Transforming industrial waste to a source of natural antimicrobials followed by semi-preparative purification to facilitate industrial applications

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Abstract

Natural antimicrobial peptides, produced by beneficial bacteria, are urgently needed to combat harmful microorganisms and fulfill consumer's preference of clean label. However, the commercial implementation of natural antimicrobial peptides is hampered by the cost and the composition of the production medium. Therefore, finding low cost production media will facilitate their industrial application. The study described here investigated the production of antimicrobial peptides in dairy acid whey using novel food-grade lactic acid bacteria (LAB). *Enterococcus durans* OSY-EGY, isolated from artisanal cheeses, and *E. faecalis* OSY-RM6, isolated from raw cow's milk, were found to successfully produce their enterocins EGY and RM6 respectively in the ferment acid whey. Additionally, to optimize enterocins EGY and RM6 production in the fermented whey, the effect of incubation time (24, 48 and 72 hours), growth temperature (30ºC and 37ºC), initial pH (4.3, 6.0, 6.5 and 7.0), the chemical composition of the whey (adding 1% yeast extract, 1% lactose or both) and the inoculation volume of the bacterial culture (1% and 2%) were explored. Finally, to facilitate further industrial application, the produced enterocins were semi purified and concentrated from the fermented whey using a new simple purification protocol using resin beads followed by ultrafiltration and freeze drying. The concentrations of EGY and RM6 in the resulted active antimicrobial powders as two final products were 512,000 AU/g and 128,000 AU/g respectively. To our knowledge, this is the first report of antimicrobial powder derived from fermented acid whey.

Key Words: Acid whey, antimicrobial peptides, fermentation, *E. durans*, *E. faecalis*

Introduction

The substantial economic burden associated with microbial diseases and the spoilage of perishable commodities can be mitigated by using natural, effective and industrially usable antimicrobials (Saeed et al., 2019). The quest for alternatives to currently used antimicrobials is urged by health professionals considering the rapid rise in resistance to preservatives and antibiotics among pathogens (Fair and Tor, 2014). Additionally, customers are increasingly avoiding products containing synthetic antimicrobials and prefer products with clean labels (Asioli et al., 2017). Driven by these urgencies, researchers are prompted to search for potent and natural antimicrobials to replace the synthetic ones and combat the emerging resistant microorganisms. For centuries, the beneficial lactic acid bacteria (LAB) have been used safely to make fermented foods with long shelf life, facilitated by the production of lactic acid and antimicrobial peptides (Hammes and Tichaczek, 1994). These natural antimicrobial peptides are considered a promising alternative to synthetic and less effective antimicrobials. The commercial implementation of the scarcely produced natural antimicrobial peptides is challenging as it requires large-scale production and purification hampered by the cost and the composition of the fermentable medium (Wibowo and Zhao, 2019). Finding low cost production media will facilitate the industrial application of these natural antimicrobials. Cheese whey is an industrial by-product obtained after the precipitation of milk casein during cheese making (Lievore et al., 2015). Worldwide, 40 million tons of cheese whey are produced every year which creates significant environmental pollution source and expensive disposal problem (Prazeres et al., 2012). Reutilizing cheese whey to produce value-added products, such as natural antimicrobials, could provide multiple economic solutions to the industry through decreasing the cost of natural antimicrobials production and the cost of cheese whey disposal. Therefore, it was selected as a cheap fermentable medium to produce the natural

antimicrobial peptides using bacteriocin-producing LAB discovered previously in our lab. The objectives of the current study were: (1) investigating the feasibility of producing antimicrobial peptides in cheese whey using selected LAB strains, (2) exploring the optimal conditions to maximize the antimicrobial peptides production, and (3) scaling up the production and developing a semi-preparative purification protocol to facilitate the industrial application.

Materials and Methods

Bacterial strains and growth conditions

The food-grade LAB strains, *E. durans* OSY-EGY, *E. faecalis* OSY-RM6, *Lactobacillus paraplantarum* OSY-TC318, were previously isolated from Egyptian artisanal cheese, raw cow's milk, and Turkish artisanal cheese, respectively (Huang et al., 2013; Hussein et al., 2019b, 2019a). A nisin-producer *Lactococcus lactis* ATCC 11454 strain was used as a positive control. The four total bacteriocin-producing LAB strains were streaked on de Man, Rogosa and Sharpe (MRS) agar, and their overnight subcultures in MRS broth were mixed in 80% sterile glycerol in a 1:1 ratio and stocked at -80°C in the food microbiology laboratory culture collection, The Ohio State University (OSU; Columbus, OH, USA). *Pediococcus pentosaceus* strain, cultivated in MRS broth and grown overnight at 30 °C, was used to measure the concentrations of the produced antimicrobial agents in the fermented whey. Selected bacterial strains (Table 2) were cultivated in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD) and potato dextrose agar (PDA) (Oxoid, Thermo Scientific, Waltham, MA, USA) to determine the minimum inhibitory concentration (MIC) and the antimicrobial spectrum of the final antimicrobial whey powder.

Antimicrobial peptides production in dairy acid whey

Cottage cheese whey (Superior Dairy, Canton, OH) was neutralized from pH 4.3 to pH 6.5 using 5M NaoH (Fisher scientific) and pasteurized at 65°C for 30 min. 1% (v/v) overnight culture of *E. durans* OSY-EGY, *E. faecalis* OSY-RM6, *L. paraplantarum* OSY-TC318, or *Lactococcus lactis* ATCC 11454 were inoculated in to 10 ml neutralized cheese whey followed by incubation at 30°C for 24-48 hr. After incubation, the antimicrobial activity was determined using spot-on-lawn method (He et al., 2007). Briefly, aliquots (10 µL) of *Pediococcus pentosaceus* overnight culture, as indicator bacterium, were transferred into 10 mL of sterile soft molten MRS agar, fortified with 0.75% agar (Fisher Scientific, St. Louis, MO, USA). The mixture was poured onto a basal MRS agar plate. After the soft agar solidified, 10 µl of fermented cheese whey were spotted on the top of the indicator lawn. After incubation at 30°C overnight, clear inhibitory zones were observed.

Maximizing the antimicrobial peptides production in cheese whey

To maximize the antimicrobial peptides production in cheese whey, the optimal conditions of cheese whey fermentation using *E. durans* OSY-EGY and *E. faecalis* OSY-RM6 were investigated as follows. The effect of initial cheese whey pH (4.3, 6.0, 6.5 and 7.0), fermentation time (24, 48 and 72 hours), fermentation temperature (30ºC and 37ºC), the chemical composition of the cheese whey (adding 1% yeast extract (w/v), 1% lactose (w/v), or both), and the bacterial culture inoculation volume (1% (v/v) and 2% (v/v)) were explored (Table 1) followed by measuring the produced antimicrobial peptides concentrations using spot on lawn method. Antimicrobial activity was expressed in arbitrary units (AU)/ml calculated as the reciprocal of the highest dilution displaying a clear zone of *P. pentosaceus* growth inhibition multiplied by 100 (Figure 1). All experiments were done in triplicate.

Preparation of active antimicrobial powder extracted from fermented cheese whey

A- Scaling up the production antimicrobial peptides production in cheese whey

Cheese whey fermentation using *E. durans* OSY-EGY and *E. faecalis* OSY-RM6 was scaled up from 10 ml to 4 L. 4 L cheese whey, divided in to two flasks (two liters each), were neutralized from pH 4.3 to pH 6.5 using 5M NaoH, fortified with 1% yeast extract for EGY production using OSY-EGY and 1% lactose for RM6 production using OSY-RM6. The fortified whey was pasteurized at 65°C for 30 min. The pasteurized whey was inoculated with 1% overnight culture of OSY-EGY strain and incubated at 30°C for 48 hr or 1% overnight culture of OSY-RM6 strain and incubated at 30°C for 24 hr. After incubation, the concentrations of the produced antimicrobial peptides in the fermented cheese whey against *P. pentosaceus* were measured as previously described.

B- Semi-preparative purification of antimicrobial peptides from cheese whey

To facilitate further industrial application, two-step purification protocol was developed to purify the produced antimicrobial peptides from the fermented whey. The first step was adding 20% w/v XAD16N resin beads (amberlite; Sigma Aldrich, St. Louis, MO, USA) to the fermented active whey. The whey and beads mixtures were shaken at 150 RPM in a shaking incubator (New Brunswick Scientific, Edison, NJ) at 25°C for 24 hr to allow the binding of the antimicrobial peptides with the resin beads. The resin beads were collected a way from the whey and washed with deionized water until the flow through water became colorless followed by washing with 45% ethanol. The antimicrobial peptides were eluted from the resin beads using equal volume of 75% 2-propanol+0.1% TFA followed by 90 % 2-propanol+0.1% TFA. All washing and elution steps were conducted at 150 RPM in a shaking incubator at 25°C for 24 hr. 2-propanol was removed from the resulted antimicrobial extract using a vacuum concentrator (Speed-Vac;

Savant SPD131DDA, Thermo scientific, USA). The purified antimicrobial extract was further concentrated through ultrafiltration using 3,000 Da MW cutoff Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA) at 4°C. The concentrated active retinate was later dried using freeze drier (Labconco, Kansas City, MO) to obtain the final active antimicrobial powder. The concentrations of the purified antimicrobial peptides in the powder were measured against *P. pentosaceus* as previously described after dissolving 1% powder (w/v) in sterile deionized water. To investigate the antimicrobial spectrum of the final whey powder product, its MIC against selected Gram-positive and Gram-negative bacteria was determined (Table 2). MIC represented as milligrams of antimicrobial powder per ml of final product required to result in a visible inhibition of growth when grown on soft agar using spot on lawn method.

Results and discussion

In addition to their roles in food fermentation and probiotic characteristics, lactic acid bacteria (LAB) are known for their ability to produce antimicrobial peptides called bacteriocins (De Vuyst and Leroy, 2007; Zacharof and Lovitt, 2012). Bacteriocins produced by generally recognized as safe (GRAS) LAB has shown to be efficient in combating bacterial food borne pathogen and food spoilage bacteria (And and Hoover, 2003). This antibacterial effect mechanism could be due to formation of cell membrane pores, inhibition of DNA synthesis , or inhibition of peptidoglycan synthesis (Bruno and Montville, 1993). The concentration of the produce bacteriocin depends on many factors including the bacterial strain and the culturing conditions (Mokoena, 2017).

Potent antimicrobial activity of the LAB strains, *E. durans* OSY-EGY, *E. faecalis* OSY-RM6, and *Lactobacillus paraplantarum* OSY-TC318 against several pathogenic bacteria including *Listeria monocytogenes*, *Staphylococcus aureus*, *E. faecalis*, and *Bacillus cereus* has been previously reported (Huang et al., 2013; Hussein et al., 2019b, 2019a). To investigate the feasibility of

utilizing dairy acid whey as a cheap medium for antimicrobial peptides production, cheese whey was fermented using these bacteriocinogenic LAB strains. Among the tested antimicrobials-producing strains, only OSY-EGY and OSY-RM6 strains were found to ferment neutralized cheese whey and successfully produce their natural antimicrobial peptides. In previous studies, OSY-EGY and OSY-RM6 were found to produce potent and stable antimicrobial peptides, designated enterocins RM6 (Huang et al., 2013) and EGY (Hussein et al., 2019b), by fermenting microbiological media and milk which inhibit antimicrobial-resistant pathogenic microorganism. Through cheese whey fermentation at 30°C, EGY was found to be produced at 100 AU/ml after fermentation for 48hr and RM6 was found to be produced at 400 AU/ml after 24 hr (Figure 2). Due to the potent antimicrobial activity of the fermented whey, *E. durans* OSY-EGY and *E. faecalis* were selected for further investigation. The antimicrobial peptides EGY and RM6 production in cheese whey was maximized by exploring optimal conditions of whey fermentation including time, temperature, pH, chemical position, and inoculation volume. The production was found to be influenced significantly by the chemical position of cheese whey (Table 1). The optimal conditions needed to increase EGY concentration in the whey from 100 AU/ml to 1600 AU/ml (Figure 2) were found to be: (i) supplementing the whey with 1% yeast extract, (ii) adjusting the whey-based medium to pH 6.5, and (iii) incubation at 30ºC for 48 hours. For RM6, the optimal conditions to increase its concentration from 400 AU/ml to 800 AU/ml (Figure 2) were found to be: (i) supplementing the whey with 1% lactose, (ii) adjusting the whey-based medium to pH 6.5, and (iii) incubation at 30ºC for 24 hours. We next explored the feasibility of scaling up the antimicrobial peptides production from 15 ml test tubes to 2 L flasks. The antimicrobial peptides produced in 4 L fermented whey for OSY-EGY and OSY-RM6 were 1600 AU/ml and 800 AU/ml, respectively which are the same concentrations obtained from fermenting 10 ml whey.

This result indicated that possibility of scaling up EGY and RM6 production. Finally, to facilitate further industrial application, the produced antimicrobial-rich fermented whey was further enriched through purification, concentration, and drying. The concentrations of the antimicrobial peptides, EGY and RM6 in the resulted active antimicrobial powders as two final products were 512,000 AU/g (Figure 3) and 128,000 AU/g (Figure 4), respectively. The antimicrobial spectrum of OSY-EGY and OSY-RM6 whey powder showed potent activity against Gram-positive and Gram-negative and spoilage bacteria (Table 2). As the inexpensive cheese whey was efficiently used as a production medium for natural antimicrobial peptides, the discussed method and final product address these challenges of their industrial production and application through reutilizing a low-cost processing waste, dairy acid whey using novel lactic acid bacteria. To our knowledge, this is the first report of antimicrobial powder derived from fermented acid whey. The final products can be used in the food and cosmetic industries as safety and shelf life enhancers.

Conclusion

In conclusion, the study described here investigated the potent antimicrobial peptides EGY and RM6 production in inexpensive dairy acid whey using two novel food-grade LAB. We explored the optimal conditions of cheese whey fermentation, using *E. durans* OSY-EGY and *E. faecalis* OSY-RM6, including time, temperature, pH, and medium composition to maximize the production. Compared to conventional microbiological media, cheese whey was found to be a cleaner medium that facilitated and simplified the purification and increased the yield of the antimicrobial peptides in the final antimicrobial powder. The developed method and final products could provide multiple economic solutions to the food industry by decreasing the cost of producing natural antimicrobials and by making it possible to utilize a product that needed costly disposal.

Additionally, it can facilitate the industrial application of natural antimicrobial peptides in the form of semi-purified active powder useful for medical, pharmaceutical and food applications.

Table 1. the investigated conditions to optimize the antimicrobial peptides production in the whey

Investigated Factor	**Fermentation conditions**	**EGY**	**RM6**
pH	pH 6.0+ incubation at 30°C for 24 hr	-/+	400
	pH 6.5+ incubation at 30°C for 24 hr	-/+	400
	pH 7.0+ incubation at 30°C for 24 hr	-/+	100
Time/ temperature	pH 6.5 + incubation at 30°C for 24 hr	-/+	400
	pH 6.5 + incubation at 30°C for 48 hr	100	200
	pH 6.5 + incubation at 30°C for 72 hr	100	-/+
	pH 6.5 +incubation at 37°C for 24 hr	-	400
	pH 6.5 +incubation at 37°C for 48 hr	-	200
	pH 6.5 +incubation at 37°C for 72 hr	-	-/+
Medium composition	pH 6.5 + incubation at 30°C for 24 hr + 1% yeast extract	800	400
	pH 6.5 + incubation at 30°C for 48 hr + 1% yeast extract	**1600**	200
	pH 6.5 + incubation at 30°C for 24 hr + 1% lactose	-/+	**800**
	pH 6.5 + incubation at 30°C for 48 hr + 1% lactose	100	400
	pH 6.5 + incubation at 30°C for 24 hr + 1% yeast extract+ 1% lactose	800	800
	pH 6.5 + incubation at 30°C for 48 hr + 1% yeast extract+ 1% lactose	1600	400

(-/+): Very weak activity (-): No activity

Table 2. Minimum inhibitory concentration (MIC) is represented as milligrams of antimicrobial powder per ml of final product required to inhibit each of the bacteria listed when grown on soft agar.

Bacteria	**Strain**	**OSY-EGY mg powder/ml product**	**OSY-RM6 mg powder/ml product**
Pediococcus pentosaceus	OSU	0.155	0.156
Listeria innocua	33090	0.625	5
Staphylococcus aureus	6538	2.5	2.5
Escherichia coli	8739	NA	NA
Candida albicans	SC5314	NA	NA
Candida albicans	10231	NA	NA
Aspergillus brasilensis	16404	NA	NA
Pseudomonas aeruginosa	9027	NA	100
Burkholderia cepacia	25416	100	NA
Klebsiella pneumoniae		NA	100
Enterobacter gergoviae		NA	100
Serratia marcescens		NA	NA

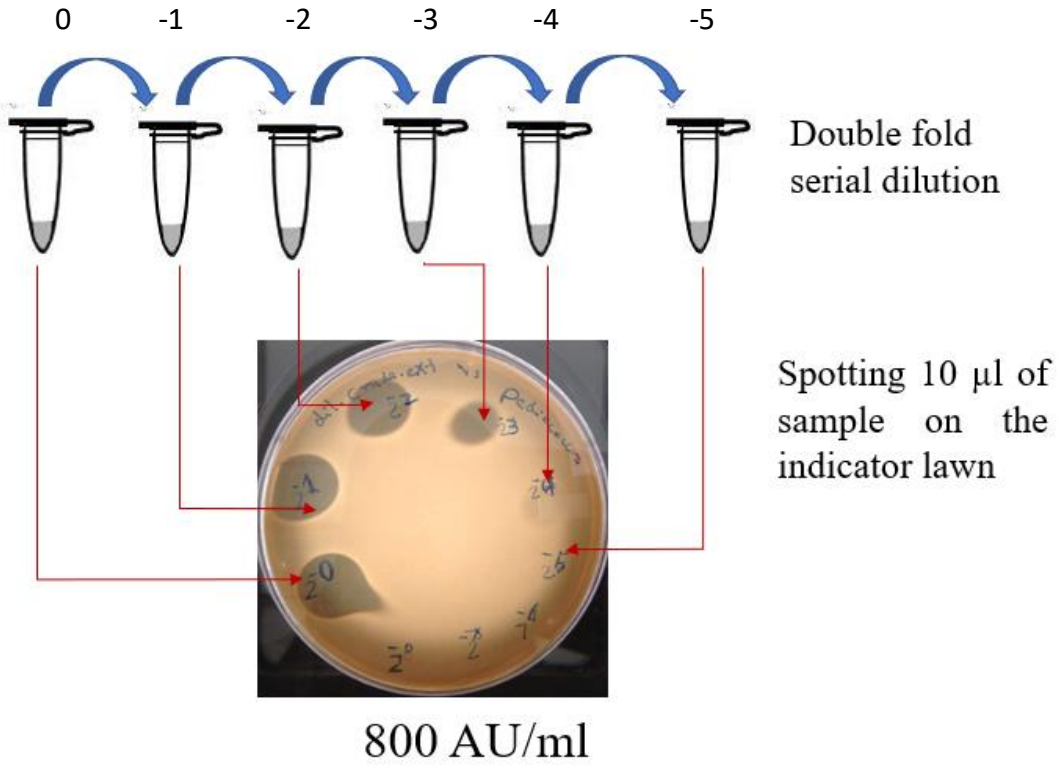

Figure 1: Measuring the antimicrobial activity using spot on lawn method

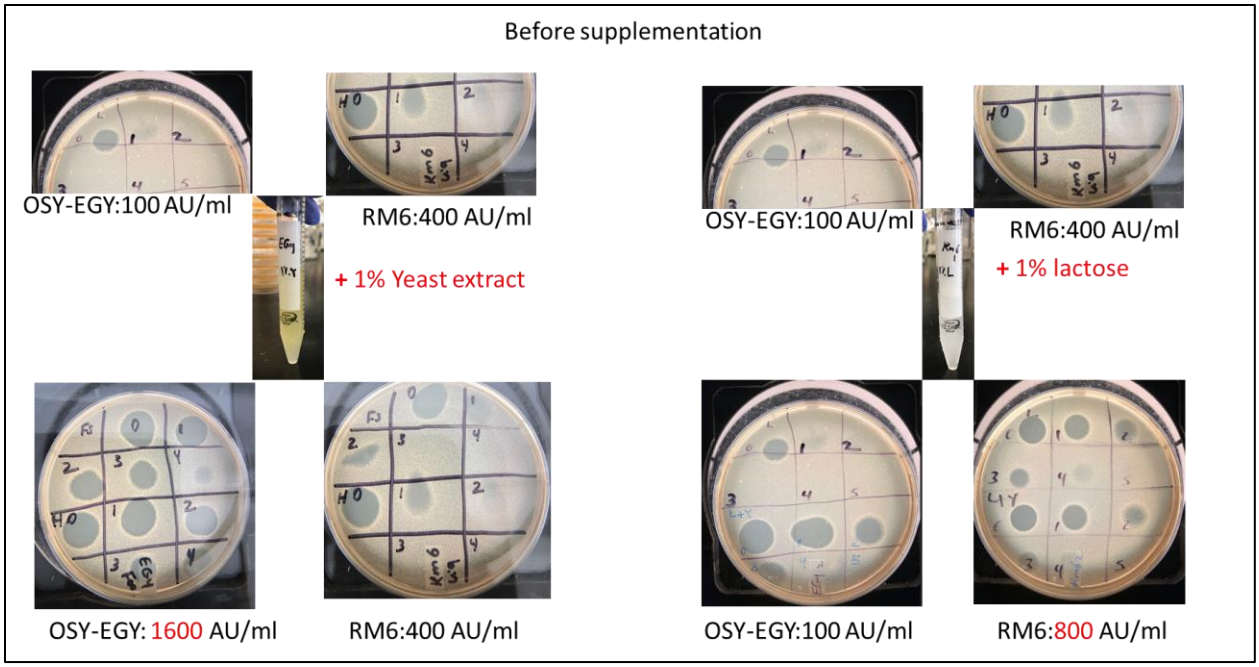

Figure 2: Maximizing the production of EGY and RM6 in cheese whey

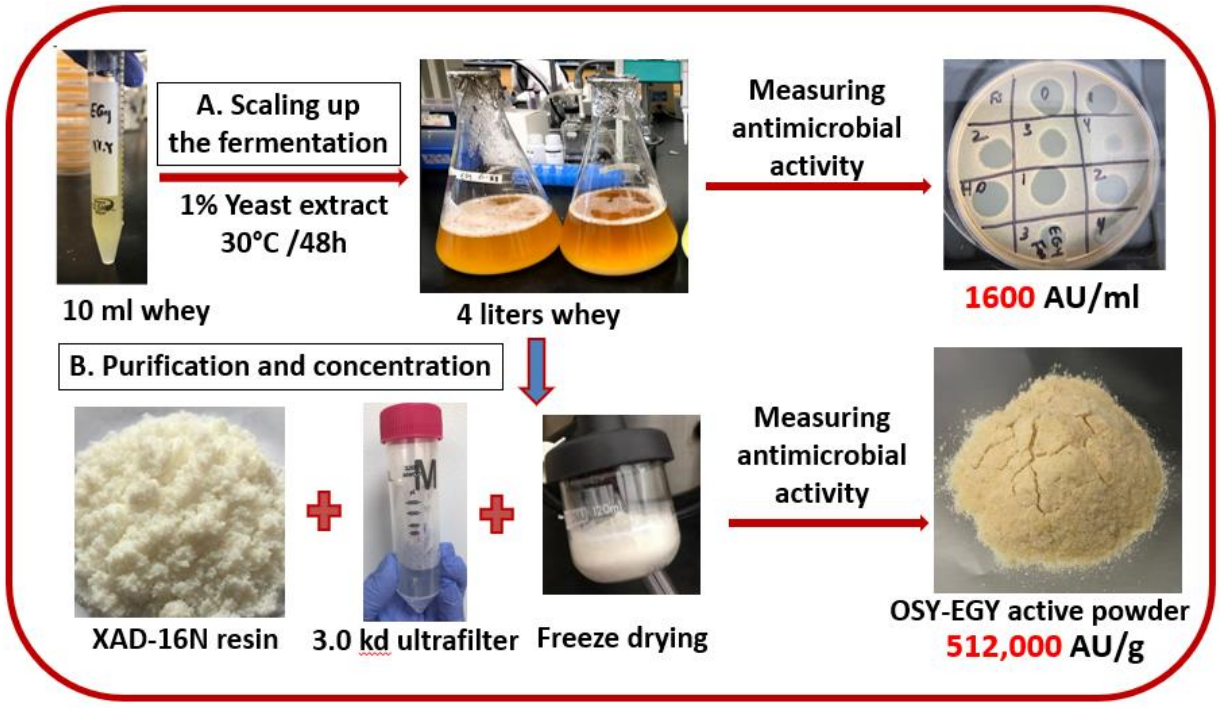

Figure 3. Producing OSY-EGY active antimicrobial powder

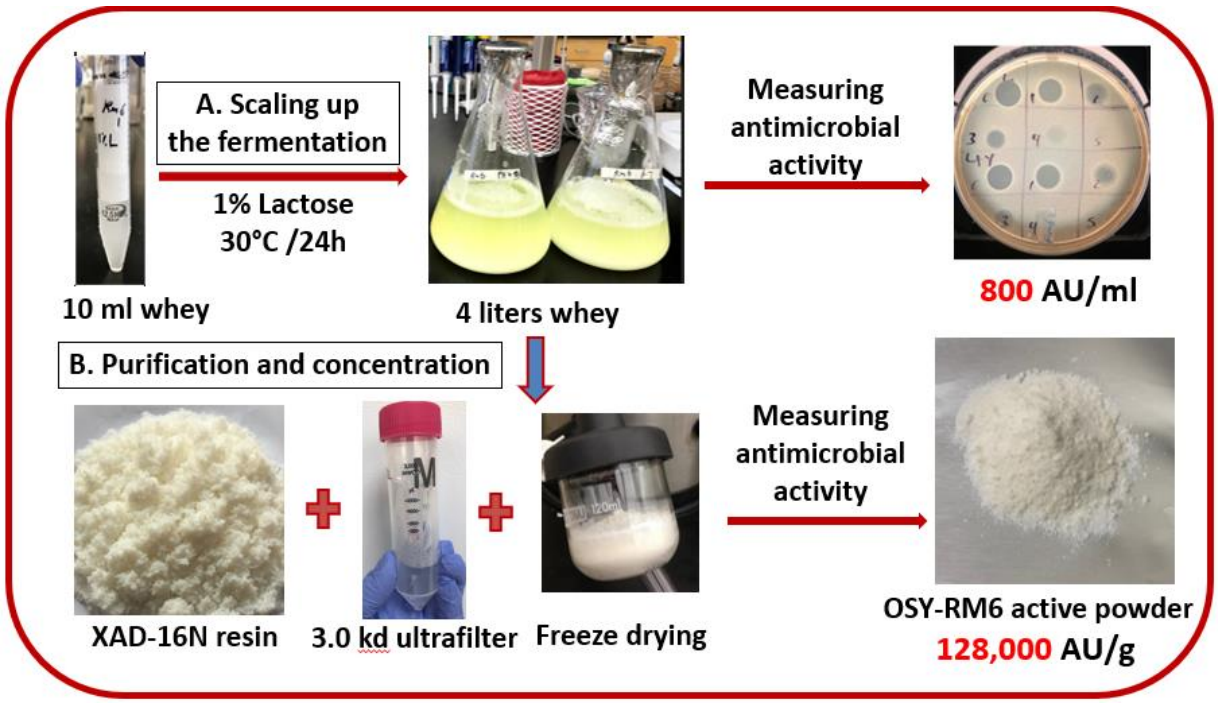

Figure 4. Producing OSY-RM6 active antimicrobial powder

References

And, H. C., and Hoover, D. G. (2003). Bacteriocins and their Food Applications. *Compr. Rev. Food Sci. Food Saf.* 2, 82–100. doi:10.1111/j.1541-4337.2003.tb00016.x.

Asioli, D., Aschemann-Witzel, J., Caputo, V., Vecchio, R., Annunziata, A., Næs, T., et al. (2017). Making sense of the "clean label" trends: A review of consumer food choice behavior and discussion of industry implications. *Food Res. Int.* 99, 58–71. doi:10.1016/j.foodres.2017.07.022.

Bruno, M. E. C., and Montville, T. J. (1993). Common Mechanistic Action of Bacteriocins from Lactic Acid Bacteriat The influence of four bacteriocins from lactic acid bacteria on the proton motive force (PMF) of sensitive. Available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC182399/pdf/aem00038-0249.pdf [Accessed June 12, 2019].

De Vuyst, L., and Leroy, F. (2007). Bacteriocins from Lactic Acid Bacteria: Production, Purification, and Food Applications. *J. Mol. Microbiol. Biotechnol.* 13, 194–199. doi:10.1159/000104752.

Fair, R. J., and Tor, Y. (2014). Antibiotics and bacterial resistance in the 21st century. *Perspect. Medicin. Chem.* 6, 25–64. doi:10.4137/PMC.S14459.

Hammes, W. P., and Tichaczek, P. S. (1994). The potential of lactic acid bacteria for the production of safe and wholesome food. *Z. Lebensm. Unters. Forsch.* 198, 193–201. doi:10.1007/bf01192595.

He, Z., Kisla, D., Zhang, L., Yuan, C., Green-Church, K. B., and Yousef, A. E. (2007). Isolation and Identification of a Paenibacillus polymyxa Strain That Coproduces a Novel Lantibiotic and Polymyxin †. *Appl. Environ. Microbiol.* 73, 168–178. doi:10.1128/AEM.02023-06.

Huang, E., Zhang, L., Chung, Y. K., Zheng, Z., and Yousef, A. E. (2013). Characterization and application of enterocin RM6, a bacteriocin from enterococcus faecalis. *Biomed Res. Int.* 2013. doi:10.1155/2013/206917.

Hussein, W. E., Huang, E., Ozturk, I., and Yousef, A. E. (2019a). Draft Genome Sequence of Lactobacillus paraplantarum OSY-TC318, a Producer of the Novel Lantibiotic Paraplantaracin TC318 . *Microbiol. Resour. Announc.* 8. doi:10.1128/mra.00274-19.

Hussein, W. E., Xiaoli, L., and Yousef, A. E. (2019b). Draft Genome Sequence of *Enterococcus durans* OSY-EGY, a Multiple-Antimicrobial-Peptide Producer Isolated from Egyptian Hard Cheese. *Microbiol. Resour. Announc.* 8. doi:10.1128/MRA.00303-19.

Lievore, P., Simões, D. R. S., Silva, K. M., Drunkler, N. L., Barana, A. C., Nogueira, A., et al. (2015). Chemical characterisation and application of acid whey in fermented milk. *J. Food Sci. Technol.* 52, 2083–2092. doi:10.1007/s13197-013-1244-z.

Mokoena, M. P. (2017). Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. *Molecules* 22, 1255. doi:10.3390/molecules22081255.

Prazeres, A. R., Carvalho, F., and Rivas, J. (2012). Cheese whey management: A review. *J. Environ. Manage.* 110, 48–68. doi:10.1016/j.jenvman.2012.05.018.

Saeed, F., Afzaal, M., Tufail, T., and Ahmad, A. (2019). "Use of Natural Antimicrobial Agents: A Safe Preservation Approach," in *Active Antimicrobial Food Packaging* (IntechOpen). doi:10.5772/intechopen.80869.

Wibowo, D., and Zhao, C. X. (2019). Recent achievements and perspectives for large-scale recombinant production of antimicrobial peptides. *Appl. Microbiol. Biotechnol.* 103, 659–671. doi:10.1007/s00253-018-9524-1.

Zacharof, M. P., and Lovitt, R. W. (2012). Bacteriocins Produced by Lactic Acid Bacteria a Review Article. *APCBEE Procedia* 2, 50–56. doi:10.1016/J.APCBEE.2012.06.010.