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Research Article

Evaluation of Microbial Recovery from Raw Materials for Pharmaceutical Use

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ABSTRACT

Microbiological quality of raw materials used in pharmaceutical manufacturing is critical attribute that affects the bioburden level of the final product. If the preliminary treatment for the microbiological analysis of the material is not suitable to recover actual microbial content, false estimation of microbial presence and/or count in raw materials may impact final product quality and safety. Accordingly a survey study was conducted on 12 selected materials that are used extensively in the manufacturing facility in order find suitable technique to recover low-level inoculums of standard strains. The basic processing and/or neutralization technique was dilution alone or in combination with chemical neutralization or less frequently filtration. Dill oil was the only raw material required combination of dilution and chemical neutralization for both of microbial enumeration and detection while this combination was necessary only in enumeration for Cetostearyl Alcohol. Although dilution method may be adopted alone for Sorbitol solution 70% yet filtration was done in combination to facilitate the visualization of commonly found yeast contaminant from the suppliers of the raw materials. Balsam Peru required the highest dilution among the tested materials for enumeration. The applied neutralization techniques were effective in detecting low level contamination in raw materials.

Key words: Raw materials- Bioburden- Dill oil- Cetostearyl Alcohol - Sorbitol.

1. Introduction

Microbial contamination of drug products causes pharmaceutical companies great financial loss. Analysis of FDA product recall data for 134 non-sterile pharmaceutical products from 1998 to September 2006 demonstrated that 48% of recalls were due to contamination by Burkholderia *cepacia, Pseudomonas* spp., or *Ralstonia picketti,* while yeast and mold contamination were found in 23% of recalls. Gram-negative bacteria accounted for 60% of recalls, but only 4% were associated with Gram-positive bacteria. Of the 193 recalls of sterile products, 78% were due to the lack of sterility assurance and 7% for yeast and mold contamination. For sterile products, Gram-negative bacteria accounted for 6% of recalls, with only 1% due to Gram-positive bacteria (Jimenez, 2007).

The majority of the contaminants of non sterile pharmaceutical products and ingredients are bacteria, yeasts, and filamentous fungi (molds). These organisms have a wide range of nutritive requirements and environmental conditions suitable for their proliferation. Many of the ingredients used in pharmaceutical formulations can become substrates for microorganisms (Clontz, 2008).

Microbial contamination of raw materials used to manufacture dry formulations (e.g. tablets) is often reduced by drug manufacturing processes such as granule drying and tablet compaction. The amount of bioburden reduction is directly dependent on the process temperature, chemical properties of the drug formulation, tablet compression pressure, and metabolic properties of the contaminating microbes. For example, bacterial spores are less susceptible to the harsh conditions encountered during tablet

processing and the survival of *Bacillus subtilis* spores found in raw materials has been studied and documented (Marshall *et al.,* 1998). Although dry formulations are less susceptible to microbial contamination, the spoilage of solid dosage form products by vegetative organisms has also been

Table (1): List of non-sterile pharmaceutical products subjected to neutralization method validation.

Raw Material	Synonyms	Chemical Nature	Pharmaceutical Use
Avicel PH 102	Microcrystalline Cellulose (INCI*), MCC, cellulose gel.	Carbohydrate of alpha-cellulose (purified and partially depolymerized cellulose made by acid hydrolysis of wood pulp).	Tablet binder.
Balsam Peru	Myroxylon pereirae (INCI*), China oil, Black, Indian, Peruvian, Surinam and Honduras balsam.	Benzoic alcohol, Benzyl benzoate and other benzoates, Benzyl acetate, Benzoic acid, Benzyl cinnamate, Benzaldehyde, benzyl salicylate, Cinnamic acid, Cinnamyl cinnamate, Cinnamic alcohol, Cinnamic aldehyde, Citrus peel, Coniferyl alcohols, Coumarin, Eugenol, Farnesol, Isoeugenol, Methylcinnamate, Nerolidol, Vanillin and resinous substances.	Possesses healing properties. It has aromatic and fixative (*i.e.*, delays evaporation) properties, and mild antiseptic, antifungal, and antiparasitic attributes.[13]
Cetostearyl Alcohol	Cetearyl alcohol, Cetylstearyl alcohol, Cetyl/stearyl alcohol.	Mixture of fatty alcohols, consisting predominantly of Cetyl and Stearyl alcohols and is classified as a fatty alcohol.	Emulsion stabilizer, opacifying agent, and foam boosting surfactant and an aqueous and nonaqueous viscosity-increasing agent. It imparts an emollient feel to the skin and used in water-in-oil emulsions, oil-in-water emulsions,
Cetyl Palmitate	Spermaceti, Palmatic acid n-hexadecyl ester, Palmitic acid n-hexadecyl ester, Palmitic acid palmityl ester, Palmitic acid hexadecyl ester, Palmitic acid cetyl ester, Palmityl palmitate, n-hexadecylpalmitat.	Ester derived from palmitic acid and cetyl alcohol.	Emulsifying and stiffening agent.
Dill Oil	Dillweed, American dill weed oil, Aneth oil, Anethum graveolens oil, Dill fruit oil, Dill oil Indian, Dill seed oil Indian, Dill seed oil.	Carvone (30 to 40%), Limonene (30 to 40%), Phellandrene (10 to 20%) and other monoterpenes.	Abortifacient, Antifungal, Aromatic, Carminative, Galactagogue, Insect repellents, Psychedelic and Stomachic.
Glycerol	Glycerin, Glycol alcohol.	1,2,3-propanetriol, Polyol (sugar alcohol).	Used as a means of improving smoothness, lubrication and as humectant. It is found in allergen immunotherapies, cough syrups, elixirs and expectorants, and mouthwashes
Kaolin Light	China clay, Bolus alba, Porcelain clay, White bole, Argilla.	Hydrated aluminum silicate.	Used internally to control diarrhea. Kaolin has also been used topically as an emollient and drying agent. It has been used as protectant for temporary relief of anorectal itching and diaper rash.
Maize Starch	Corn flour, Corn Starch.	Amylose (20 to 25%) and Amylopectin (75 to 80%).	Disintegrant and binder.
Sodium Croscarmellose	Cross-linked Sodium Carboxymethylcellulose, Sodium CMC.	Cellulose Derivative.	Disintegrant.
Sorbitan Monostearate	Synthetic wax, Span 60, D-Glucitol, Anhydro-monooctadecanoate, Anhydrosorbitol stearate, Sorbitan monooctadecanoate.	Ester of sorbitan (a sorbitol derivative) and stearic acid.	Emulsifying agent with dispersing and wetting properties. Used in hemorrhoid creams and creams to treat dry skin.
Sorbitol 70%	D-Glucitol, D-Sorbol, Sorbit.	Sugar alcohol.	Sweetener or 7umectants.
Xanthan Gum	Xantham, xanthan, Keltrol f, Rhodopol 23, Glucomannan, Xanthate gum.	Polysaccharide composed of pentasaccharide repeat units, comprising glucose, mannose, and glucuronic acid in the molar ratio 2.0:2.0:1.0.	Thickening, stabilizing, suspending and Controlled release agent.

*= International Nomenclature of Cosmetic Ingredients.

observed, especially in tropical and humid climates. As reported in the *Pakistan Journal of Scientific and Industrial Research* (Cundell, 2002), various types of tablets, both coated and noncoated, were found to be contaminated with bacteria such as *Klebsiella aerogenes*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Fungi were also isolated from the samples tested and those included *Penicillium chrysogenum*, *Aspergillus flavus*, *Candida albicans*, and *Saccharomyces* spp. The total bacterial count in the contaminated products ranged from 2.0×10^3 to 8×10^7 colony forming units (CFU) per mL of sample preparation, and the total fungal count ranged from 20 to 1.4×10^2 CFU per mL of sample preparation. This article indicated that the contaminating microbes were capable of proliferating in the product by utilizing the chemicals in the drug formulations as sole carbon and energy sources.

The current work was aimed to study the recovery of low level of microbial contamination from the selected raw materials that are commonly used in the manufacturing of drug products and those with antimicrobial properties and to establish treatment methods and processing techniques for the selected raw materials based on each nature and situation as a part of risk assessment study in pharmaceutical industry.

2. Material and Methods

Standard strains and neutralizing broth (NB) were prepared as described by Eissa *et al*., 2014 with modification of adding Lecithin 7.0 g/L to NB. Tryptone Soya Broth (TSB) was supplemented with Lecithin and Tween (80 5.0 and 40.0 g/L respectively). Tests for microbial enumeration and detection of specific pathogens were done according to USP <61>, 2014 and USP <62>, 2014. List of tested raw materials, their chemical composition and their use is listed in table (1). Both liquid and agar media were growth promotionally tested according to USP <61>, 2014. Method of sample treatment was modified when conventional technique yield results that are unsatisfactory for one or more of the tested microorganisms in either enumeration or specific microbes detection. All test procedures were conducted in biological safety cabinet (BSC). Control media were incubated along with test media to ensure sterility. All statistical analysis was performed using GraphPad Prism version 6.01 for Windows. Any interpretation or complex calculation was performed using Microsoft Excel 2007. Moreover environmental monitoring (EM) samples from surfaces and air were taken with every campaign test performed in BSC to ensure appropriate cleaning, disinfection and aseptic behavior under laminar air flow (LAF) conditions. Bacterial visualization was facilitated using colorless Triphenyltetrazolium Chloride (TTC) dye which turns red by viable cells. Cultures purity and identity were confirmed by isolation and identification (Eissa, 2014; Estridge *et al*., 2000). Interpretation of microbial recovery was based on 0.3 Log_{10} variability as described by Eissa and Nouby, 2014.

3. Results and Discussion

All prepared media passed the growth promotion test of culture media. Initial assessments of bioburden quality of the tested raw materials –using conventional methods- revealed that the tested products were clean microbiologically. Chemical neutralization method was found to be non toxic and microbial recovery was ≥70% from the viability control group. This finding is supported by similar finding of Eissa *et al*., 2014 for the chemical neutralizers used. Negative controls and EM samples showed no growth indicating appropriately sterilized media and aseptic handling. Results in table (2), (3) and (4) – all passed the method suitability test - were obtained after applying suitable treatment for the samples using dilution, filtration and chemical neutralization methods as indicated in table (5). Sorbitol 70% solution has previous history of out of specification (OOS) results from some suppliers in total yeast mold count (TYMC) due to contamination with slow growing white yeast that appeared after 7 days using pour plate technique. This necessitates the modification illustrated in table (4) as filtration allowed for earlier detection of microorganisms than direct plating. Balsam Peru, Cetosearyl Alcohol, Dill Oil and to much lesser extent Glycerol possessed inhibitory effect on low level inoculums when prepared and homogenized to 1:10 w/v dilution in either media or diluent. This is an area that needs more investigation. Block 1991 demonstrated that many organic alcohols, acids and esters possess antimicrobial properties. However, Avicel PH102, Sodium Croscarmellose and Xanthan Gum (hydrophilic macromolecules) formed solid gels and undispersed limps before attaining 1:10 ratio in saline or TSB. This necessitates performing higher dilution to achieve reliable sample homogeneity, easier sample processing for further testing and maintain appreciable water activity for microbial survival especially for Gram-negative bacteria and attenuated microorganisms. This is not strange in view of that Clontz 2008 discussed similarly that Syrups containing a high concentration of sugar (approximately 85%) resist bacterial growth owing to the exosmotic effect on microorganisms. Marshall 1998 and Cundell *et al*., 2002 demonstrated that although the microbial limit tests call for the use of the Sabouraud Dextrose Agar (SDA) for the recovery of fungi, studies performed over the years have demonstrated that all-purpose media are capable of recovering a wide range of bacteria, yeasts, and molds. This was shown in the study by using both *Candida albicans* and *Apergillus niger* with SDA (20 - 25°C) and the all purpose media Tryptone Soya Agar (TSA) (30 - 35°C) in Figure (1). Actually although *C. albicans* passed the test, it showed relatively lower recovery if compared with other standard strains. Two-Way ANOVA without replication was used to analyze transformed microbial recovery from the tested materials.

Table (2): Screening for the neutralization procedures applied for insurance of non-toxicity of the method applied in microbiological testing against tested microorganisms.

Raw Material	Ratio of Microbial Recovery						
	Staphylococcus aureus	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Candida albicans*		*Aspergillus niger*	
	30-35°C	30-35°C	30-35°C	20-25°C	30-35°C	20-25°C	30-35°C
Avicel PH 102	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Balsam Peru	0.027	0.032	-0.009	0.018	-0.127	0.000	0.000
Cetostearyl Alcohol	0.004	0.114	0.000	0.000	0.036	0.060	-0.049
Cetyl Palmitate	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Dill Oil	-0.025	0.004	0.000	-0.090	-0.061	-0.179	-0.173
Glycerol	0.000	-0.009	0.066	0.000	0.000	0.000	0.000
Kaolin Light	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Maize Starch	0.022	0.036	0.114	0.000	0.000	0.000	0.000
Sodium Croscarmellose	0.000	0.000	0.000	-0.100	0.000	0.000	0.000
Sorbitan Monostearate	0.004	0.114	0.000	0.000	0.036	0.097	-0.049
Sorbitol 70%	0.000	0.000	0.000	-0.049	0.161	0.102	0.194
Xanthan Gum	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table (3): Screening for enumeration of the microbial recovery from raw materials that are used in pharmaceutical products manufacturing after applying appropriate neutralization procedures.

Raw Material	Ratio of Microbial Recovery						
	Staphylococcus aureus	*Bacillus subtilis*	*Pseudomonas aeruginosa*	*Candida albicans*		*Aspergillus niger*	
	30-35°C	30-35°C	30-35°C	20-25°C	30-35°C	20-25°C	30-35°C
Avicel PH 102	-0.072	0.071	0.131	0.244	0.174	-0.017	-0.076
Balsam Peru	-0.057	0.018	0.027	-0.033	0.000	0.102	0.000
Cetostearyl Alcohol	0.194	-0.130	0.066	0.194	0.066	0.292	-0.127
Cetyl Palmitate	-0.121	-0.041	-0.072	0.076	0.301	0.131	-0.009
Dill Oil	-0.037	-0.093	0.032	0.108	-0.045	-0.045	0.060
Glycerol	-0.134	0.018	0.022	-0.025	0.143	0.066	-0.114
Kaolin Light	-0.004	0.174	-0.152	0.268	0.125	-0.230	-0.114
Maize Starch	-0.083	0.125	0.009	0.027	0.276	0.114	-0.212
Sodium Croscarmellose	-0.029	0.018	-0.004	0.056	0.081	-0.097	0.000
Sorbitan Monostearate	-0.053	-0.013	0.004	0.102	-0.013	0.155	-0.173
Sorbitol 70%	0.022	0.013	-0.013	-0.029	-0.090	0.237	0.022
Xanthan Gum	-0.057	0.004	-0.017	0.222	-0.086	0.155	-0.241

Table (4): Survey study for specific microorganisms detection from raw materials that are used in pharmaceutical products manufacturing after applying the neutralization procedures.

Raw Material	Detection of Specific microorganisms					
	Staphylococcus aureus	*Salmonella enterica*	*Pseudomonas aeruginosa*	*Escherichia coli*	*Candida albicans*	Bile Tolerant Gram-negative Bacteria
Avicel PH 102	+	+	+	+	+	+
Balsam Peru	+	+	+	+	+	+
Cetostearyl Alcohol	+	+	+	+	+	+
Cetyl Palmitate	+	+	+	+	+	+
Dill Oil	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+
Kaolin Light	+	+	+	+	+	+
Maize Starch	+	+	+	+	+	+
Sodium Croscarmellose	+	+	+	+	+	+
Sorbitan Monostearate	+	+	+	+	+	+
Sorbitol 70%	+	+	+	+	+	+
Xanthan Gum	+	+	+	+	+	+

Table (5): Applied technique(s) in method suitability for each tested raw material showing the inoculums level used of the test microorganisms for each material.

Raw Material	Inoculum Range	Neutralization for Enumeration			Inoculum Range	Neutralization for Detection of Specific Microorganisms		
		Dilution	Filtration	Chemical		Dilution	Filtration	Chemical
Avicel PH 102	23-97	+++	-	-	25-98	+++	-	-
Balsam Peru	42-93	+++++	-	-	25-98	+++	-	-
Cetostearyl Alcohol	35-71	+	-	+	25-98	+	-	-
Cetyl Palmitate	30-97	+	-	-	25-98	++	-	-
Dill Oil	32-90	+	-	+	25-98	++++	-	+
Glycerol	23-97	++	-	-	25-98	++	-	-
Kaolin Light	23-97	+	-	-	25-98	+	-	-
Maize Starch	35-91	+	-	-	25-98	+	-	-
Sodium Croscarmellose	23-97	+++	-	-	25-98	+++	-	-
Sorbitan Monostearate	35-71	+	-	-	25-98	++	-	-
Sorbitol 70%	22-60	+	+	-	25-98	+	-	-
Xanthan Gum	23-97	++++	-	-	25-98	++++	-	-

+=1:10 (w/v). ++=1:20 (w/v). +++= 1:40 (w/v). ++++=1:100 (w/v). +++++=1:1000 (w/v).

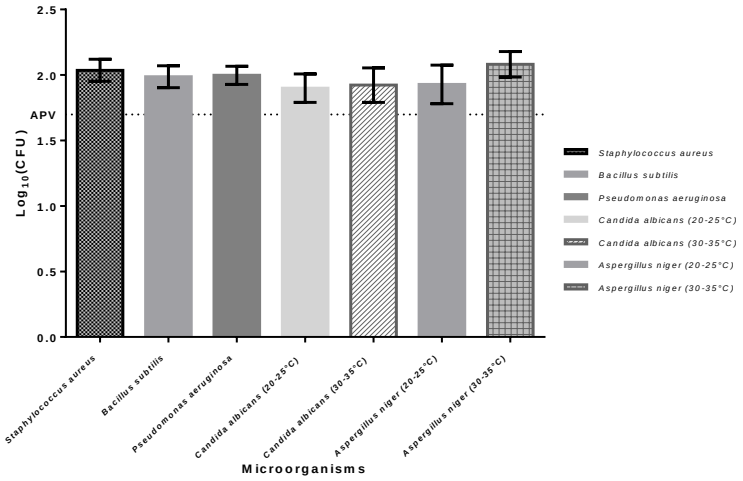

Figure (1): Relative microbial recovery of Log_{10} transformed CFU of each standard strain for 12 tested raw materials. (Graph generated using GraphPad Prism v6.01 for Windows) APV= Acceptable Plating Variability.

Data analysis showed that raw materials (each with its special treatment method) did not possess significant difference from each other (raw factor). On the other hand, there was significant difference between microorganisms groups (column factor). Column Factor accounts for 26.63% of the total variance with F = 4.29, DFn=6, DFd=66 and the P value = 0.0010. Row Factor accounts for 5.06% of the total variance with F = 0.44, DFn=11, DFd=66 and the P value = 0.9294. According to USP Chapter <1227> 2014, Validation of Microbial Recovery from Pharmacopeial Articles as the number of CFUs decreases the error as percent of the mean increases so inoculums controls of 22 CFUs or more but less than 100 CFUs were used in order to minimize errors in enumeration i.e. error as percent of mean plate count is less than 21%.

4. Conclusion

The established neutralization techniques for the tested samples were effective in recovering low level of aerobic mesophilic microbial contamination of representative microorganisms from bacteria and fungi. These results could be useful in applying bracketing or matrixing for materials. For example, the treatment method applied for Avicel or Starch could be applied to other types or varieties of them.

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