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Behavior of Native Microbial Populations of WPC-34 and WPC-80 Whey Protein Stored at Different Temperatures

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Abstract

Whey protein (WPC34 and 80) has been used as food ingredients and as a base for making biodegradable product. However, there is limited information on the behavior of native microflora associated with these products. The objective of this study was to estimate classes of native microflora of WPC34 and WPC80 using different agar media and then investigate how storage temperatures (5, 10, 15, 22 and 30°C) for 7 days would affect survival and growth of each class of the bacteria estimated. Immediately after receiving WPC34 and WPC80 from the manufacturer the initial populations of aerobic mesophilic bacteria, coliform, yeast and mold, lactic acid bacteria including lipolytic bacteria were enumerated and bioluminescent Adenosine Triphosphate (ATP) assay was used to estimate ATP level corresponding to the overall microbial populations associated with WPC34 and WPC80 products. Total microbial populations in WPC34 and WPC80 determined immediately and after storage for 7 days averaged 6.8 log and 7.1 log CFU/g, respectively and the corresponding ATP values associated with the total microbial populations averaged 62 and 73 RLU, respectively. Class of microorganism estimated from WPC80 averaged 2.8 log CFU/g for aerobic mesophilic bacteria, below detection (<2 CFU/g) for yeast and mold and coliform bacteria, as well as 2.6 and 2.4 log CFU/g for lipolytic and lactobacillus bacteria, respectively. For WPC34, aerobic mesophilic bacteria, yeast andmold, coliform bacteria, lipolytic and lactobacillus bacteria determined averaged 3.0, 1.5, below detection, 2.0 and 3.0 log CFU/g, respectively. Storage temperatures did not cause significant ($p>0.05$) changes in total microbial populations of WPC34 and WPC80 and bioluminescent ATP assay can effectively be used to estimate total microbial populations in WPC34 and WPC80 products.

Keywords: ATP values; Survival; Total plate count; WPC34 and WPC80 whey protein; Temperature; Storage

Introduction

Whey protein is an excellent food base ingredient that can be used for other functional foods or products. Functionalized healthy food ingredients were developed using micro-texturing and micro structuring processes such as micro articulation, micro-shear, and extrusion texturization processes [1]. Such developed food ingredients were noted to have improved texture with enhanced physical modifications including ex/in vivo functionality [2]. There is a lot interest in biodegradable product and for food packaging. The U.S. Food & Drug Administration's (FDA) regulation 177.1520 requires greater purity standards for materials used for biodegradable food packaging than plastics used for non-food packaging. In earlier study, Onwulata and coworkers, reported on a technology for making new and improved Dairy Based Bioplastic (DBP) from whey protein isolate and corn starch material [3]. Chemical and biological composition of biodegradable products are known however, information concerning the level of microbial populations and their survival rate during storage of whey protein isolate and corn starch materialis limited. It has been reported that the microflora of all food items are of practical significance to producers, processors and consumers [4,5].

Physical and chemical treatments are used in food processing to eliminate or at least reduce the presence of pathogenic and spoilage microorganisms in foods [6-10]. The Specific Mechanical Energy (SME), expansion indices (radial, longitudinal and volumetric), pasting viscosity, water absorption index (WAI) texture properties, and microstructure of Whey Protein Concentrate (WPC) and Corn Starch Material (CM) during extrusion processing have been reported [7]. The authors suggested that changes in structural conformation reported on CM and WPC extrudates may have contributed to the actual surviving bacterial population.

Microorganisms subjected to environmental stresses undergo metabolic injury, and in most cases cannot form colonies on selective agar plates were uninjured cells can survive and grow [11-14]. During processing, bacteria undergo sub lethal cellular injury from avariety of treatment processes leading to some survival and viability loss [4, 15]. The level of sanitation and the microbiological load of finished products are of primary importance to the quality, shelf stability and microbial safety. Previously, we reported that the initial bacterial count in post process food items includes both surviving and injured cells [7-10,12-15].

Estimation of microbial numbers in foods by conventional microbiological techniques takes atleast two to three days, before

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results are achieved and there is a need for faster methods that can give results in minutes [16]. Several rapid methods for microbial determination in foods have been reported. Hennlich et al. [17] reported use of polarographic measurement of oxygen consumption in a tight bacterial cell as mean of rapid microbial determination. Other rapid method includes but not limited to flow cytometry, DNA based tests, bioluminescent Adenosine Triphosphate (ATP) [18]. Depending on the type of food and or environment, the assay method takes 30 min to 20 h from sample preparation to the time results are achieved. An ATP methods gives result in minutes (≤ 40 min) therefore is considered as one of the fastest among other methods reported. Several studies have used bioluminescent ATP technique to estimate the presence of bacteria in various media [16,18-26]. Its use for rapid measurement of microbial cell numbers is based on the premise that all living things contain ATP, and that the intracellular ATP levels have to be constant for cells to maintain normal physiological activities [22,24].Therefore, the level of ATP determined in a sample is proportional to the actual cell number [23,27]. The assay does not require incubation, and therefore, is very rapid and sensitive and provides results in minutes.

The knowledge of the level of native microflora on WPC 34 and WPC80 whey protein should help processors in understanding storage variables needed to maintain longer shelf life and in implementing HACCP plans and Good Manufacturing Practices (GMP). WPC34 andWPC80 whey protein derived from milk, there are low moisture food ingredients with low water activity [7]. The low moisture and water activity content may encourage survival of residual native microbial populations but may not encourage growth of the microorganism. There is limited information on the level of microbial populations in these product and their survival during storage of whey protein isolate and corn starch material [7,15].

Therefore the purpose of this study was to : 1) Determine the total microbial populations of WPC34 and WPC80 whey protein; 2) Estimate different class of microorganism within the total microbiota including spoilage bacteria in WPC34 and WPC80 whey protein; 3) Monitor the behavior of these classes of native bacteria during storage at cold and warm temperatures for a week; and 4) Investigate possible use of bioluminescence ATP assay to estimate total microbial populations of WPC34 and WPC80 whey protein during storage at different temperatures.

Materials and Methods

Microbial enumeration

The WPC34 and WPC80 samples collected were stored at 5, 10, 20 and 30°C for 7 days. Prior to storage and at the end of 7 days storage, microbial populations in the samples were determined as described below. Approximately, 50 g of WPC-80 and WPC-34 whey protein sample was withdrawn at each time of investigation from the storage temperatures stated above for microbial analysis. The 50 g sample was placed in a Stomacher® bag with 450 ml of 0.01 % peptone water(PW, BBL/Difco) and blended for 30 s in a Stomacher (model 400; Dynatech Laboratories, Alexandria, VA, USA) set at 250 rpm. Aliquot (100 µL) of the sample was plated on a range of agar media to estimate the populations of native microbiota on the samples. The following media plates per type of bacteria were used: trytic soy agar (TSA, BD/Difco) plates with incubation at36°C for 24 h were used for estimating the populations of mesophilic aerobic bacteria [28,29]. Spoilage Yeast and Mold population was estimated by plating 0.1 ml of the samples on Dichloran Rose Bengal Chlortetracyclin (DRBC, BD/ Difco) agar plates with incubation at room temperature at 23°C for 5 days. Agar plates were wrapped with aluminum foil to provide darkness and the plates were stored without turning the plates upside down to avoid condensation. For enumeration of Coli form /E. coli bacteria, samples were plated on Violet RedBile Agar (VRBA, Difco, Detroit, MI), and incubated at 37°C for 24 h [30,31]. The choice of media and use of incubation temperatures listed in this study allowed for enumeration of native emicroflora that may grow well at the specific agar plate per temperature. For example Lipolytic microorganism (psychrotrophic bacteria etc) that can affect flavor of milk were also estimated by plating 0.1 ml on spirit blue agar (BBL/Difco, MD), while lactobacillus and leuconostoc bacteria, both a class of lactic acid bacteria were enumerated on APT agar (BBL/Difco, MD). All microbial enumeration were performed according to the FDA bacteriological analytical manual, 7th ed, [30].

Bioluminescence ATP assay for native microflora

Several ATP extractants [0.1 MTris-EDTA, pH 7.75, Trychloro acetic acid-water (TCA, 1.5 % v/v); and Extra light (Turnerdesign, Sunnyville, CA)] was studied to determine which solution would give the highest ATP yield. The method of Anhsen and Nilsson [20], as modified by Ukuku and Shelef [27], was used to determine level of bacterial ATP in each sample. Aliquots (1ml) of samples taken for microbiological analysis was also added to 4 ml of each of the extracting solution listed above. The samples were mixed, followed by heating in boiling water for 5 min, and cooling to room temperature in an ice-bath before ATP determination. ATP extraction involving the Extra light was not subjected to the heating but was vortexed for 1 min. The ATP content was determined by the luciferin-luciferase reaction using the ATP bioluminescent assay kit (Sigma ChemicalCo., St. Louis, MO). Bioluminescence ATP assays were performed with 20/20 Luminometer (Turner Design, CA). The generated light signal referred to as Relative Light Units (RLU) was measured after a 16s delay time and a 60s integration time. Any possible inhibition of the luciferase reaction by extracts was corrected by adding known amounts of ATP standard into thereaction vial and based on the results of the ATP extraction study, the TCA solution was betterand therefore was used throughout the study.

Statistical analyses

All experiments were done in triplicate with duplicate samples analyzed at each sampling time. Data were subjected to the Statistical Analysis System (SAS; SAS Institute, Cary, NC) for analysis of variance (ANOVA) and the Bonferroni LSD method [32] to determine if there were significant differences ($p<0.05$) between mean values of number of cells recovered at each day of determination.

Results and Discussion

The initial native microbiota associated with WPC34 and WPC80 whey protein were enumerated on a range of agar media incubated at optimum growth temperatures for each class of microorganism (Tables 1 and 2). The populations of aerobic mesophilic bacteria, yeast and mold, coliform, lipolytic and lactobacillus bacteria determined immediately in WPC-34 and after storage at different temperatures are shown in Table 1. The initial averaged populations of aerobic mesophilic bacteria, yeast and mold, coliform, lipolytic and lactobacillus bacteria determined was 3.0 log CFU/g, 1.5 log CFU/g, below detection (<2 CFU/g), 2.0 log CFU/g and3.0 log10 CFU/g, respectively. The populations for each class of organism decreased slightly ins amples stored at 5, 10, 22 and 30°C. Again, the population of coliform bacteria was below detection (<2 CFU/g) in WPC-34 stored at 5, 10, 22 and 30°C for 7 days. For the

Populations (Log_{10} CFU/g)					
Background bacteria	Initial	5°C	10°C	22°C	30°C
Aerobic mesophilic bacteria	3.0 ± 0.2^{A}	2.8 ± 0.1^{A}	2.4 ± 0.2^{B}	3.1 ± 0.2^{A}	2.2 ± 0.10^{B}
Yeast and Mold	1.5 ± 0.10^{A}	1.2 ± 0.10^{A}	1.4 ± 0.05^{A}	0.9 ± 0.10^{B}	0.3 ± 0.1^{C}
Coliform	BD	BD	BD	BD	BD
*Lipolytic*bacteria	2.0 ± 0.2^{A}	1.8 ± 0.1^{A}	1.5 ± 0.2^{B}	1.9 ± 0.1^{A}	2.2 ± 0.1^{A}
Lactobacillus	3.0 ± 0.2^{A}	2.8 ± 0.1^{A}	1.9 ± 0.2^{B}	2.1 ± 0.1^{B}	2.0 ± 0.1^{B}

[a]Values represent means ± SD for data from three experiments with duplicate determinations per experiment
Means in the same row not followed by the same letter are significantly ($p<0.05$) different
BD = Below detection (< 1 CFU/g)

Table 1: Aerobic Mesophilic Bacteria, Yeast And Mold And Coliform Of Wpc-34 Stored At Different Temperatures For 7 Days.

Populations (Log_{10} CFU/g)					
Background bacteria	Initial	5°C	10°C	22°C	30°C
Aerobic mesophilic bacteria	2.8 ± 0.1^{A}	2.6 ± 0.2^{A}	2.5 ± 0.2^{B}	2.4 ± 0.2^{A}	3.3 ± 0.10^{B}
Yeast and Mold	BD	BD	2.0 ± 0.05^{A}	1.4 ± 0.10^{B}	$1.7 \pm 0.1A^{B}$
Coliform	BD	BD	BD	BD	BD
*Lipolytic*bacteria	2.6 ± 0.1^{A}	2.4 ± 0.2^{A}	2.0 ± 0.1^{B}	1.4 ± 0.1^{C}	2.2 ± 0.2^{A}
Lactobacillus	2.4 ± 0.1^{B}	2.2 ± 0.2^{B}	2.2 ± 0.1^{B}	1.7 ± 0.1^{C}	3.3 ± 0.2^{A}

[a]Values represent means ± SD for data from three experiments with duplicate determinations per experiment
Means in the same row not followed by the same letter are significantly ($p<0.05$) different
BD = below detection (< 1 CFU/g)

Table 2: Aerobic Mesophilic Bacteria, Yeast And Mold and *Coliform*of Wpc-34 Stored At Different Temperatures for 7 Days.

WPC80samples, the populations of aerobic mesophilic bacteria, yeast and mold, coliform, lipolytic and lactobacillus bacteria determined immediately and after storage at different temperatures are shown in Table 2. The initial aerobic mesophilic bacterium was 2.8 log and this value was slightly lower than the numbers in WPC34. Unlike the WPC34 samples, the yeast and mold population and coliform bacteria determined in WPC80 were below detection (<1 CFU/g) atinitial time and after storage at 5°C for 7 days. Only coliform bacterium in WPC80 samples was below detection after storage at all temperatures tested. Storage of samples at 10°C and aboveincreased the populations of yeast and mold in WPC80. The initial population of lipolytic bacteria in WPC80 was approximately 0.6 log higher than the numbers in WPC34. Storage of WPC 34 and WPC80 whey protein at 5°C or at abusive 30°C temperature did not cause significant ($p>0.05$) changes in the populations of each class of bacteria. The level of total mesophilic aerobes and coliform bacteria enumerated in all initial samples, and at all temperatures tested were within the reported values by the manufacturers.

Initial ATP level of WPC34 and WPC80 and after storage

In another study designed to investigate the sensitivity of using the ATP methods to estimate total bacterial populations in the samples, Escherichia coli bacteria was grown overnight in TSB and the samples were diluted to 102 with sterile distilled deionized water(ddH_2O). The ATP values determined from each dilution is shown in Figure1. The results suggest that the accuracy of ATP determination can be correctly made at 4 log CFU/ml and above and this is in agreement with earlier studies [20,26,27]. The initial total plate count of native bacteria determined in WPC34 and WPC80 averaged 7.2 and 6.8 log CFU/g, respectively. These numbers determined suggests that the total background microflora in the WPC34 andWPC80 samples tested is higher than the minimum threshold of 4 log reported here for ATP determination. Therefore ATP method was used to estimate total bacterial populations in WPc34and WPC80.

The initial total microbial count and the corresponding ATP values determined immediately in WPC 34 samples and after storage at 5, 10, 22 and 30°C for 7 days are shown in Figure 2. A slightly higher bacterial population and ATP value was determined in WPC34samples stored at 30°C for 7 days but the numbers were not significantly ($p>0.05$) different from samples stored at 10, 15 and 22°C. Though, the ATP values of WPC34 whey protein was slightly lower than the log CFU/g, the ATP values and the log CFU/g determination followed the same trend suggesting that ATP methods can be used to estimate bacterial populations in the sample. For the WPC80 sample, a slightly lower ATP value was determined compared to the log CFU/g values observed in samples stored at 10°C for 7 days (Figure 3). In WPC80 samples stored at 15°C and above, both ATP values and log CFU/g numbers determined were similar and again were not significantly ($p>0.05$) different within the two methods of bacterial estimation.

A linear relationship between cell numbers determined by total plate count method at 4 log10 CFU/g and above to the bioluminescence ATP values was established (Figure 1). With the bioluminescence ATP assay, a 43 RLU value was established as the threshold or the baseline value at which the microbial load on WPC34 and WPC80 can be measured effectively. Below this threshold, the ATP determination showed a large variation to the log CFU and therefore is considered unreliable. Griffith [23] reported an approximate 70% agreement between the traditional plating techniques and the ATP assay rapid method. In our study, our data shows approximately 89% agreement between the traditional plating techniques and the ATP assay rapid method. Karl [24] reported that vacuum filtration can be used to increase the sensitivity of the ATP assay to <104 CFU/ml and we never applied this technique to our method. The bioluminescence ATP assay measures cellular components and metabolic by- products or actual growth; hence it can be used to estimate bacterial numbers [19-23]. The Relative Light Units (RLU) value of any bacterial bioluminescent is directly related to the ATP extracted and thus to the number of microbial cells from which it originated [26]. Griffiths [23] reported that ATP estimation of cell numbers by bioluminescence may not accurately be agreement with colony count on an agar plate for Gram- positive cocci (staphylococci and streptococci). For example, the ATP count may show 105 CFU /

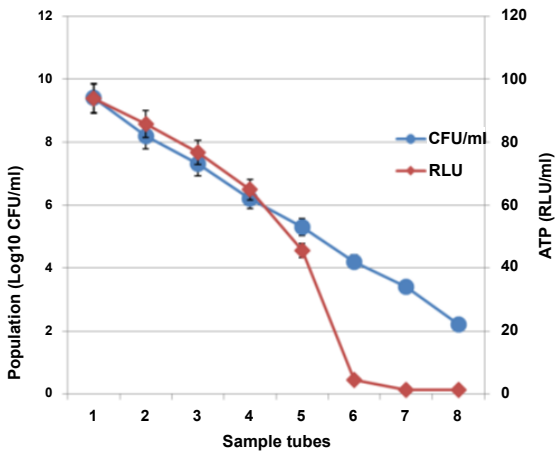

Figure 1: ATP values of *Escherichia coli* bacteria in sterile distilled deionized water. Values are means ± SD of three experiments with duplicate determinations.

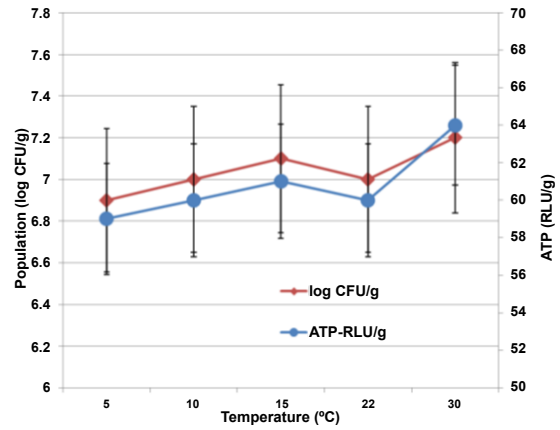

Figure 2: Colony forming units for total native microflora of WPC34 whey protein and the corresponding ATP values determined at different storage temperature. Values are means ± SD of three experiments with duplicate determinations.

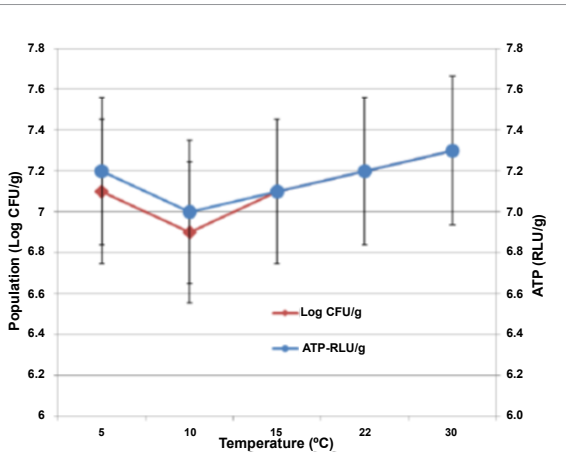

Figure 3: Colony forming units for total native microflora of WPC80 whey protein and the corresponding ATP values determined at different storage temperature. Values are means ± SD of three experiments with duplicate determinations.

ml while the colony count shows 104 CFU /ml or less. The reason for such a discrepancy is that staphyloccus and streptoccus organisms grow in bunches and chains, respectively. Therefore, each CFU of these organisms may in fact represent 10 to 20 bacteria. This phenomenon may not apply in our study since the blending process of WPC34 and WPC80 utilized in this study may have dispersed any bacterial clumps in the samples before the ATP extraction and determination. However, the bioluminescence ATP assay cannot distinguish ATP values from individual classes of microflora from WPC34 and WPC80 therefore, it can only be used where microbial estimation is designed to investigate total microflora in food. The ATP assay provides an indication of the total microbial load, which may help establish a threshold for GMP and HACCP guidelines.

In conclusion, the total plate count of native bacteria in WPC34 and WPC80 determined immediately were not ($p>0.05$) significantly different. Aerobic mesophilic bacteria, yeast and mold, Lipolytic bacteria and lactobacillus were determined in WPC34. Only yeast and mold populations including coliform bacteria were not determined in WPC80 stored at 5°C. Coliform bacteria was not determined in WPC34 and WPC80 stored at 5, 10, 15, 22 and 30oC for 7 days while populations of other class of bacteria slightly decreased or increased but were not significantly ($p>0.05$) different from the control. This observation suggests that storage temperature up to 30°C did not change microbial populations of each class of bacteria except the yeast and mold population in WPC34. Similarly, the total ATP values determined in both samples were not significantly ($p>0.05$) different between them and RLU values showed similar trend to the log CFU values at each storage temperature suggesting that the ATP method can be used to estimate total bacterial count in WPC34 and WPC80. Also, the ATP method can be used to estimate the hygienic nature of WPC34 and WPC80 after preparation and during storage.

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