A REVISED BBP[1] PROPOSAL ON THE REGULATORY STATUS OF THE STERILE LIQUID AND SOLID FRACTIONS RESULTING FROM ALKALINE HYDROLYSIS OF ANIMAL CARCASSES

[1] BBP (Belgian Biosafety Professionals) is the Belgian section of the European Biosafety Association

Introduction

Alkaline hydrolysis has become of increasing interest as an alternative inactivation method for (microbiologically) contaminated organic waste, including animal carcasses. Its effectiveness, relative ease to use, low impact on the environment and relative low operational costs make it the method of choice compared to the classical inactivation techniques such as autoclaving, incineration or rendering (see Annex). Since the 1990-ies, tissue digesters for alkaline hydrolysis have been installed in many US high containment facilities to dispose animal carcasses (Kaye, 2003) and new digesters are being installed with increasing frequency. In Europe, tissue digesters have become operational since 2011 in high containment large animal facilities in Spain and Germany. Additional digesters are planned in high containment facilities in the UK, the Netherlands, Norway and potentially other European countries. In Belgium, a 300 - 500 kg capacity tissue digester will be installed in 2013-2014 at the BSL3 facility of the Faculty of Veterinary Medicine, Ghent University and at the BSL3 facility of the CODA-CERVA in Machelen.
Despite its proven effectiveness in terms of inactivation, the implementation of alkaline hydrolysis is hindered because of overlapping legislation, i.e. the contained use legislation, the waste management legislation and the animal by-products legislation, that do not apply the same criteria.

Alkaline hydrolysis and regulation

The European Directive 2009/41/EC describes the rules concerning the **contained use** of genetically modified microorganisms (GMMs). In Belgium, this directive has been implemented to cover not only GMMs, but also other genetically modified organisms (GMOs) and pathogens. The three Belgian regions have implemented this EU directive in their environmental legislation using the same principles and based upon a common risk assessment framework. In all three regions it is required that hazardous biological materials from BSL3 animal facilities are inactivated within the facility using a validated inactivation method.
The **regional waste management legislation** aims to protect the environment and public health against potential negative effects of waste in general. According to this legislation, hazardous biological materials that have been inactivated using a validated means are (residual) industrial waste.
The requirements for handling **animal by-products,** which aim to prevent infection risks by animal by-products, are stipulated in the EU regulations 1069/2009 and 142/2011 that are applicable in all member states. There is additional legislation on the regional level, but this mainly organizes a number of practical and administrative matters and formulates a number of additional requirements. This set of regulations stipulate that animal carcasses of experimental animal facilities are a category 1 animal by-product if the competent authorities have decided that such material has the potential to pose serious health risks to humans or to other animals (Point (a)(iv) of article 8 of regulation 1069/2009, as changed by EU directive 2010/63)[2]. If the

[2] Point (a)(iv) of article 8 of regulation 1069/2009/EC now reads: "*animals used in a procedure or procedures defined in Article 3 of Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, in cases where the*

competent authorities decide that such material does not have the potential to pose serious risks, then the material is either a category 2 or a category 3 animal by-product. Before the change of regulation 1069/2009, animal carcasses of experimental animal facilities were category 1 animal by-products by default and had to be disposed of as described in article 12 of the regulation. In Belgium the competent authorities have not (yet) taken a decision on whether or not animal materials stemming from experimental animal facilities have the potential to pose serious health risks to humans or to other animals, and hence still apply the requirements from before the alteration of the EU regulation, namely that such materials are category 1 animal by-products by default. This has a number of consequences. It implies that the derived products, such as the liquid and solid fractions resulting from alkaline hydrolysis, have to be eliminated and destroyed by an approved waste treatment company (Rendac for Belgium), even though sufficient evidence is available to substantiate that the end-product of alkaline hydrolysis is sterile and non-hazardous (see Annex). As such, the way the Belgian legislator treats the sterile products resulting from alkaline hydrolysis creates an unnecessary burden in terms of financial costs, energy consumption and impact on the environment (further processing (incineration) of an already safe product, special transport requirements). A similar issue has emerged in other European countries where tissue digesters are being or will be installed.

Disposal of the end products from alkaline hydrolysis: a proposal by BBP

Proposal: Categorize the products resulting from alkaline hydrolysis of animal carcasses as not posing any infectious risk to the health of humans, other animals or the environment, and hence declassify them to category 3 animal by-products.

Facts to substantiate this position:

1. Alkaline hydrolysis is a process that involves the use of physical parameters (alkaline concentration, pressure, temperature and duration) that can easily be controlled, validated and monitored. For large animal carcasses it is preferred over autoclaving as it appears a more effective and reliable method of inactivation. Indeed, autoclaving of large animal carcasses requires a shredding step prior to autoclaving to allow uniform heating, but shredding involves a high risk of generating infectious aerosols that are difficult to contain. Because of the effect of chemicals, no shredding step is required in alkaline hydrolysis, limiting the biological risk near the source.
2. The sterility of the products resulting from alkaline hydrolysis, and hence their inability to pose any threat to human or animal health, or the environment.
3. The contained use and regional waste management legislations classify materials that have been inactivated using a thoroughly validated means no longer as hazardous (it is residual industrial waste).
4. Article 10 of regulation 1069/2009 makes clear that materials that do not show any signs of disease, and which do not pose a health risk, should be

competent authority decides that such animals or any of their body parts have the potential to pose serious health risks to humans or to other animals, as a result of that procedure or those procedures without prejudice to Article 3(2) of Regulation (EC) No 1831/2003

categorized as a class 3 animal by-product. Recital 35 of regulation 1069/2009, explicitly states that submitting certain animal by-products as category 2 would severely hinder their possible uses, while not necessarily being proportionate to the risks involved.

5. In Germany, where a tissue digester recently became operational in a BSL3/4 animal facility, the liquid fraction resulting from alkaline hydrolysis is classified as non-hazardous material because this fraction is germ free. They no longer regard this material as an animal by-product (personal communication by Prof. Dr. Jens Teifke, Biorisk officer at the Friedrich Loeffler Institute - Bundesforschungsinstitut für Tiergesundheit, Germany).
6. In the US, products resulting from alkaline hydrolysis performed in high containment facilities can be disposed off via a wastewater treatment facility (http://www.ars.usda.gov/research/publications/publications.htm?seq_no_115=139881&pf=1).

Accepting this proposal would lead to the following beneficial consequences:

1. The interpretation of the contained use, regional waste management and animal by-products legislations in Belgium would lead to a scientifically sound, pragmatic and consistent implementation that would meet the purposes of this set of legislation.
2. The sterile hydrolysate and sterile solid fraction resulting from alkaline hydrolysis would no longer need to be shipped and destroyed at high and unjustified financial and environmental costs.
3. The sterile hydrolysate and sterile solid fraction could then be processed further, as other biological waste are, in a useful manner in a biogas installation without any risk for the environment and without unjustified administrative or logistic burden.

ANNEX

Alkaline hydrolysis: a general overview

Hydrolysis is a naturally occurring chemical process in which a water molecule is added to a target molecule resulting in the split of that target molecule. One fragment of the target molecule gains a hydrogen ion (H+) from the water molecule. The other portion of the target molecule collects the hydroxyl group (OH-). By doing so, complex molecules can be broken down into their basic building blocks. Hydrolysis is catalyzed by enzymes, metal salts, acids or bases. The latter process is called alkaline hydrolysis. Alkaline hydrolysis has recently been introduced as a technology for biological waste disposal.

The process of alkaline hydrolysis

During alkaline hydrolysis, sodium hydroxide (NaOH) and/or potassium hydroxide (KOH) catalyze the breakdown of biological material into a sterile aqueous solution containing small peptides, amino acids, sugars and soaps (Kaye *et al.*, 2004; Thacker, 2004). In Table 1 an overview is presented of the chemical and biological effects of the process. Alkaline hydrolysis is accelerated by heat and high pressure.

Table 1. Effect of alkaline hydrolysis on biological material

Biological material	Chemical effect of hydrolysis	Biological effect of alkaline hydrolysis
Proteins	Degradation into small peptides (max. 7 to 9 amino acid residues) and subsequently into sodium or potassium salts of free amino acids, destruction or racemization of amino acids, release of carbohydrate side chains from glycoproteins	Destruction of protein constituent in animal cells and tissues
Lipids	Hydrolysis of ester bonds into sodium or potassium salts of fatty acids (simple fatty acids), hydrolysis of amide groups in glycolipids, molecular rearrangement and subsequent destruction of polyunsaturated fatty acids and carotenoids	Destruction of sterol esters, phospholipids and glycolipids of cell membranes
Carbohydrates	Removal of carbohydrate present in glycoproteins, glycosaminoglycans and glycolipids, break down of polysaccharides into monosaccharides Large carbohydrate molecules (e.g. cellulose) are resistant to alkaline hydrolysis	Destruction of principal carbohydrates of connective tissue as well as exoskeletons of invertebrates No destruction of large carbohydrate molecules, but completely sterilized
Nucleic acids	Hydrolysis of phosphodiester bonds	Destruction of the genetic constituent of the cell (RNA, DNA)
Inorganic components (e.g. bone, teeth)	Not digestible	No destruction, but completely sterilized

Application of alkaline hydrolysis for the destruction of biological material

The first use of alkaline hydrolysis for destruction of biological material dates from the late 19th century when Amos Herbert was granted the U.S. Patent 394.982 for the process. Since the early 1990-ies, there has been a significant increase in the application of alkaline hydrolysis. The company WR^2 was the first commercial enterprise to market the technique for waste disposal. Subsequently, several other US enterprises followed.

Because of its effect on a wide range of biological materials, alkaline hydrolysis has been used for the disposal of many difficult-to-handle biological and biohazardous wastes. These include, but are not limited to low-level radioactive biological waste, aldehyde-containing fixatives and embalming fluids, waste water, infectious waste, prions, recombinant organisms and molecules as well as chemotherapeutic agents (Neyens *et al.*, 2003; Kaye *et al.*, 2004). More recently, the technique has also been applied for the disposal of human remains, as it seems more ecologically favorable than cremation. The process is being marketed worldwide as an alternative to the traditional options of burial or cremation. It remains outside the scope of this document to discuss all applications of alkaline hydrolysis. Hence, it will focus on the use of the process for the disposal of infectious animal waste where alkaline hydrolysis is performed in a so-called tissue digester.

Alkaline hydrolysis for the disposal of infectious animal waste

Disease agents considered

In general, alkaline hydrolysis will interfere with pathogen structure. Its effects include but are not limited to the destruction of viral envelop and (glyco)proteins, of bacterial and parasitic membranes, of the genetic constituent of microorganisms and of peptide bonds in prions. Consequently, the pathogens will lose their ability to interact with the host and, as such, become inactivated.

Most research on the effect of alkaline treatment on pathogen inactivation has been performed for bovine or **transmissible spongiform encephalitis (TSE)**, a fatal neurodegenerative disease in humans and animals, particularly transmitted via meat co-products such as meat-and-bone meal (MBM). In order to guarantee food safety and health of consumers, complete inactivation of TSEs in meat co-products is a prerequisite. Several scientific studies demonstrate that alkaline hydrolysis as such may not be completely effective for the inactivation of TSEs (Prusiner et al., 1984; Tamai et al., 1988; Tateishi et al., 1988; Diringer and Braig, 1989; Ernst and Race, 1993; Taylor et al., 1994; Siedel et al., 2006; Bruederle et al., 2008). However, combining the process with increased temperature and pressure has proven effective to destroy infectivity (Ernst and Race, 1993; Taylor et al., 1997; Murphy et al., 2009), although care should be taken when interpreting the results. Not all studies clearly address the sensitivity of the TSE detection method (EC report, 2003) and sensitivity of the bioassays was reduced in some studies because the necessity to dilute the samples to abolish toxic side effects for the recipient animals (Taylor, 2000).

Kaye *et al.* (1998) assessed the effect of high temperature (110-120°C) and pressure alkaline hydrolysis with NaOH on a number of pure cultures of **bacteria and yeast** using a commercially available tissue digester. Microorganisms included *Staphylococcus aureus*, *Mycobacterium fortuitum*, *Candida albicans*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, *Mycobacterium bovis BCG*, MS-2 bacteriophage, and *Giardia muris*. They were contained in dialysis bags, separate from the animal tissues. It was found that none of the samples obtained from the dialysis bags or the hydrolyzate yielded bacteria or yeast after the digestion. Murphy *et al.* (2007) evaluated the effect of KOH at high temperature and pressure on *G. stearothermophilus* spore suspensions and also found a complete inactivation (<1 log CFU/ml).
Homer et al. (2012) carried out a similar series of experiments in a BSL3 animal facility. Using the manufacturer's recommended cycle parameters, a high concentration of chemically resistant bacterial spores, used as a surrogate for the infectious agents, was inactivated by the process. Moreover, animal tissues were completely digested into a non-infectious liquid effluent. Reducing the cycle time by 50% still inactivated all spores, although a small amount of tissue remained undigested.

Also recently, Dixon *et al.* (2012) investigated the use of alkaline hydrolysis at ambient temperature for inactivation of selected **fish pathogens** in fish tissues. They included infectious salmon anaemia virus (ISAV) and *Lactococcus garvieae*, which previously showed to be the most resistant virus and bacteria to pH 12 from a wide range of viruses and bacteria tested. They were spiked at high titres into fish extracts that were then treated with 1 M sodium hydroxide. Complete inactivation was observed after 1 and 24 hours for *L. garvieae* and ISAV, respectively. Also, fish that died because of infectious pancreatic necrosis virus in the field were treated by alkaline hydrolysis at ambient temperature. A significant reduction in viral titres was observed after 24 hours and no virus was detected after 48 hours of treatment.

Environmental impact of the process

Alkaline hydrolysis of animal carcasses results in a sterile aqueous solution and a solid fraction. Reduction in volume and weight of the solid material can be as much as 97%. The solid fraction consists of the inorganic components of the carcass, i.e. bone and teeth (calcium phosphate). The typical characteristics of the residual effluent solution are presented in Table 2.

Table 2. Typical characteristics of animal carcass hydrolysate digested in an alkaline tissue digester (adapted from Das, 2008)

Characteristic	Average value
Biological oxygen demand (BOD) (mg/l)	70.000
Chemical oxygen demand (COD) (mg/l)	105.000
Total suspended solids (mg/l)	1000
Organic N (mg/l)	8000
Ammonia N (mg/l)	1000
Total P (mg/l)	400
pH	10.9

Because of its high pH, BOD and COD, disposal of effluent raises some environmental concerns. The high pH of the effluent can nevertheless be lowered easily by bubbling carbon dioxide into the hydrolysate at the end of the digestion to obtain a pH of 8 or less (Thacker, 2004). The high BOD has been considered suitable to provide nutrients for microorganisms of large municipal wastewater treatment plants (Thacker, 2004; Das, 2008). However, when operating the tissue digester at small wastewater treatment plants, disposal of the high BOD and COD effluent will provide a concern because of over-loading limitations. Alternative applications for the effluent include co-composting with low nutrient substrates such as yard trimmings (Das, 2008), rich natural fertilizer, enrichment of manure slurry, nutrient feedstock for anaerobic digesters, biofuel or biogas production and landfill disposal (www.bioliquidator.com).

Even though the conditions applying to the alkaline hydrolysis process seem suitable to generate toxic chemicals (i.e. alkaline environment, temperature in excess of 150 °C, presence of chloride and/or organochlorine compounds), most of the emitted volatile organic compounds were reported to be below the given chemical detection limits (Malcolm Pirnie Inc., 2002). Detailed studies performed by WR^2 show that no dioxins were detected in the emitted air, nor were chlorophenols and other polychlorinated hydrocarbons found in the residual fluid. Moreover, the process did not generate dioxins or polychlorodibenzofurans in the alkaline tissue digest beyond any dioxins already contained in the original animal carcass tissue. As such, the Scientific Steering Commission assigned by the European Commission considers that, although the data were obtained from laboratory scale studies and experiments and the levels of chloride were probably low, the results provide sufficient reassurance that the levels of dioxin release into the environment are unlikely to constitute a risk (EC report, 2003). This is in contrast to incineration of animal waste, where generation of dioxin and other volatile toxics is a serious public concern (Kaster and Phebus, 2004).

Economics of the process

McClaskey *et al.* (2004) summarized the economic implications and costs of different disposal techniques, including alkaline hydrolysis. The average cost of operation for alkaline hydrolysis has been estimated between 40 - 320 $ per ton (or 0.02 - 0.16 $ per pound) of carcass material disposed including costs for power, chemical inputs, personnel, sanitary sewer expenses, maintenance and repair. In comparison, incineration and rendering, two other current techniques for disposal of infectious animal waste, were estimated at 35 - 2000 $/ton and 40 - 460 $/ton, respectively.

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