes such as nonionic surfactants at lower costs than the conventional chromatography method (Iqbal et al., 2016). However, long settling times in ATPS which could be overcome by centrifugation consume high energy which is the drawback of this method. This strictly limits the development of biomanufacturing process especially in purification of valuable bioproducts. Furthermore, poor understanding of the conventional ATPS give rise to the problem

of optimizing and sorting out the mechanism of the biomolecules partitioning in ATPS (Grilo et al., 2014).

The drawbacks in ATPS could be defeated or prevailed by combination of ATPS with impregnated resin principle which was reported in our previous study by Tan et al (2018). In aqueous PEG impregnated resin system, an aqueous polymer phase is impregnated into the solid materials while the aqueous salt phase represents the surrounding bulk phase. This will create the mass transfer between the two phases. Systemic and detailed information on this new extraction behavior such as impregnation stability and adsorption kinetics needs to be studied to make this system to extract more types of protein.

The aim of this study was to evaluate the extraction behaviors of aqueous PEG impregnated resin system in terms of impregnation stability and recovery via protein impregnated resin interactions on BSA as exemplary target product. This method implies the concept of hydrophobicity of polymer that being impregnated into solid materials and the hydrophilicity of sodium citrate as salt aqueous solution to extract biomolecule. Furthermore, the influencing factors such as molecular weight and concentration of PEG, pH and concentration of sodium citrate, on BSA partitioning were examined and the final recovery yield was evaluated. The information gathered from the study was used to validate the competency of the system in purification of biomolecules.

3.2 Materials and Methodology

3.2.1 Materials

Polyethylene glycols with different average molecular weights of 2000 g/mol, 4000 g/mol, 6000 g/mol and 8000 g/mol were purchased from Merck (City, USA). Potassium citrate was obtained from HmbG (City, Germany). Sodium chloride, bovine serum albumin and Amberlite XAD4 were sourced from Sigma Aldrich (City, USA). The porous VitraPor glass pellets with two different particle sizes of 4.0 mm 8.00 mm were supplied from ROBU Glassfilter-Gerate GmbH (City, Germany). PageBlue protein staining solution, silver staining kits and protein loading buffer were all from Fermentas (St. Leon-Rot, Germany).

3.2.2 Methodology

3.2.2(a) Experimental Design

Two different aqueous solutions, PEG solution and sodium citrate solution were prepared as described by Tan et al., (2018). The PEG and sodium citrate aqueous solution were chosen as the base of this separation system because these aqueous solutions are nontoxic, inflammable and inexpensive. Dry impregnation method was used to impregnate PEGs to resins. Briefly, the PEG solution was added drop by drop into the resins until all the resins were fully impregnated with PEG. A total of 0.4 g of 40% (w/w) of PEG was needed to fully impregnate 2 g of VitraPor glass pellets or 0.3 g of Amberlite XAD4. The Amberlite XAD4 was chosen due to its good physical and chemical properties such as porosity, high surface area, high durability and high purity (Berdous and Akretche, 2013). The impregnated resins were added into 2.5 g of bulk aqueous salt aqueous salt solution containing 10% (w/w) of sodium citrate, 4% (w/w) of NaCl and 1% (w/w) of BSA and agitated at 150 rpm, incubated at 30˚C for 30 min to enhance the mass transfer rate. For back extraction, the impregnated porous solids containing the BSA were filtered out from the citrate solution using a sieve. The excess salt solutions on the solids surface were dried and were suspended into aqueous