Alexander Technological Educational Institute of Thessaloniki, Faculty of Food Technology and Nutrition, Department of Food Technology

THESIS

STUDY OF THE AEROBIC BIOLOGICAL TREATMENT OF BAKER'S YEAST BEET-MOLASSES WASTEWATER COMING FROM THE ANAEROBIC EFFLUENT

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Dedicated to my family, to my parents who gave me life,
and to Xrysa for sharing life with her…

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Abstract

The yeast production wastewater contains a relatively considerable amount of concentrated organic substances with processed sugar as a main constituent of the pollutants. Colored compounds contained in molasses are predominantly melanoidins. These are brown recalcitrant compounds hardly to be biodegraded. They lead to the eutrophication of water courses due to their high pollution load. Here, they are examined the factors that enhance the microorganisms ability to biodegrade these high strength agro-industrial wastewater. The treatment methods which were tested were: a) poplar wood chips series: 1. Sterilized wood chips and sterilized wastewater, 2. Both sterilized with the presence of activated sludge (AS), 3. None of the both is sterilized with the presence of AS, b) ultrasounds (≈0.2W/ml, 10min) and c) plastic carriers (K3 Anoxkaldnes, 50% occupied of the filled volume of the reactor). The best results were obtained from woodchips series 1. It was noticed a gradual stable decrease (57.4%) from the first week in the COD values, comparing to those of the influent COD, which are very close to those of the control. Series 2 resulted in 9.6% higher COD values, then, after 3 months approximately, an increase was watched indicating that the woodchips started to become saturated. This means that only the woodchips degrade the molasses wastewater through sorption mechanisms. In fact AS hinders the wastewater sorption from the woodchips leading to their saturation and no biodegradation due to the activated sludge microorganisms is observed. On the contrary, it can be said that AS creates a system that promotes the wastewater sorption from the woodchips since the beginning of the experiment. In series 3 the COD variation is similar to that of series 2 with the exception of observing two differences: an initial gradual COD decrease due to the microorganisms adaptation and a fluctuation in the COD values (494 – 807ppm) which can be attributed to the competition between the microorganisms living on the woodchips and the molasses wastewater, that can tolerate the high pH values. Non sterilized woodchips work as a microbiological support. So it can be deduced that there is an effect of sterilization wastewater molasses, which act as contaminating microorganisms. Ultrasound implementation failed for different reasons: 1. Due to the presence of bicarbonate ions which are strong inhibitors of reactions between hydroxyl radicals and the organic content, 2. The AS used, was probably out of the optimal range of solids content. 3. The ultrasound intensity was probably insufficient to enhance the biological activity, 4. The irradiation period might not be appropriate, 5. Low biological activity because of the alkaline pH. Plastic carrier implementation also failed for different reasons: 1. The alkaline pH values had as a consequence a decreased retention of bacteria onto the carriers and a decreased microorganisms activity, 2. Decreased retention of bacteria due to the low temperature (25^{o}C), 3. The selected carrier might not be the appropriate one. Due to the low biological activity in the last two methods and the increased pH, accordingly, a repolymerization of the coloured compounds took place leading to a COD increase.

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Περίληψη

Τα απόβλητα ζύμης αρτοποιίας περιέχουν μια σχετικά σημαντική ποσότητα συμπυκνωμένων οργανικών ουσιών με την επεξεργασμένη ζάχαρη ως κύριο συστατικό των ρυπαντών. Οι έγχρωμες ενώσεις που περιέχονται στη μελάσσα είναι κυρίως μελανοϊδίνες. Αυτές είναι καφέ ανθεκτικές ενώσεις δύσκολα βιοδιασπόμενες. Οδηγούν στον ευτροφισμό των υδάτων λόγω του υψηλού ρυπαντικού φορτίου τους. Εδώ, εξετάζονται οι παράγοντες που ενισχύουν την ικανότητα των μικροοργανισμών να βιοδιασπούν αυτά τα υψηλής αντοχής αγρο-βιομηχανικά απόβλητα. Οι μέθοδοι επεξεργασίας που εξετάστηκαν ήταν: α) πριονίδι λεύκης σειρά: 1. Αποστειρωμένο πριονίδι και αποστειρωμένα απόβλητα, 2. Και τα δύο αποστειρωμένα παρουσία ενεργού ιλύος (Ε.Ι.), 3. Κανένα από τα δύο αποστειρωμένα παροουσία Ε.Ι., β) υπερήχοι (≈ 0.2W/ml, 10min) και γ) πλαστικό πληρωτικό υλικό (K3 Anoxkaldnes, καταλαμβάνεται το 50% του πληρωμένου όγκου του αντιδραστήρα). Τα καλύτερα αποτελέσματα ελήφθησαν από το πριονίδι σειρά 1. Παρατηρήθηκε μια μεγάλη βαθμιαία σταθερή μείωση (57.4%) από την πρώτη εβδομάδα στις τιμές COD, σε σύγκριση με εκείνες του COD εισόδου, που είναι πολύ κοντά σε εκείνες του μάρτυρα. Η σειρά 2 οδήγησε σε 9.6% υψηλότερες τιμές COD, και στη συνέχεια μετά από 3 μήνες περίπου, παρατηρήθηκε μία αύξηση υποδεικνύοντας ότι το πριονίδι άρχιζε να φτάνει σε κορεσμό. Αυτό σημαίνει ότι μόνο το πριονίδι αποικοδομεί τα απόβλητα μελάσας διαμέσου μηχανισμών ρόφησης. Στην πραγματικότητα η Ε.Ι. παρεμποδίζει τη ρόφηση των αποβλήτων από το πριονίδι οδηγώντας σε κορεσμό τους και δεν παρατηρείται βιοαποικοδόμηση λόγω των μικροοργανισμών της Ε.Ι.. Αντιθέτως, μπορεί να λεχθεί ότι η Ε.Ι. δημιουργεί ένα σύστημα που προάγει την ρόφηση των αποβλήτων από το πριονίδι από την έναρξη του πειράματος. Στην σειρά 3 η μεταβολή του COD είναι παρόμοια με εκείνη της σειράς 2 με εξαίρεση ότι παρατηρούνται δύο διαφορές: μια αρχική σταδιακή μείωση του COD λόγω της προσαρμογής των μικροοργανισμών και μια διακύμανση στις τιμές COD (494 - 807ppm) αποδιδόμενη στον ανταγωνισμό μεταξύ των μικροοργανισμών που ζουν στο πριονίδι και στα απόβλητα μελάσσας, οι οποίοι μπορούν να ανεχθούν τις υψηλές τιμές pH. Το μη αποστειρωμένο πριονίδι λειτουργεί ως μικροβιολογική υποστήριξη. Έτσι μπορεί να εξαχθεί το συμπέρασμα ότι υπάρχει μια επίδραση της αποστείρωσης των αποβλήτων μελάσας, τα οποία δρουν ως μικροοργανισμοί επιμόλυνσης. Η εφαρμογή των υπερήχων απέτυχε για διάφορους λόγους: 1. Λόγω της παρουσίας των διττανθρακικών ιόντων τα οποία είναι ισχυροί αναστολείς των αντιδράσεων μεταξύ των ριζών υδροξυλίου και του οργανικού περιεχομένου, 2. Η χρησιμοποιηθείσα ενεργός ιλύς, πιθανώς ήταν εκτός του βέλτιστου εύρους περιεκτικότητας σε στερεά. 3. Η ένταση υπερήχων ήταν πιθανώς ανεπαρκής ώστε να ενισχυθεί η βιολογική δραστηριότητα, 4. Ο χρόνος ακτινοβόλησης μπορεί να μην ήταν ο κατάλληλος, 5. Χαμηλή βιολογική δραστηριότητα λόγω αλκαλικού pH. Η εφαρμογή του πλαστικού πληρωτικού υλικού απέτυχε επίσης για διάφορους λόγους: 1. Το αλκαλικό pH είχε ως συνέπεια την μειωμένη κατακράτηση των βακτηρίων επί των μεταφορέων και την μειωμένη δραστηριότητα των μικροοργανισμών, 2. Μειωμένη κατακράτηση των βακτηρίων λόγω της χαμηλής θερμοκρασίας (25^{o}C), 3. Ο τύπος του πληρωτικού υλικού ίσως να μην ήταν ο κατάλληλος. Λόγω της χαμηλής δραστηριότητας των μικροοργανισμών στις δύο τελευταίες μεθόδους και του αυξημένου pH, ευνοήθηκε ο επαναπολυμερισμός των έγχρωμων ενώσεων οδηγώντας σε αύξηση του COD.

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Chapter 1: Introduction

Molasses is the main by-product of beet sugar process (Pena et al., 2003) and one of the by-products of cane sugar production (Jiranuntipon et al., 2009). In the sugar industry, during extraction from either beet or cane, large volumes of highly concentrated waste are produced. Such waste, known as molasses, is complex and its composition depends on the substrate used for sugar production. Since approximately, half of the organic compounds is made up of biodegradable sugars, this co-product is used profitably as a substrate in other industries and biotechnology (Battimelly et al., 2010). Molasses has a high commercial value due to its use as a carbon source for fermentation industries (e.g. commercial production of baker's yeast and ethanol (Amber & El Sayaad, 2010; Pena et al., 2003), biofertilizer and feed for domestic animals (Amber & El Sayaad, 2010; Jiranuntipon et al., 2009). In ethanol production, industries generate large quantities of waste generally known as vinasses, stillages or molasses spent wash. These vinasses can be used as fertilizer due to their nutrient content, mainly calcium and potassium and their high organic material content (Espana-Gamboa et al., 2011) which is common practice in Brazil (cane based vinasse, i.e. stillage from sugar-based feedstocks), Eastern Europe and in some of the western European countries (Cibis et al., 2011; Lutosławski et al., 2011). Molasses vinasse (from sugar and ethanol plants) can also be used for the production of glutamic acid and betaine (Olbrich, 1963). Stillage from sugar-based feedstocks, e.g. beet molasses vinasse and cane based vinasse, can be used in agriculture as fodder. However, compared with stillage from starch-based feedstocks which is characterized by a high potassium content they have a lower nutritive value (Cibis et al., 2011). Belitz et al. (2009) report that molasses are utilized in the production of citric, lactic and gluconic acid and for glycerol, butanol and acetone.

Citric acid fermentation of cane-molasses by submerged fermentation in 15 L stirred fermentor (working volume 9 L) was carried out by Haq et al. (2002). In the present study, the mutant strain of *Aspergillus niger* GCMC-7 supported

maximum production of citric acid (106.65 g/L) without supplements which is substantial. The addition of nitrogen sources and minerals like calcium and phosphate may further increase the production of citric acid, as required for an industrial process.

Mostafa (1995) studied among others, the utilization of molasses for glycerol production using a local yeast (*Saccharomyces cerevisiae*). The author recommends the molasses as a substrate for glycerol production and concluded that stirring increased the glycerol production by about 18.5% and calcium ions result in an increase in glycerol production of about 6%. Also, calcium ions in presence of both Na_2SO_3 and HCl give a more efficient process and a higher yield of glycerol.

The biochemical utilization of molasses sugar has been witnessed in Germany in the preparation of rum from beet molasses which is the combination of acidulation processes with alcoholic fermentation. Manufacture of rum is typically and traditionally indigenous to the cane sugar regions (Olbrich, 1963).

Moreover, molasses can also be used for the production of itaconic acid – Kautola et al. (1985) report that Aspergillus Terreus fermented beet molasses to itaconic acid in a yield of 66.2%-, dextran (Olbrich, 1963), aminoacids (Belitz et al., 2009), as already mentioned for the glutamic acid, and succinic acid. Liu et al. (2008) propose an economical succinic acid production from cane molasses by Actinobacillus succinogenes. In their work, succinic acid production from cane molasses by A. succinogenes CGMCC1593 using cane molasses as a low cost carbon source was developed for the first time. In anaerobic bottles fermentation, succinic acid concentration of 50.6 ± 0.9 g/L was attained at 60 h using an optimum medium containing molasses pretreated with sulfuric acid, resulting in a succinic acid yield of 79.5 ± 1.1% and sugar utilization of 97.1 ± 0.6%. They concluded that the inexpensive cane molasses could be utilized for the economical and efficient production of succinic acid by A. succinogenes. However, in the study of Chan et al. (2012), a high level of succinic acid production by metabolically engineered E.coli

KJ122-pKJSUC-24T was demonstrated in a low-cost medium containing sucrose, and sugarcane molasses without any complex nutrients under simple-batch fermentations. Therefore, KJ122-pKJSUC-24T could be a potential strain for economic succinate production from renewable substrates. Productions compared favorably or exceed titers of A. succinogenes or other published biocatalysts.

Finally, Yan et al. (2011) demonstrated that waste molasses (defined as a by-product in sugar refinery) alone can displace glucose-based medium for microalgal fermentation towards cost-saving biodiesel production. This work is the first to illustrate the feasibility that waste molasses served as a fermentable carbon source for biodiesel production using C. protothecoides. It has been demonstrated that waste molasses could be used as a cheap feedstock to lower costs of C. protothecoides culture. Improved biomass and oil content have been achieved under optimized culture conditions, either in batch or fed-batch cultures. Waste molasses is raised as a promising feedstock for algae-based cost-saving biodiesel production.

At this point it must be stressed out that in the sugar industry, with the extraction from either beet or cane, large volumes of highly concentrated waste are produced and among the possible alternatives which have already been mentioned, this highly concentrated product can be made profitable on-site by anaerobic digestion, which produces methane while removing more than 80% of the organic load (Battimelly et al., 2010). Methane may be burned as a supplementary energy source (Chiu, 1990) or it can be used as fuel to generate the energy required for the industrial plant itself (Espana-Gamboa et al., 2011).

The present thesis is organized as following:

In chapter 2 the problem of the molasses wastewater is stated, following by the literature survey (chapter 3). In chapter 3 the following issues are addressed:

- What are the molasses wastewater
- How these are produced
- What is their problem
- What are the melanoidins
- Which treatment methods are applied with emphasis in the investigated methods
- What problem cause the molasses wastewater before and after their treatment

In chapter 4 the objectives of this thesis are presented. The materials and methods are referred in chapter 5. Then, in chapter 6 the results and discussion are mentioned. The thesis is completed with the conclusions, the suggestions for future work and the references, chapters 7, 8, and 9 respectively.

The aim of this thesis is to examine the factors that enhance the microorganisms ability to biodegrade the high strength agro-industrial beet-molasses wastewater.

Chapter 2: Statement of the problem

Most food processing wastewater contains biodegradable organic compounds which are commonly removed in biological processes such as anaerobic, aerobic and anoxic biodegradation. The activity of microorganisms results in the removal of pollution through assimilation of the biodegradable substances. A variable fraction of refractory compounds remains after biodegradation, even if the biological treatment is advanced. These compounds are often present in the original effluent or may also be produced during the biological processes (Battimelly et al., 2010).

Zhang et al. (2011) reported that the capacity of a wastewater treatment plant ultimately depends on two features: the metabolic capabilities of the

microorganisms in the system and the effectiveness of solid/liquid separation at the last stage of the treatment process.

As it was mentioned above, the factors that enhance the microorganisms' ability to biodegrade the high strength agro-industrial beet-molasses wastewater are examined here.

Chapter 3: Literature Survey

3.1. Baker's yeast wastewater

3.1.1 Brief process description

Baker's yeast *Saccharomyces cerevisiae* is an essential ingredient in bakery products produced by dough fermentation (yeast biomass is generally grown in aerobic fedbatch culture systems). The properties of baker's yeast cells in commercial products, such as fermentation ability, stress tolerance, and flavor formation, depend on both the cultivation conditions and the genetic constitution of yeast strains (Tokashiki et al., 2011). The production of baker's yeast includes processes such as cultivation, fermentation, separation, rinsing and filtration (Ersahin, et al., 2011a; Catalkaya & Sengul, 2006). An example is shown in Figure 1 (Ersahin et al., 2011b). Figure 1 is a process flow diagram for the production of baker's yeast. A variety of processes are used in producing baker's yeast. Most processes, however, are a variation of the Zulauf process, which was introduced in the early 1900's (EPA, 1994).

The principal raw materials used in producing baker's yeast are the pure yeast culture and molasses. Cane and beet molasses are used as the principal carbon sources to promote yeast growth. Molasses contains 45 to 55 by weight fermentable sugars in the forms of sucrose, glucose, and fructose. Other sources of sugar, such as corn grits or raisins, are available (EPA, 1994), but molasses is the least expensive source of sugar known (Ersahin, et al., 2011a; EPA, 1994). Usually, a blend consisting of both cane and beet molasses is used in the fermentations (EPA, 1994).

It is important to be mentioned that Zub (2007) and the final report for compressed yeast and active dry yeast (ADY) of EPA (1994) reported that once the feed materials are blended, the pH of the molasses mixture is adjusted because an alkaline solution hampers bacteria growth. Bacteria growth occurs under the same conditions as yeast growth, making pH monitoring very important. Optimum yeast yields are obtained at pH values between 4.5 and 5.0. The pH of the fermentation mixture is partly controlled by the feed rate of ammonia to the fermentation.

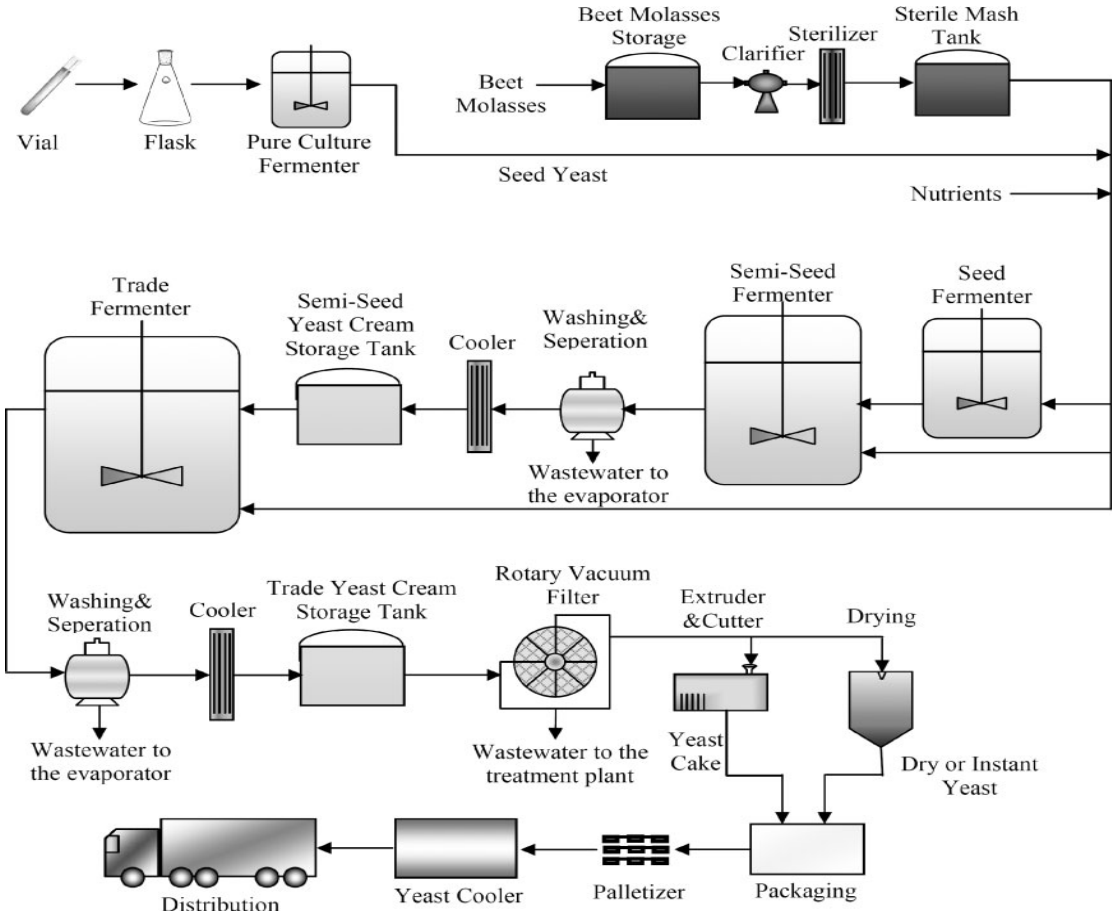

Figure 1: The production scheme of the proposed processing line by Ersahin et al. (2011b) for dry or instant baker's yeast.

3.1.2. Wastewater sources and characterization

The yeast production wastewater contains a relatively considerable amount of concentrated organic substances with processed sugar as a main constituent of the pollutants. A small amount of non-sugar oxidized substances in the form of biologically unprocessed metabolites are also found in wastewater making it dark brown (Zak, 2005). It must be pointed out that both beet and cane molasses are used in yeast fermentation. The aromatic nature of molasses is the result of phenolic compounds. Colored compounds contained in molasses are predominantly melanoidins (sugar–nitrogen complexes), caramel substances, polyphenol-iron compounds and to a lesser extent plant pigments. The humic non-sugars (or polymerized forms of melanoidins) are claimed to be particularly relevant to the colour of yeast produced (Pala & Erden, 2005). During yeast fermentation, the sugars contained in the molasses are a source of carbon and energy. However, a major part of the non-sugar substances in the molasses are not assimilated by the yeast and are released unchanged to the processing wastewater. These compounds represent the principal waste from the yeast production process. In addition, the chemicals added during fermentation (e.g. various salts, antifoams, propionic acids, brine, etc.), yeast metabolites, and residual yeast cells are in the wastewater (Blonskaja & Zub, 2009; Shi et al., 2009; Kobya & Delipinar, 2008; Blonskaja et al., 2006).

Baker's yeast fermentation produces high strength wastewaters that are characterized by high concentrations of organic materials difficult to be degraded by biological processes (Zhou, 2010; Kobya & Delipinar, 2008). More specific, effluents generated from baker's yeast industries are characterized by the low levels of readily degradable sugars, acids, and high concentrations of BOD_5 (biological oxygen demand), (40,000–50,000 mg/L), COD (chemical oxygen demand) of 80,000–100,000 mg/L, TKN (total Kjeldahl nitrogen), total dissolved solids (TDS), trimethylglycine (betaine), sulfate (6,000-8,000 mg/L), potassium, variable phosphorus content and non-biodegradable organic pollutants are generated in high concentrations too. The other major characteristic of this process is the generation of strongly

acidic (pH 4–5) wastewaters with strong odour that contain a large amount of colorants that give dark brown color and high COD (Ersahin et al., 2011b; Gengec et al., 2011; Zhou et al., 2008; Blonskaja et al., 2006; Catalkaya & Sengul, 2006; Zak, 2005; Lo & Liao, 1990). Residuals present in the wastewater and making it dark are of stable character and are not subject to the processes of biological treatment. It probably attributes to their natural characteristics, particularly in the presence of humus fractions and Maillard reaction products (melanoidins), (Zak, 2005).

Zub (2007) also informs that depending on the type of yeast fermented (commercial baker's yeast, seed yeast or yeast for special products), the yeast fermentation wastewaters are characterized by high content of COD (10,000-80,000 mg/L), strong nitrogenous (1,500-2,500 mg/L total N), sulfate-rich (2,000-10,000 mg/L), phosphorus (30-60mg/L), resistant to biodegradation and highly coloured (melanoidins etc.) substances.

After a multistage biological treatment, most of the organic load is removed. However, due to the nature of the colorants known as melanoidins which are resilient in nature to the biological treatment and resistant to biodegradation, the brown color in these effluents is imparted by this pigment and does not disappear and it can even increase due to repolymerization of colorants (Gengec et al., 2011; Zhou et al., 2008).

There are basically two types of wastewater; one is high strength process wastewater that originates from yeast separators, such as centrifuges and rotary vacuum filters, and the other one is low and medium strength process wastewater that originates from tank, floor washing, and equipment cleaning operations. (Ersahin et al., 2011b; Gengec et al., 2011; Catalkaya & Sengul, 2006; Mutlu et al., 2002).

High strength process wastewaters are high in chemical oxygen demand (COD) and color, but low in quantity. On the other hand, low strength wastewaters are very high in quantity but low in COD and color (Mutlu et al., 2002). High strength process wastewater that is high in COD and color can be

treated anaerobically (Catalkaya & Sengul, 2006). Aerobic treatment of this wastewater is not possible without substantial dilution, since aerobic microorganisms can only degrade wastewaters with COD concentrations in the order of a few thousand milligrams per liter. Anaerobic digestion, however, can treat wastewaters of high organic strengths, requires little energy to operate and yields methane (Chiu, 1990). During anaerobic digestion, the majority of biodegradable compounds is removed; a subsequent biological polishing treatment is carried out in order to reduce the remaining pollution: it is based on activated sludge, generally combined with denitrification (Battimelly et al., 2010).

It must be emphasized that the presence of high concentrations of sulfate in the wastewater causes concern in relation to anaerobic fermentation. High concentrations of sulfurous compounds have been known to either lower the anaerobic digestion efficiency or, in worse cases, hamper the process altogether. Nevertheless, the problem of sulfate toxicity is not impossible to overcome (Chiu, 1990). Sulfate is utilized by sulfate reducing bacteria (SRB) as an electron acceptor in the anaerobic process to produce hydrogen sulfide. The production of hydrogen sulfide inhibits anaerobic treatment when it is present at high concentrations (Lo & Liao, 1990). Moreover, sulfate reducing bacteria compete with methane producing microorganisms for the organic carbon available (Koplimaa et al., 2010; Zub et al., 2008; Lo & Liao, 1990). Since the pathway for the sulfate reducing bacteria is more energetically favored, electrons are diverted from the production of methane. This results in lower methane production (Lo & Liao, 1990). However, Koplimaa et al. (2010) reported that their previous experiments established that, during the anaerobic degradation of baker's yeast wastewater much more methane, as compared to the theoretical value, was produced. Kalyuzhnyi et al. (2005) attributed the observed higher methane production, when they examined the performance of a UASB (Up-flow Anaerobic Sludge Blanket) reactor treating the simulated general effluent of yeast factory, to the presence of betaine in the influents, which is not measured in the COD analysis.

Zhou et al. (2010); Kobya & Delipinar (2008); Catalkaya & Sengul (2006) & Mutlu et al. (2002) also reported that a two-stage (anaerobic + aerobic) treatment system has been used in order to treat this effluent efficiently. Specifically, high strength process wastewaters are treated anaerobically first, combined with low strength wastewaters and then sent to the aerobic treatment stage. The final effluent discharged from the treatment facility is relatively low in organic matter content, but very high in color. The same strategy is described by Altinbas et al. (2003) who reported that the total COD removals of the two-stage treatment plant are ranging at about 80–90%. Most of the COD reduction takes place during the anaerobic degradation. However, the current technology is still not optimal as the total treatment efficiency in terms of COD is only about 80% (Kobya & Delipinar, 2008; Blonskaja et al., 2006).

Kalyuzhnyi et al. (2005) inform that in spite of acidic influent (pH 4.01-4.99) fed to the UASB reactor treating the simulated general effluent of yeast factory, the effluent pH was close to 8 as a result of VFA (Volatile Fatty Acids) consumption and mineralization of nitrogenous species to ammonia. Moreover, they found that the concentration of phenolic compounds and colour almost did not change (data not shown) showing that phenols and substances responsible for colour are persistent in anaerobic conditions. Their conclusion was that the UASB reactor was quite efficient for removal of bulk COD (52–74%) from simulated general effluent of baker's yeast production. The aerobic-anoxic biofilter can be used for removal of remaining BOD and ammonia from anaerobic effluents; however, it suffered from COD-deficiency to fulfil denitrification requirements. To balance COD/N ratio, some bypass of raw wastewater should be added to the biofilter feed.

3.1.2.1. Betaine

As a by product of sugar manufacturing, molasses has 45 to 50% of residual sugars, 15 to 20% of non-sugar organic substances, 10 to 15% of ash (minerals) and about 20% of water (Blonskaja & Zub, 2009; Kobya & Delipinar, 2008; Zub, 2007; Blonskaja et al., 2006). The residual molasses

after cane sugar processing contains no betaine, but unlike beet molasses, contains about 5% aconitic acid (on dry basis) (Belitz et al., 2009). The stillage of sugar beet molasses is rich in glycerol (6 g/L) and betaine (15–20 g/L) as it can be seen from Table 1 (Decloux et al., 2002). In some sugar plants betaine is recovered as another value added product (Polematidis et al., 2010).

Table 1: Typical composition of stillage in USA

	Beet	Cane
pH	4.6–4.9	4.6–4.9
Solids, g/L	70–95	70–90
Glycerol, g/L	6.5–9.0	24–30
Betaine, g/L	15–20	—
Succinic acid, g/L	0.8–0.9	1.5–2.0
Inositol, g/L	0.4–0.9	0.9–2.8
Aconitic acid, g/L	—	0.9–1.3
Itaconic acid, g/L	—	0.7–1.1
BOD_5 (in O_2), g/L	30–40	28–38

(Decloux et al., 2002)

Molasses is used as a substrate in a wide range of industrial fermentations as it has referred above, for example, in the production of alcohols, acids and yeast cells. The sugar-beet molasses used as a growth medium for yeast contains high amounts of betaine (up to 6% dry solids, DS), also known as N,N,N-trimethyl glycine (Figure 2), a soluble nitrogenous compound (Koplimaa et al., 2010; Zub et al., 2008; Zub, 2007; Thalasso et al., 1999).

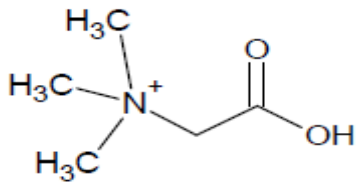

Figure 2: Chemical structure of betaine (N,N,N-trimethyl glycine), (Koplimaa et al., 2010).

This highly water-soluble compound (maximum solubility of 1600 g/dm^3 of water) is extracted with sucrose from sugar-beet pulp and is carried through

the subsequent processing stages into the molasses fraction (Zub, 2007; Thalasso et al., 1999).

Betaine is not consumed to any signifcant extent during baker's yeast fermentation and appears to largely pass through the subsequent processing stages, becoming a signifcant constituent of wastewaters produced by yeast industry processing beet molasses. The presence of betaine in wastewaters from fermentation plants using sugar-beet molasses as substrate has been reported by Koplimaa et al. (2010); Zub et al. (2008); Zub (2007) and Thalasso et al. (1999).

Koplimaa et al. (2010) reported that the wastewater from baker's yeast production contains low levels of readily degradable sugars and acids and high levels of trimethylglycine and sulfate (2,900 mg/L). The average concentration of organic pollutants by total chemical oxygen demand (COD) is 25,000 mg/L of which up to 33% is accounted for betaine.

Betaine in common with several other compounds, such as pyridine, resists oxidation during the standard dichromate method for Chemical Oxygen Demand (COD) determination. Consequently, the COD assay may significantly underestimate the organic content of the wastewater, with the result that the organic load to the digester is also underestimated or in other words this will lead to a significant overloading of wastewater treatment plants (Koplimaa et al., 2010; Thalasso et al., 1999). Furthermore, betaine is a nitrogenous compound, its complete anaerobic degradation can result in the increase of effluent ammonia concentration. This will raise the risk of ammonia inhibition of the anaerobic stage by free ammonia (Zub et al., 2008, Zub, 2007; Thalasso et al., 1999), unless strict pH control is practised. Release of ammonia during anaerobic treatment of betaine containing wastewaters also entails a requirement for subsequent nitrification / denitrification stages prior to discharge of the treated wastewater to receiving waterbodies (Thalasso et al., 1999).

Lutosławski et al. (2011), studying the biodegradation of beet molasses vinasse by a mixed culture of micro organisms, determined betaine using the colorimetric method (Focht et al., 1956). Since dichromate analysis of COD fails to detect betaine, the parameter COD_{sum} was defined as the sum of the stillage COD and the theoretical COD of betaine (2.097 g O_2/g betaine) and it was introduced as one of the pollution load parameters of the stillage examined.

Considering that betaine is not present at significant concentrations in sugarcane molasses, these potential problems do not apply to wastewaters plants treating effluents from sugar-cane molasses fermentation plants (Zub et al., 2008; Zub, 2007; Thalasso et al., 1999).

3.2 Ethanol wastewater

3.2.1 Brief process description

Biological feedstocks that contain appreciable amounts of sugar –or materials that can be converted into sugar, such as starch or cellulose– can be fermented to produce alcohol (bioethanol) to be used in gasoline engines (Malca & Freire, 2006). Technically, ethanol can be produced from a wide variety of renewable feedstock, which can be roughly classified into three main groups (Basso et al., 2011; Jiranuntipon, 2009; Lin & Tanaka, 2006; Malca & Freire, 2006): (1) those containing considerable amounts of readily fermentable sugars (sugar cane, sugar beets, cane juice, sweet sorghum), (2) starches and fructosans (corn, potatoes, rice, barley, wheat, cassava, root crops, agave). Lin & Tanaka (2006) reported that starches feedstock firstly must be hydrolyzed to fermentable sugars by the action of enzymes from malt or molds unlike fructosans which can be converted to ethanol directly and (3) cellulosic materials (stover, grasses, corn cobs, wood, trees, forestry processing residues, agricultural residues, sugar cane bagasse, municipal solid wastes, waste sulfite liquor from pulp and paper mills) must likewise be converted into sugars, generally by the action of mineral acids (Lin & Tanaka, 2006). From the point of industrial ethanol production, the sucrose-based substrates such as sugar cane and sugar beet juices present many

advantages including their relative abundance and renewable nature. Molasses has additional advantages: it is a relatively inexpensive raw material, readily available, and already used for industrial ethanol production (Ergun & Mutlu, 2000).

Typically sugar cane contains 12 to 17 percent total sugars on a wet weight basis, with 68 to 72 percent moisture. The sugars comprise about 90 percent sucrose and 10 percent glucose plus fructose. Sugar beet normally contains 16 to 18 percent sugar, which is slightly higher than sugarcane. All these three sugars are readily fermented by the yeast *Saccharomyces cerevisiae* to produce ethanol (Drapho et al., 2008). The *Saccharomyces cerevisiae* is widely used as a biocatalyst in bioconversion processes and is suitable for production of ethanol from molasses under certain conditions (Ergun & Mutlu, 2000). Molasses can be used for ethanol production after adjustment of the sugar concentrations. Removal of suspended solids prior to fermentation may also be needed. Both sugarcane juice and molasses normally have sufficient nutrients to support ethanol fermentation (Drapho et al., 2008).

Former Soviet Union and Eastern Europe once used surplus sugar-beets or frozen and perishable sugar-beets for alcohol (bioethanol) production (Qi Zhou et al., 2011).

As it has referred above, ethanol can be produced directly from sugarcane juice or from molasses produced during the sugar making process (Fig. 3).

Sugar production starts with sugarcane milling to extract juice, and this juice is clarified, filtered and concentrated. The concentrated juice is then crystallized, and sugar crystals are separated from molasses by centrifugation. This process may be repeated up to three times. Products of the first crystallization/centrifugation stage are termed ‘‘A’’ Sugar and ‘‘A’’ molasses. Second stage products are termed ‘‘B’’ sugar and ‘‘B’’ molasses. Third stage products are termed ‘‘C’’ sugar and ‘‘C’’ or final molasses, and these ‘‘C’’ products do not contain recoverable sucrose but still have 50% fermentable

sugars. Final molasses are fermented by yeast cultures, and the wine that is obtained is distilled to obtain ethanol (Garcia et al., 2011).

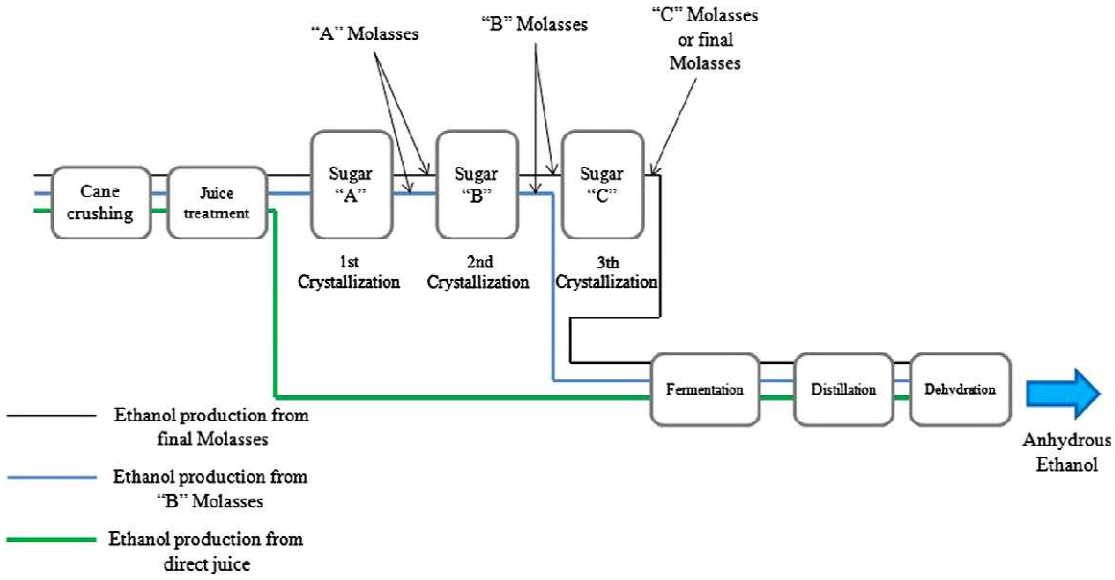

Figure 3: Industrial processes of ethanol production using sugarcane (Garcia et al., 2011).

At this point it must be emphasized that since molasses contains microorganisms which can disturb the fermentation, the molasses is taken first to the sterilizer and then to the fermentor (Lin & Tanaka, 2006). At the beginning, the molasses is diluted with water to the mass fraction of 10±18% for two reasons: 1) to reduce its viscosity in the pipeline (Krajnc et al., 2007; Lin & Tanaka, 2006) and 2) a very high concentration of sugar can give too much ethanol and results in a prolonged fermentation time and an incomplete sugar conversion (Lin & Tanaka, 2006). Now the molasses is sterilized by direct steam injection. This stream is fed into the fermentation system (Krajnc et al., 2007). Dias et al. (2009) reported that clarified juice contains around 15 wt.% diluted solids, so it must be concentrated before fermentation to achieve an adequate ethanol content that allows reduction of energy consumption during product purification steps. In order the juice to be sterilized, it is heated up to 130^{o}C for about 30 min and then rapidly cooled down to fermentation temperature.

Jiranuntipon (2009) informs for the molasses that pH is adjusted below 5 using sulfuric acid before fermentation. It is then supplemented with assimilable nitrogen source like ammonium sulfate or urea. If necessary, it is also supplemented with phosphate.

The ethanol conversion phase consists of three steps: (a) fermentation/distillation (distillery unit), (b) dehydration processes (anhydrous ethanol plant), and (c) effluent treatment plant (Fig. 4).

First, the distillery unit produces 95% (v/v) of hydrous ethanol (rectified spirit). Fermentation of sugar-rich molasses with yeast in the presence of nutrients (fermented mash) and repeated distillation of the fermented mash (lean aqueous solution) occur in the distillation unit to get hydrous ethanol (Khatiwada and Silveira, 2011; Nguyen, et al., 2008). Fermentation of molasses takes place under the action of yeast to produce hydrous ethanol. During the fermentation reactions, sucrose ($C_{12}H_{22}O_{11}$) is hydrolyzed into fructose ($C_6H_{12}O_6$) and glucose ($C_6H_{12}O_6$), which are converted into ethanol and carbon dioxide (Chauhan et al., 2011; Dias et al., 2009; Khatiwada and Silveira, 2009; Gonsalves, 2006).

Fermentation of sucrose is performed using commercial yeast (such as *Saccharomyces ceveresiae*). Chemical reaction is composed of enzymatic hydrolysis of sucrose followed by fermentation of simple sugars. First, invertase (an enzyme present in the yeast) catalyzes the hydrolysis of sucrose to convert it into glucose and fructose (Kanimozhi & Vasudevan, 2010; Gnansounou & Dauriat, 2005).

$$\underset{\text{Sucrose}}{C_{12}H_{22}O_{11}} + H_2O \xrightarrow{\text{invertase}} \underset{\text{Glucose}}{C_6H_{12}O_6} + \underset{\text{Fructose}}{C_6H_{12}O_6}$$

Then, another enzyme (zymase), also present in the yeast, converts the glucose and the fructose into ethanol and CO_2 (Kanimozhi & Vasudevan, 2010; Gnansounou & Dauriat, 2005).

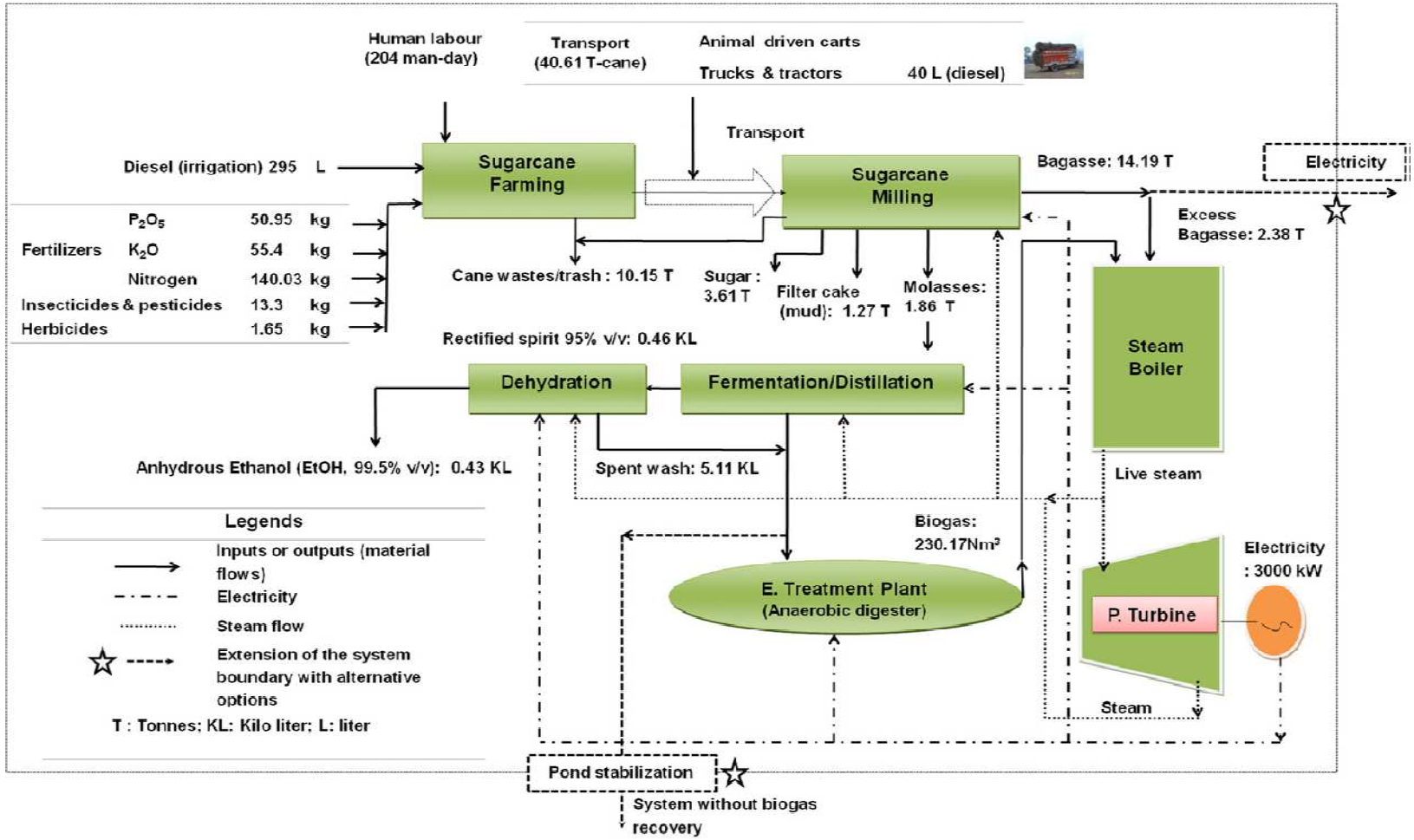

Figure 4: System boundary and material flows (per hectare) for sugarcane based systems in Nepal (Khatiwada and Silveira, 2011)

$$C_6H_{12}O_6 \xrightarrow{zymase} 2\,CH_3CH_2OH + 2\,CO_2$$

Glucose or Fructose → *Ethanol*

In the simplest form, production of ethanol from glucose can be expressed by the following equation (Drapho et al., 2008):

$$C_6H_{12}O_6 + 2\,Pi + 2\,ADP \rightarrow 2\,C_2H_5OH + 2\,CO_2 + 2\,ATP + 2\,H_2O$$

Glucose → 2 ethanol + 2 carbon dioxide + energy

The alcohol concentration during fermentation remains up to 7–9% (v/v). In the distillation column, continuous distillation of the lean aqueous solution is performed to get 95% (v/v) concentrated rectified spirit alcohol (Khatiwada and Silveira, 2009).

Special techniques (e.g. azeotropic distillation, extractive distillation, molecular sieve/pressure swing adsorption, membrane separation) are used to get passed the azeotropic point (95% ethanol concentration), (Gonsalves, 2006).

Second, the dehydration process generates 99.5% anhydrous ethanol (EtOH), (Khatiwada and Silveira, 2011). Production of EtOH from rectified spirit is achieved with molecular sieve dehydration pre concentration column: The rectified spirit, before sending it for the dehydration process, is heated and water is stripped in the pre concentration column through a pre-heater. The mixture of alcohol and little water is sent to molecular sieve in the form of vapour. It is the physical property of the sieves, which make them useful for the separation of mixture of ethanol and water. Water molecules can invade the inner structure of the molecular sieve beads and be absorbed thereon, while the ethanol molecules are too large and pass out of the vessel leaving the water behind, Thus, dehydration of ethanol takes place in the molecular sieve technology (Dharani Sugars and Chemicals LTD, 2012). However, pervaporation has also been employed in Brazil and India for this purpose (Satyawali & Balakrishnan, 2008). Later anhydrous vapor of EtOH is

condensed and cooled down to get the bioethanol (Khatiwada and Silveira, 2011).

Third, ethanol production emits the distillation residue (residue mash) which is called stillage or spent wash. This stillage is refined as biogas with the help of advanced anaerobic digestion system, e.g.: UASB reactors. By using open anaerobic system, there are two advantages energy (in the form of biogas) secured and CH_4 emission to the atmosphere is avoided. Ethanol conversion has several processes such as fermentation with yeast, distillation, dehydration, and produce residue mash (Fig. 4) which can be used for the production of biogas (energy production) from anaerobic digestion in UASB reactors and remaining stillage is stabilized in ponds (Chauhan et al., 2011). The residue mash can also be used as fertilizer and animal feed (Nguyen, et al., 2008).

The production of ethanol from sugar beet (Fig. 5) comprises of similar steps. These are categorized by Malca & Freire (2006) in two steps: (i) green juice and green syrup (GS) are produced at the sugarhouse, by subjecting biomass to a sequence of processes, namely washing and diffusion to obtain green juice and afterward purification, evaporation and crystallization to obtain GS from green juice, with sugar as a co-product; (ii) ethanol is produced both from green juice and GS at the distillery through the following processes: fermentation using yeast, followed by distillation to increase ethanol concentration and, finally, dehydration to obtain anhydrous ethanol.

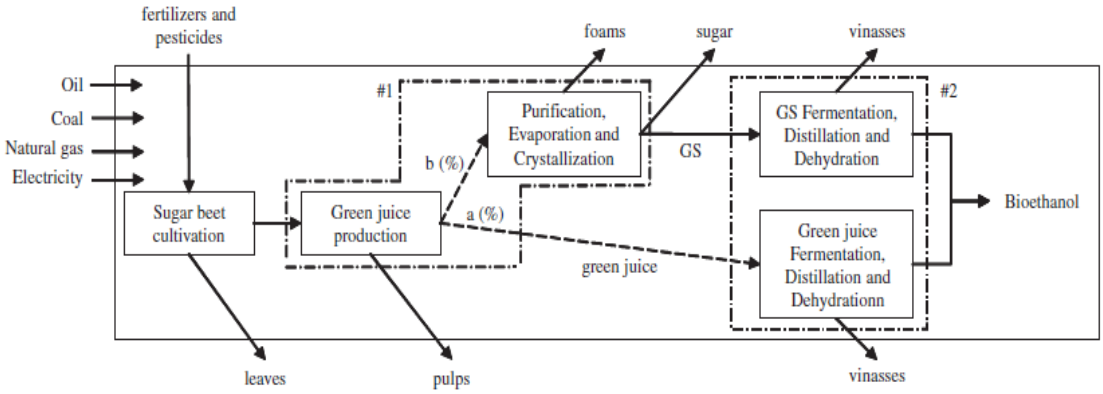

Figure 5: Flow chart illustrating the bioethanol production chain from sugar beet (Malca & Freire, 2006).

3.2.2. Wastewater sources and characterization

Vinasse, a hydrophilic viscous liquid waste with strong objectionable odour (Chavan et al., 2006) comprises a by-product of ethanol production from molasses which is a high-strength effluent with a high content of organics, mainly organic acids, reducing substances, betaine (only in the case of beet-vinasse), colored matter and glycerol (Ryznar-Luty et al., 2008). Rajasundari & Murugesan (2011); Pant & Adholeya (2007); Sangave et al. (2007a); Sangave & Pandit (2006) report that the wastewater is characterized by a high dissolved solid loading (of which 50% may be present as reducing sugars), high ash content, dark brown colour and high percentage of dissolved organic and inorganic matter (Thakur et al., 2009, Thakur, 2006). Distillery wastewaters contain phenolic compounds, mainly gallic acid, *p*-coumaric acid and gentisic acid, which impart high antibacterial activity. Organic acids such as lactic acid (29% v/v), tartaric acid (27% v/v), succinic acid (26% v/v), acetic acid (10% v/v) and malic acid (8% v/v) also documented in distillery waste water. Apart from these, distillery waste water also contains soluble proteins (Rajasundari & Murugesan, 2011), polysaccharides, lignin, waxes (Kaushik & Thakur, 2009), hemicelluloses, dextrins and resins (Sangave & Pandit, 2004). In the distillation process, ethanol ranges from 5% to 12% by volume, hence it follows that the amount of waste varies from 88% to 95% by volume of the alcohol distilled. An average molasses based distillery generates 15 L of spent wash / L of alcohol produced (Pant & Adholeya, 2007). Ryznar-Luty et al. 2008; Jimenez et al. 2003 report that the production of vinasses in a traditional alcohol factory is in the range of 9 - 14 L of wastewater per L of ethanol obtained. Specifically, in a typical batch process, 11–13 L of spent wash is generated per liter of alcohol distilled, whereas in a continuous process, this reduces to about 8–10 L of spent wash per liter of distilled alcohol (Sangave & Pandit, 2006). The amount and composition of distillery wastewater may exhibit major daily and seasonal variations due to the nature of the alcohol-producing industry, the substrate used, the specific production process utilised (Mohana et al., 2009; Gie, 2007; Pant & Adholeya, 2007; Sangave & Pandit, 2006) and the location of the industry (Gie, 2007; Sangave & Pandit, 2006). It is generally characterised by an extremely high organic

content (chemical oxygen demand (COD) in the range of 100,000 – 150,000 mg/L) and biochemical oxygen demand (BOD) of the class 35,000 – 50,000 mg/L (Pant & Adholeya, 2007; Sangave et al., 2007a), acidic pH (4 – 5), (Jimenez et al., 2003), high total solids (TS) content (20,500 – 52,000 mg/L) and high total suspended solids (TSS) content (± 10,000 mg/L), (Gie, 2007). Dry molasses spentwash contains water (90-93%), solids (7-10%), sugar (2-20%) and protein (10-11%). The metals present in molasses spentwash are Fe (348 mg/L), Mn (12.8 mg/L), Zn (4.61 mg/L), Cu (3.65 mg/L), Cr (0.64 mg/L), Cd (0.48mg/L) and Co (0.08 mg/L) with electrical conductivity in the range of 15 - 23 dS/m (Thakur, 2006). Washing water used to clean the fermenters, cooling water and boiler water further contribute to its variability (Jiranuntipon, 2009; Mohana et al., 2009; Pant & Adholeya, 2007). More specific, during the production of alcohol, large volumes of water (25 – 240 L water/L alcohol produced) are required, mainly for cleaning and cooling purposes, and large amounts of heavily polluted distillery wastewater are generated (Gie, 2007). The low strength process wastewater or diluted wastewater that originates from the floor washing and equipment cleaning has low concentration of COD, BOD and color (Jiranuntipon et al., 2008).

The main source of wastewater is the distillation step wherein large volumes of dark brown effluent (termed as spentwash, stillage, slop or vinasse) is generated with a temperature range of 70–80℃, acidi c (low pH), and with high concentration of organic materials and solids (Jiranuntipon, 2009; Mohana et al., 2009). Sangave & Pandit (2006) reported that the temperature can reach 104℃. Effective control over the distillatio n process, which ensures that the wastewater contains less than 0.2% (v/v) ethanol, is imperative, because each percent of ethanol remaining in the wastewater increases the chemical oxygen demand (COD) by 20,000 mg/L (Gie, 2007).

Apart from high organic content, distillery wastewater is also characterized by high concentrations of nitrogen, potassium, calcium, chloride and sulphate ions, and a high temperature at the moment of generation. The water content that ranges between 91 and 94% reduces the applicability of the stillage to direct grazing, as this is concomitant with the production of large amounts of

liquid manure whose treatment is far more troublesome than the treatment of vinasse itself. The high potassium content is responsible for the laxative properties of vinasse (Ryznar-Luty et al., 2008). Turkdogan-Aydınol & Yetilmezsoy (2010); Kumar et al. (2009); Satyawali & Balakrishnan (2008) inform that distillery effluents from sugar cane factories using molasses as a raw material contain high concentration of nutrients in the form of nitrogen (1660–4200 mg/L), phosphorus (225–3038 mg/L) and potassium (9600–17,475 mg/L) that can lead to eutrophication of receiving water bodies. Further, its dark color hinders photosynthesis by blocking sunlight and is therefore deleterious to aquatic life. They are also characterized by moderately acidic pH (4–5), very high total chemical oxygen demand (TCOD) (65,000–130,000 mg/L), high concentration of mineral salts and has a bad smell and dark brown color as the melanoidin pigment (Turkdogan-Aydınol & Yetilmezsoy, 2010). Table 2 summarizes the typical characteristics of untreated and anaerobically treated distillery effluent.

Table 2: Characteristics of untreated and anaerobically treated distillery effluent.

Parameters	Values of distillery effluent	Values of anaerobically treated effluent
pH	3.0–4.5	7.5–8
BOD_5 ($mg\,L^{-1}$)	50,000–60,000	8000–10,000
COD ($mg\,L^{-1}$)	110,000–190,000	45,000–52,000
Total solid (TS) ($mg\,L^{-1}$)	110,000–190,000	70,000–75,000
Total volatile solid (TVS) ($mg\,L^{-1}$)	80,000–120,000	68,000–70,000
Total suspended solid (TSS) ($mg\,L^{-1}$)	13,000–15,000	38,000–42,000
Total dissolved solids (TDS) ($mg\,L^{-1}$)	90,000–150,000	30,000–32,000
Chlorides ($mg\,L^{-1}$)	8000–8500	7000–9000
Phenols ($mg\,L^{-1}$)	8000–10,000	7000–8000
Sulphate ($mg\,L^{-1}$)	7500–9000	3000–5000
Phosphate ($mg\,L^{-1}$)	2500–2700	1500–1700
Total nitrogen ($mg\,L^{-1}$)	5000–7000	4000–4200

(Mohana et al., 2009)

Wastewater from molasses processing presents a large amount of colored substances that give dark brown color and high organic load to the effluents (Pena et al., 2003). During anaerobic treatments and due to repolymerization, the brown pigment in the molasses wastewater is hardly degraded and also increased during the conventional treatments. Phenolics (tannic and humic acids) from the feedstock, melanoidins from Maillard reaction of sugars (carbohydrates) with proteins (amino groups), caramels from overheated

sugars, and furfurals from acid hydrolysis (decomposition products such as hydroxyl methyl furfural (Thakur, 2006)) mainly contribute to the brown colour of the effluent (Rajasundari & Murugesan, 2010; Pant & Adholeya, 2007). Melanoidin is known to constitute about 2 per cent of vinasse (Kanimozhi & Vasudevan, 2010; Naik et al., 2010; Kaushik & Thakur, 2009; Satyawali & Balakrishnan, 2007). Spentwash is believed to resemble humic acids in its properties (Naik et al., 2008). On the basis of the similarities of the physical and chemical properties of the melanoidins and humic acids, melanoidins could be precursors of nitrogen-containing humic acids (Kim et al., 2004). Organic compounds present in vinasse are of humic in nature, similar to those in soil, except that fulvic acid predominates over humic acid (Naik et al., 2008, Thakur, 2006). Philp et al. (1992); Suzuki & Philp (1990) report that this complex polymeric material, called melanoidin, is considered by many to be an important precursor of geopolymers such as fulvic acids, humic acids and proto-kerogens (melanoidin-like geopolymers). Melanoidins are highly recalcitrant (Naik et al., 2008) and have antioxidant properties, which render them toxic to many microorganisms (Rajasundari & Murugesan, 2010; Naik et al., 2010; Naik et al., 2008; Mohana et al., 2009; Chavan et al., 2006), including those present in wastewater treatment processes (Mohana et al., 2009; Naik et al., 2008; Chavan et al., 2006). As a result, conventional biological processes such as biomethanation and activated sludge treatment process are insufficient to treat melanoidin containing wastewater released from distilleries and fermentation industries (Singh & Dikshit, 2011; Ojijo et al., 2010; Chandra et al., 2008; Miyata et al., 2000). Only 6%–7% degradation of the melanoidins has been achieved in the conventional anaerobic–aerobic effluent treatment processes, hence, alternative treatment processes have been explored (Ojijo et al., 2010; Satyawali et al., 2010; Blonskaja & Zub, 2009; Sreethawong & Chavadej, 2008; Pena et al., 2003) in order to remove color from molasse effluents and prevent the serious environmental problems that colored wastewaters can promote in river courses (Pena et al., 2003). Because melanoidins prevent sunlight penetration and reduce both photosynthetic activity and dissolved oxygen concentration (Liang et al., 2009; Pena et al., 2003). However, Liang et al. 2009; Ryan et al. (2009) inform that combined anaerobic/aerobic biological treatments are usually able to reduce

stillage BOD to acceptable standards in a cost-effective manner but are unable to decolourise it. Significant concentrations of melanoidins and phenolic/humic compounds contribute both to the heavy colour of stillage and a typical residual COD loading of 4,000–10,000 mg/L (Ryan et al., 2009). Due to the presence of putriciable organics like skatole, indole and other sulphur compounds which are not decomposed by yeast during distillation, the molasses spent water disposed in canals or rivers produces obnoxious smell (Rajasundari & Murugesan, 2011; Jiranuntipon, 2009; Pant & Adholeya, 2007).

3.3 Melanoidins formation and structure

Wastewater from fermentation industries such as ethanol production, bakery yeast processing and breweries is characterized by large volumes of dark brown colored effluent with a high organic content. The dark brown color of fermentation industry wastewater is mainly attributed to the presence of a class of compounds termed melanoidins (Satyawali et al., 2010). Melanoidins have conjugated carbon–carbon double bonds –C=C– in their structure that are responsible for their brown colour (Kanimozhi & Vasudevan, 2010). Recently, the empirical formula of melanoidin has been suggested as $C_{17-18}H_{26-27}O_{10}N$ (Kanimozhi & Vasudevan, 2010; Prasad & Srivastava, 2009; Pant & Adholeya, 2007). The concentration of nitrogen compounds in sugar beet juices is higher than in cane sugar juices and, therefore, the formation of melanoidins is more important in sugar beet processing (Coca et al., 2004).

Melanoidins belong to the so called Maillard reaction products (MRP's), (Dolphen & Thiravetyan, 2011), such as aroma compounds and ultra-violet absorbing intermediates (Kim & Lee, 2008). The MRP's are a particularly complex mix of various compounds of different molecular weights. They include not only aldehydes, ketones, dicarbonyls, acryl amides, and heterocyclic amines, all of which contribute to flavour, but also melanoidins and advanced glycation end products (AGE's), which are polymeric products formed at the advanced steps of maillard reaction (Wang et al., 2011).

Melanoidins are macromolecules (Venir et al., 2009) with very heterogeneous structures and different chromophore functional groups (Fiedler & Kroh, 2007) originated by the Maillard reaction (Venir et al., 2009), but also originated directly from by-products from yeast fermentation and contain large amounts of refractory organic nitrogen (Battimelli, et al., 2010). They are referred as the *non-enzymatic browning reaction* (Smaniotto et al., 2009), and formed primarily by interactions between carbohydrates, typically reducing sugars (Venir et al., 2009) or their degradation products (Morales, 2002) and compounds characterised by a free amino group (Venir et al., 2009; Morales, 2002) of an amino acid or in proteins mainly the ε-amino group of lysine, but also the α-amino groups of terminal amino acids (Martins et al., 2001). The resulting carbon chain is a cyclic based structure with nitrogen bound in amine rather than nitro forms (Ojijo et al., 2010; Dwyer & Lant, 2008). Melanoidins are negatively charged, highly coloured humic organics (Dwyer & Lant, 2008). Analytical separation of melanoidins has been attempted through gel or paper electrophoretic techniques according to the belief of an amphoretic nature. However, later studies using capillary zone electrophoresis (CZE), have shown that these compounds possess a partially anionic character in solution over a wide pH range (Morales, 2002). Therefore, due to the net negative charge of melanoidins, different heavy metal ions (Cu^{2+}, Cr^{3+}, Fe^{3+}, Zn^{2+}, Pb^{2+} etc.) form large complex molecules with melanoidins, amino acids, proteins and sugars, which in acidic medium are precipitated (Chandra et al., 2008). Melanoidins colour, nitrogen and carbon content is related to the degree of polymerisation, aromaticity and saturation that occurs as a result of reaction conditions (Dwyer & Lant, 2008). The colour intensity increases with the polymerisation degree. The degree of browning, usually measured via absorbance at 420 nm, is often used to follow the extent of the Maillard reaction (Coca et al., 2004). From the point of view of color, melanoidins can be built up of subunits in two contrasting ways. One possibility is that melanoidins are formed by more or less random reaction of low molecular weight reaction intermediates (which may inherently be colored or not). Alternatively, a repeating unit (which may be colorless or contribute little to color) may form the backbone of melanoidins, which chromogenic low molecular weight structures attaching themselves to this backbone, resulting

in high-molecular weight colored structures. The development of color is an extremely important and obvious feature of the extent of the advanced Maillard reaction. This stage characterized by the formation of unsaturated, brown nitrogenous polymers and copolymers, although nitrogenfree polymers are also formed from condensation reactions from furfurals or dehydroreductones (Kim & Lee, 2008) as it can be seen from Figure 6 (Martins et al., 2001). Depending on their molecular weight, melanoidins can be divided into two classes: low-molecular weight (LMW) below 1000 Da (Wilska-Jeszka, 2007), consisting of two to four linked rings that contain extended double-bond conjugation (Kim & Lee, 2008), and high-molecular weight (HMW) up to 150,000 Da (Wilska-Jeszka, 2007) possessing discrete chromophore groups (Kim & Lee, 2008). Considering that the molecular weight of melanoidins produced from Maillard reactions is highly dependant on the heating intensity, HMW melanoidins are produced at longer reaction periods (>24 h). It is possible that in the initial stages of the Maillard reaction, LMW chromophoric melanoidins are formed, which subsequently polymerise or cross-link with other MRP's to produce HMW melanoidins during the later stages of the Maillard reaction (Wang et al., 2011).

Melanoidins have been defined as nitrogen-containing brown polymers and can be considered as the final products of glycation (advanced glycation end products (AGE's), Hayase et al., 2008) formed either in foods or *in vivo*, for example, in diabetic subjects because of the high level of circulating glucose (Smaniotto et al., 2009). The formation mechanism of melanoidins is complex because of many reactants, such as osones, unsaturated osones, furfurals, pyrrolyl aldehydes (pyrraline) and carbonyl compounds are generated by cleavage of reducing sugars, and various amino compounds are involved (Hayase, 1996). The formation of melanoidins (elemental composition and structure of melanoidins, Cammerer et al., 2002) is estimated to be affected by various factors such as types of reducing sugars and amino compounds (Coca et al., 2004; Hayase, 1996), their concentrations (Venir et al., 2009; Smaniotto et al., 2009; Morales, 2002; Hayase, 1996), types of catalysts and buffers (Hayase, 1996), reaction temperature and time, pH, (Venir et al., 2009; Chandra et al., 2008; Smaniotto et al., 2009; Coca et al., 2004; Morales, 2002;

Hayase, 1996), water activity (Smaniotto et al., 2009; Coca et al., 2004; Morales, 2002), sugar reactivity, glass transition temperature (Venir et al., 2009) and the presence of oxygen and metals (Hayase, 1996) affecting the yield and type of products (Smaniotto et al., 2009). With increasing reaction time and temperature, the total carbon content increases at the cost of the nitrogen ratio, thus promoting the aromatic character and the unsaturation of melanoidins (Coca et al., 2004; Cammerer & Kroh, 1995). Heating of melanoidins (90^{o}C) under aerobic conditions not only causes decolourisation but also produces a development of fluorescence. A heat treatment at 90^{o}C, under anaerobic conditions, causes an increase in both colour and reductone content. The development of fluorescence does not take place under these conditions (Coca et al., 2004).

Melanoidins are similar to humic acids (also called as synthetic humic acids / substances, Cosovic et al., 2010; Obretenov & Verninb, 1998) in terms of their elemental composition, structure, chemical properties and environmental stability. Both are dispersed colloids possessing negative charge due to the dissociation of carboxylic and hydroxyl groups (Satyawali et al., 2010). At this point, it must be stated that ‘‘browning products” produced by Maillard reaction from sugars only, i.e., characterised by lack of nitrogen are called ‘‘pseudomelanoidins” (Cosovic et al., 2010).

The Maillard reaction has three basic phases (Fig. 6):

1. The initial reaction is the condensation of the carbonyl group of reducing sugars with a free amino acid group, which loses a molecule of water, to form N-substituted glycosylamine.
2. The unstable glycosylamine undergoes the Amadori rearrangement to form ketosamines (1-amino-1-deoxy-2-ketoses).
3. The ketosamine can then react in different ways: One is simply dehydration (loss of $2H_2O$ molecules) into reductones and dehydroreductones, which are powerful antioxidants. A second is the production of shortchain hydrolytic fission products, such as diacetyl, acetol, and pyruvaldehyde. A third path is the Schiff’s base or furfural

path. This involves the loss of $3H_2O$ molecules and then a reaction with amino acids and water. All these products react further with amino acids to form the brown nitrogenous polymers and copolymers called melanoidins (Wilska-Jeszka, 2007). The molecular weight of coloured compounds increases as browning proceeds (Coca et al., 2004).

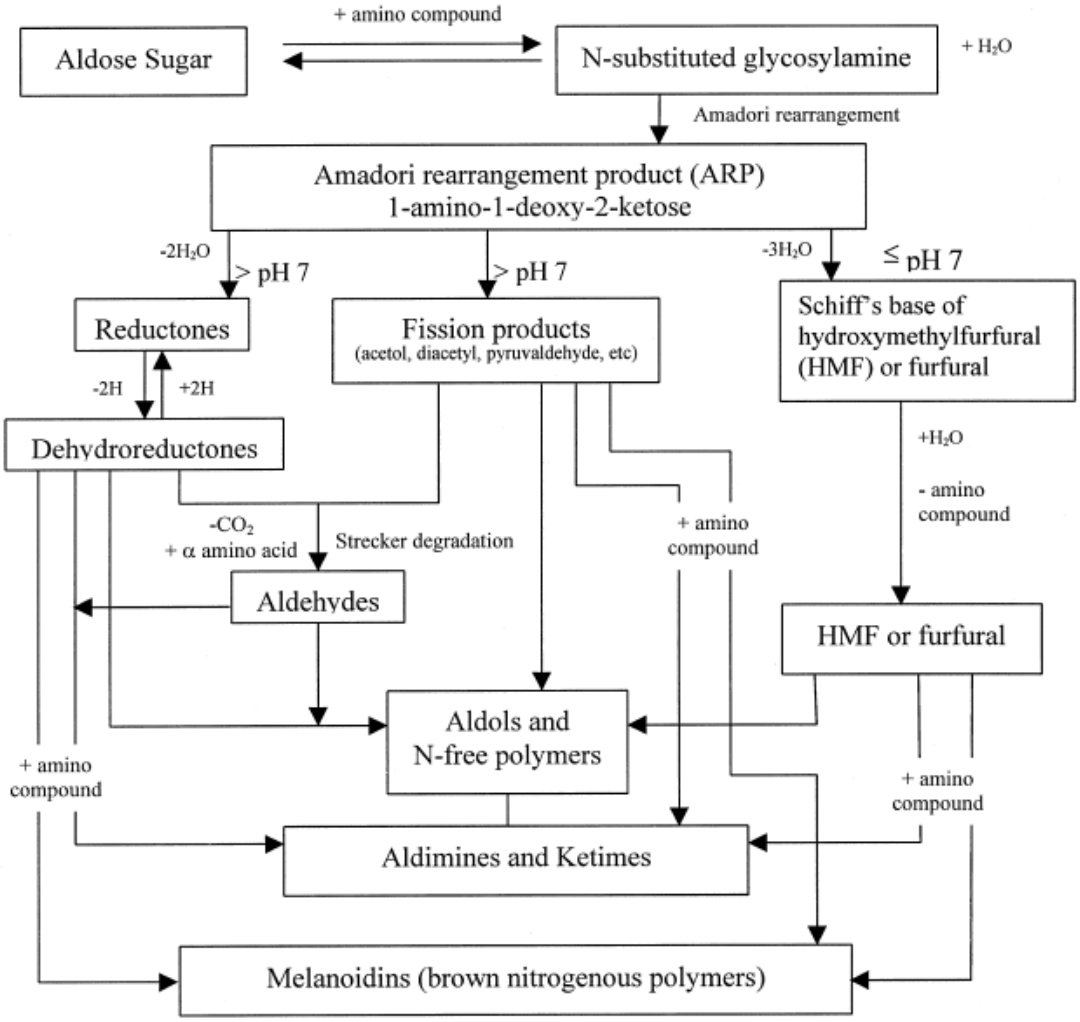

Figure 6: The maillard reaction scheme (Martins et al., 2001)

In model Maillard reaction systems, pH appears to be an important factor that influences the structure of the chromophoric melanoidins, while the reaction temperature and time dictate the molecular weight of melanoidins (Wang et al., 2011). The subsequent degradation of the Amadori product is dependent on the pH of the system. At pH 7 or below, it undergoes mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or

hydroxymethylfurfural (HMF) (when hexoses are involved) (Martins et al., 2001). At pH >7 the degradation of the Amadori compound is thought to involve mainly 2,3 enolisation, where reductones, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one (HMF^{one}), and a variety of fission products, including acetol, pyruvaldehyde and diacetyl are formed (Martins et al., 2001).

The references are scarce in the literature with regard to the physical structure of melanoidins possibly due to their complexity and heterogeneity (Venir et al., 2009). Despite extensive investigations using techniques such as infrared (IR) spectroscopy, mass spectrometry and advanced multidimensional nuclear magnetic resonance (NMR) spectroscopy analyses, the structures of melanoidins are still not completely understood (Dolphen & Thiravetyan, 2011). Many research efforts have been done to determine the structure and chemical properties of melanoidins but since none has been isolated and characterised yet, this information is still lacking. An approximation on its structure has been gained through the techniques mentioned previously (Morales, 2002). This is of relevance since some among the most important roles of melanoidins, as for most biopolymers, related to flavour and texture, are strongly affected by physical changes and structure transitions (Venir et al., 2009). Coca et al. (2004) reported that changing reaction conditions play an important role in the fundamental structure of melanoidins. This means that it cannot be assumed that melanoidins have a regular composition with repeating units. Moreover, Herzfeld et al. (2011) reported that the amorphous and insoluble nature of the melanoidin polymers has made them resistant to conventional structural characterization. Ojijo et al. (2010) reported that most of the studies about melanoidins have been done on model melanoidins, since natural and synthetic melanoidins both have similar elemental (CHON) compositions, spectroscopic properties and electrophoretic mobilities at various pH values.

There are currently three main proposals for the structure of melanoidins:

(a) a polymer consisting of repeating units of furans and/or pyrroles, formed during the advanced stages of a Maillard reaction, and linked by

polycondensation reactions (Fig. 7), (Brudzynski & Miotto, 2011; Cammerer et al., 2002). In other words, HMW coloured structures are formed by polymerisation of LMW Maillard reaction intermediates, such as furans, pyrroles, pyrrolopyrroles, and/or their derivatives, in the later stages of the reaction (Wang et al., 2011). Tressl & Rewici (1999) reported that in a previous research it was assumed that the LMW precursors; which channel the maillard reaction effectively and irreversibly into coloured macromolecules, will not be accumulated during the reaction cascade and must be associated with the most reactive intermediates. For several reasons, especially for the superior browning activity of pentoses and tetroses, research was focused on N-substituted C_4- and C_5-pyrrole and furan derivatives, which can easily be generated from pentoses, hexozes and disaccharides as shown in Figure 7.

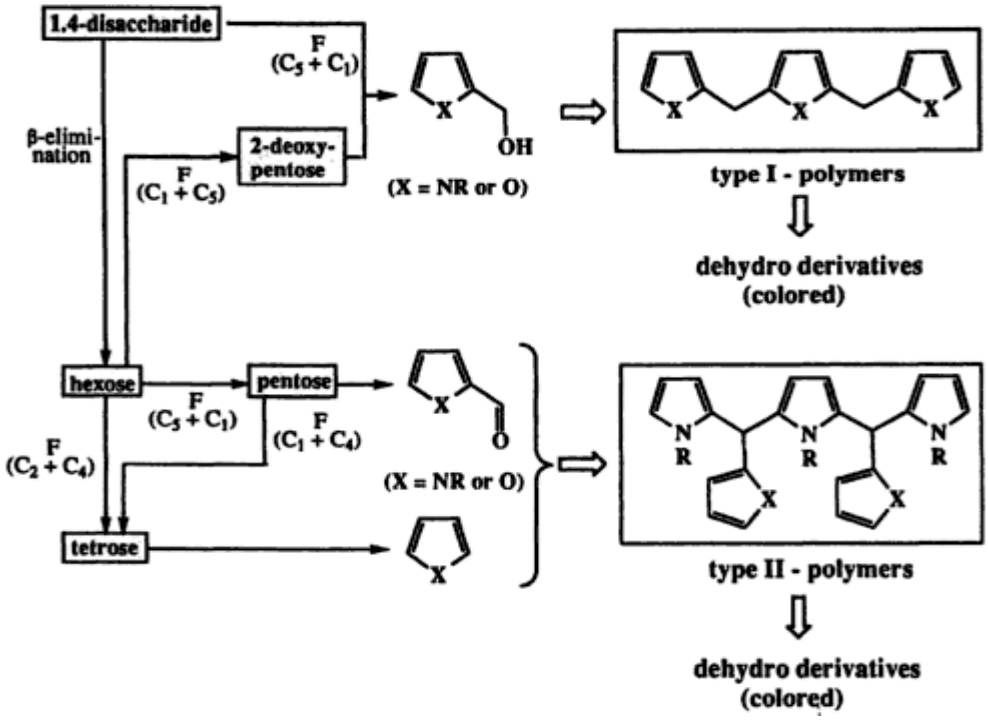

Figure 7: Routes to melanoidin-type polymers in sugar/amine model systems. Proposed structures for melanoidin polymers: linear and branched polymers in which bridging carbons link furan and pyrrole units (Nursten, 2005; Tressl & Rewici, 1999)

By the use of MALDI-TOF-MS (Nursten, 2005), linear oligomers (or the corresponding dehydro-oligomers) of up to a dodecamer were identified. However, Herzfeld et al. (2011) by making use of solid-state NMR methods,

including selective ^{13}C substitution, ^{1}H-dephasing and double quantum filtration showed that the spectra and their interpretation, are simplified by relying exclusively on hydronium for catalysis. The results for polymers derived from ribose, deoxyribose, and fructose indicating diverse pathways to furans, suggest a simple route to pyrroles in the presence of amines, and reveal a heterogeneous network-type polymer in which sugar molecules cross-link the heterocycles (Fig. 8). For example, the furans produced in browning are embedded in complex heteropolymers, rather than homopolymers of the sort as depicted in Figure 7.

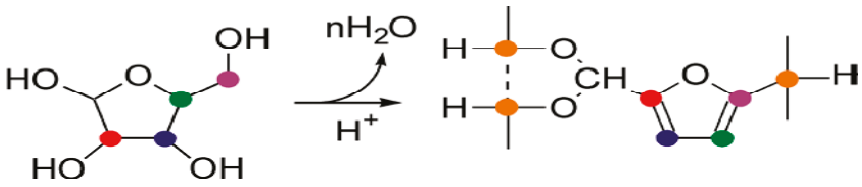

Figure 8: Heterogeneous network-type polymer in which sugar molecules cross-link the heterocycles (Herzfeld et al., 2011)

As it was mentioned above ‘‘browning products” produced by Maillard reaction from sugars only, i.e., characterised by lack of nitrogen are called ‘‘pseudomelanoidins”. Guan et al. (2011) reported that glucosone or 3-deoxyglucosone could form some furan and its derivatives such as carboxylic acid which might polymerize through esterification, oxidation and aldol condensation, which might form some non-nitrogen polymers (Fig. 6).

(b) low-molecular-weight colored substances, which were able to cross-link proteins via ε-amino groups of lysine or arginine to produce high-molecular-weight colored melanoidins and

(c) in a third structure proposal, the melanoidin skeleton is mainly built up of sugar degradation products, formed in the early stages of a Maillard reaction, polymerized through aldol-type condensation, and possibly linked by amino compounds (Brudzynski & Miotto, 2011; Cammerer et al., 2002). Guan et al. (2011) reported with certainty that the carbonyl compound reacts mainly via

the Amadori product to form several deoxyosones which are able to react with each other in an aldol-type condensation to form a basic melanoidin skeleton of amino-branched sugar degradation products. For example, the nucleophilic attack of the carbanion in the C3 position of a 3-deoxyhexosulose can take place on the C1 of another molecule of deoxyhexosulose. It has been demonstrated in aqueous systems that melanoidins are formed by aldol condensations of highly reactive a-dicarbonyl compounds, which are the main intermediates during the early stages of the Mallard reaction and partially branched by amino compounds (Wang et al., 2011).

Rajasundari & Murugesan (2011); Pant & Adholeya (2007) reported that from studies using ^{13}C and ^{15}N CP-NMR spectrometry, it has been confirmed the presence of olefinic linkages and conjugated enamines which were suggested to be important for the structure of the chromophores in melanoidin.

In spite of these studies, the melanoidins chromophore has not yet been identified. Hence, the chemical structure of the so-called melanoidin is still not clear but probably it does not have a definite one and there exist various types of melanoidins differing in structure depending on parent reactants and reaction conditions as pH, temperature and reaction time. Moreover, it further needs intensive investigations with more refined recent and advanced techniques for the elucidation of chromophore structure to deduce the main skeleton of melanoidin polymer (Chandra et al., 2008).

3.4 Enviromental hazards from molasses wastewater

Wastewaters containing molasses are generated by fermentation industries (such as ethanol production and bakery yeast processing), sugar mills and other molasses-based industries (Verma et al., 2011). These are characterized by large volumes of dark brown colored effluent with a high organic content (Satyawali et al., 2010). The resistance of melanoidins to degradation is apparent from the fact that these compounds escape various stages of wastewater treatment plants and finally enter into the environment (Mohana et al., 2009). Melanoidins are brown recalcitrant compounds present

in the effluents of the fermentation processes that use molasses as carbon source and are hardly biodegradable (Ojijo et al., 2010). Melanoidins in wastewaters released from these industries may cause aquatic pollution and several studies of the decolorization and degradation of melanoidins have been performed (Dolphen & Thiravetyan, 2011). When released in water bodies they prevent sunlight penetration and reduce both photosynthetic activity of aquatic plants and dissolved oxygen level of surface waters (Agarwal et al., 2010; Naik et al., 2010; Liang et al., 2010; Liang et al., 2009; Guimarães et al., 2005; Pena et al., 2003). This will therefore create an anaerobic condition thereby killing most of the aerobic marine animals (Özgunerge, 2008). Mohana et al. (2009) reported hematological alterations in fresh water catfish, *Channa punctatus*, exposed to distillery effluents. Moreover, they lead to the eutrophication of water courses due to their high pollution load (Rajasundari & Murugesan, 2011; Agarwal et al., 2010; Pant & Adholeya, 2007). From the presence of putriciable organics like skatole, indole and other sulphur compounds which are not decomposed by yeast during distillation, the molasses spent water that is disposed in canals or rivers produces obnoxious smell (Rajasundari & Murugesan, 2011; Jiranuntipon, 2009; Mohana et al., 2009; Pant & Adholeya, 2007).

When released in soil they reduce the soil alkalinity and manganese availability, inhibit seed germination and affect vegetation (Verma et al., 2011; Agarwal et al., 2010; Kanimozhi & Vasudevan, 2010). Further due to the possibility of complexation reaction of introduced melanoidin with metal ions, they could influence the biogeochemical cycle of many constituents in natural water, which are highly resistant to microbial attack (Agarwal et al., 2010). Besides causing unaesthetic discoloration of water and soil, melanoidin pigments are also toxic to microorganisms present in soil and water (Verma et al., 2011).

The effects of distillery wastewater are given in Table 3 (part of the table from Kanimozhi & Vasudevan, 2010).

Table 3: Effects of distillery wastewater

Study organism	*Effluent conc.*	*Effects studied*
Oryza sativa	5, 10, 20 and 50 (v/v)	Coleoptile emerged before the emergence of the root primordial. Greening of coleoptile delayed with increased effluent concentration
Cicer arietinum L	100% effluent concentration	No germination
Phaseolus mungo L	–	Heavy metal contents were increased in different parts of P.mungo. Zinc and Copper accumulation was maximum vs. other heavy metals
Mung bean (*Vigna radiata*)	20% (v/v)	Decreased activity of membrane transport enzymes, Alkaline phosphatase and Ca^{2+}-Mg^{2+}-ATPase Decline in membrane structural constituents – total hexose, sialic acid and phospholipids content
Vegetable crops (Tomato, Chilli, bottle gourd, Cucumber and Onion)	75% and 100%	Complete failure of germination

(Kanimozhi & Vasudevan, 2010)

3.5 Molasses wastewater treatment technologies

The objective of wastewater treatment process is to remove pollutants from wastewater to improve the wastewater quality to a point where it may be reused or disposed off without detrimental environmental impacts (Shaban et al., 2009). Most food processing wastewater contains biodegradable organic compounds which are commonly removed in biological processes such as anaerobic, aerobic and anoxic biodegradation (Battimelli et al., 2010). It has already been mentioned that conventional anaerobic-aerobic treatment processes can accomplish the degradation of melanoidins only up to 6% or 7%. As a result, the COD and color of the effluents after aerobic treatment can still highly exceed the national discharge regulation. Therefore, it is necessary to further remove these recalcitrant organic matters by polishing treatment. Several treatment processes have been used in decolorization of yeast

wastewater (Zhou et al., 2008). Majority of these methods remove colour by either concentrating the colour into sludge or by partial or complete breakdown of the colour molecules (Agarwal et al., 2010). The process of removing the contamination involves physical, chemical and biological processes and produces residues which must be managed by design a wastewater treatment station (Shaban et al., 2009).

Physical processes are widely used in the water and wastewater treatment plants. These physical techniques are based on the separation of one or more compounds from the waste stream. Because of the separation, the pollutant is transferred from one phase to another. Therefore, further treatment is required for the degradation of the contaminants in the second phase. Physical methods are employed mainly to separate large settleable and floating matter, clarify turbid solutions, recover and recycle valuable substances utilized in the main processes and separating inorganic materials. The conventional and advanced physical techniques include filtration, adsorption, gas stripping (Mohajerani et al., 2009), screening, sedimentation and skimming. Chemical treatment methods such as precipitation, pH adjustment, coagulation etc., remove toxic materials and colloidal impurities (Naik et al., 2008).

Biosorption, an alternative to physico-chemical treatment is recommended by several researchers for treatment of colored effluents. Live or dead microbial biomass of algae, yeast, bacteria and fungi has been used for this purpose (Verma et al., 2011). The same authors using heat-killed wet biomass of white-rot fungus decolorized the effluent further in a three-step technology for treatment of four molasses-based raw industrial effluents. Sequential steps involved in this treatment are; (1) sonication of the effluents, (2) whole-fungal treatment of these by a ligninolytic marine fungus (basidiomycetous fungus NIOCC #2a) and (3) biosorption of the residual color with heat-inactivated biomass of the same fungus.

Physico-chemical treatments which have been tested are evaporation (Ersahin et al., 2011a, b; Ojijo et al., 2010; Zhang et al., 2009; Jimenez et al., 2003) - condensation with or without combustion (Zhang et al., 2009; Jimenez

et al., 2003), thermochemical liquefaction (Oller et al., 2011), coagulation/flocculation (Gengec et al., 2011; Dolphen & Thiravetyanb, 2011; Liang et al., 2009) using various coagulants like aluminum sulfate ($Al_2(SO_4)_3 \cdot 14H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$), sodium aluminate, aluminum chloride and ferric sulfate (Prasad & Srivastava, 2009), adsorption using activated carbon (Dolphen & Thiravetyanb, 2011; Zhou et al., 2008; Mutlu et al., 2002), coal fly ash (Ojijo et al., 2010; Prasad & Srivastava, 2009), organic resin (Verma et al., 2011) or chitin nanofibers (Dolphen & Thiravetyanb, 2011), membrane-based pressure driven processes such as microfiltration (MF) (Shi et al., 2009; Mutlu et al., 2002), ultrafiltration (UF), nanofiltraton (NF), (Zhou et al., 2008; Mutlu et al., 2002) and reverse osmosis (Gengec et al., 2011) (Fig. 9), electrocoagulation (Gengec et al., 2011; Battimelli et al., 2010),

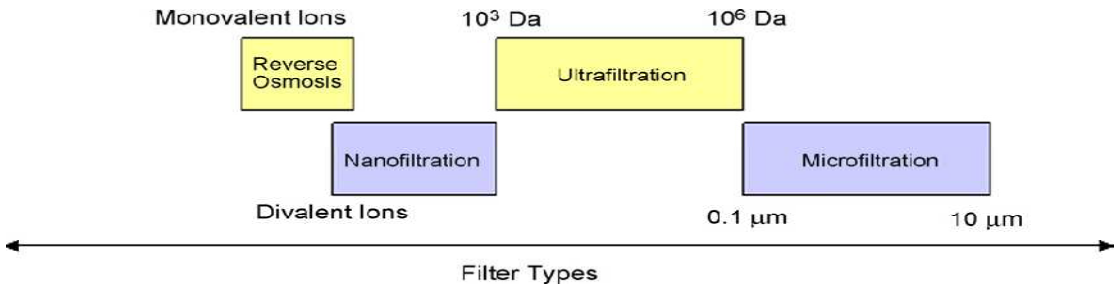

Figure 9: Membrane options and appropriate pollutant 'size' ranges (Ryan et al., 2009)

internal-electrolysis (Shi et al., 2009), oxidation and advanced oxidation processes (AOP's) such as ozonation, Fenton reagent (hydrogen peroxide activated with Fe(II) salts, Anjaneyulu et al., 2005), UV/H_2O_2-based oxidation (Battimelli et al., 2010), ultrasound (sonication) (Verma et al., 2011), wet-air oxidation processes (WAO), (Oller et al., 2011), or known as thermal liquid-phase oxidation (Ryan et al., 2009), (supercritical water oxidation (Liang et al., 2009)), oxidation using hypochlorite, hydrogen peroxide (H_2O_2), (Gengec et al., 2011; Zhou et al., 2008; Mutlu et al., 2002) and UV radiation (Satyawali et al., 2010). Alternative physico-chemical treatment methods which have been tried more recently are photocatalytic oxidation, electro-oxidation, catalytic thermolysis, electro-coagulation (EC) and electrofenton (EF) processes.

These advanced oxidation processes could result in a decrease in the COD of up to 97% and result in a 92% reduction of the color present in melanoidin-containing distillery wastewater (Satyawali et al., 2010). Satyawali et al. (2010) also examined electrolytic treatment combined with activated carbon adsorption for the removal of melanoidins.

The combination of chemical and biological processes offers an advantageous alternative for COD removal and profitable reuse (Battimelli et al., 2010). ‘’To improve treatment efficiency, chemical oxidation may be coupled with a filtration step prior to biological treatment, as illustrated in Figure 10. The rationale behind this is that the partially oxidized effluent is passed over a membrane module, where larger (and less biodegradable) molecules are recycled into the chemical oxidation reactor, while the permeate containing the smaller (and potentially more biodegradable) compounds is fed to the biological treatment. In this respect, process efficiency increases since membrane selectively retains large molecules to undergo further chemical oxidation’’ (Mantzavinos & Psillakis, 2004).

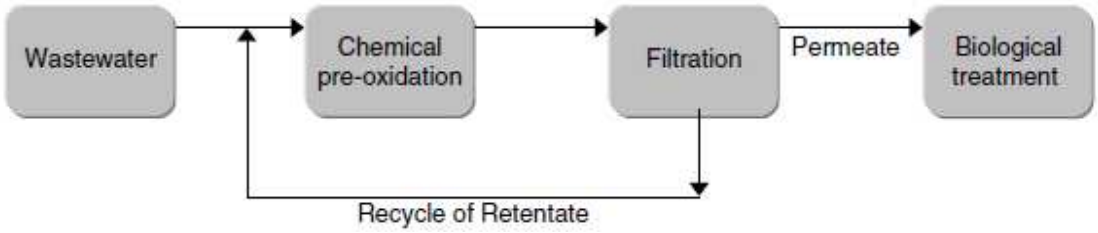

Figure 10: Coupling chemical, physical and biological treatment (Mantzavinos & Psillakis, 2004)

3.5.1 Biological treatment methods

Biological treatment techniques are classified into two main groups: aerobic and anaerobic. Aerobic processes could be carried out by suspended (activated sludge), attached (biofilm reactor, trickling filter, and rotating disk contactor) or combined (moving bed biofilm reactor) depending on the operating conditions and wastewater characteristics. Wastewater can also be treated by anaerobic processes such as up-flow anaerobic sludge blanket

(UASB), anaerobic fluidized bed reactor (AFBR), expanded granular sludge bed (EGSB), and anaerobic baffled reactor (ABR). Anaerobic techniques are usually employed for treating a concentrated municipal and industrial wastewater (Mohajerani et al., 2009).

Naik et al. (2008) reports that ‘’physical or chemical methods of decolorization are invariably cost intensive and can not be employed in industries. Hence, in recent years, the importance of biological wastewater treatment systems has attracted the attention of workers all over the world and has helped in developing efficient low cost waste treatment systems’’. Sirianuntapiboon & Prasertsong (2008) reported that most of Thai-alcohol factories have attempted to treat molasses wastewater by anaerobic methods such as anaerobic pond, anaerobic contact digester or UASB systems and followed by aerobic treatment such as activated sludge system, aerated lagoon or oxidation pond. But by these conventional treatment processes, most of melanoidin pigment in molasses wastewater still remained and the COD of the treated wastewater was higher than the standard permission value. So, increased attention has been directed towards utilization of microbial activity for the mineralization and decolorization of spentwash (Naik et al., 2008).

Biological decolourisation using fungi such as Coriolus, Aspergillus, Phanerochaete (Chandra et al., 2008), Rhizoctonia (Nakajima-Kambe et al., 1999) and certain bacterial sp. as Bacillus, Alkaligenes and Lactobacillus have been successfully achieved and thus can be applied as bioremediation techniques (Chandra et al., 2008). Nakajima-Kambe et al. (1999) reported that biological treatments using these fungi degrade molasses pigment aerobically or anaerobically under mesophilic or thermophilic conditions. The decolorization rates of these aerobic and anaerobic microorganisms have been reported to be 60-90% and 28%, respectively. However, the long growth cycle and spore formation limit the performance of the fungal system. In contrast, bacterial decolourization is usually faster, but it may require a mixed culture to decolourize molasses wastewater through combined metabolic modes of bacterial strains (Jiranuntipon et al., 2009).

Several studies regarding the degradation of melanoidins, humic acids, and related compounds by microorganisms have also suggested the participation of different categories of enzymes (Chandra et al., 2008). There are a good number of reports showing the role of fungi in the decolourization of melanoidins by adsorption to mycelia as well as the role of ligninolytic enzymes (Jiranuntipon et al., 2009). Generally, fungi are known for their ability to remove different pollutants either by excreting a protein to chelate it, excreting an enzyme that can decompose or degrade the pollutants (Gad & Sayaad, 2010). Bioremediation of melanoidin-containing waste waters with white-rot fungi and their lignin-degrading enzymes have been reported. These ligninolytic enzymes are non-specific in their substrate requirement (Verma et al., 2011).

3.5.1.1 Anaerobic treatment process

The final objective of the biological treatment of wastewater is the transformation of dissolved and particulate organic constituents into acceptable end products such as carbon dioxide, methane and new organic materials. In industrial wastewater, some of the constituents may be toxic to microorganisms (melanoidins in our case), so some type of pre-treatment could be required prior to the biological treatment (Korsak, 2008).

Under anaerobic conditions, organic pollutants in wastewater are degraded by wastewater microbes, producing methane (CH_4) and carbon dioxide (CO_2) (Fung & Liu, 2001). The removal and stabilization of dissolved and particulate carbonaceous organic matter found in wastewater are carried out biologically using a variety of micro-organisms, principally bacteria. In anaerobic digestion, these micro-organisms convert organic matter into simple end products and additional biomass following the general equation for anaerobic biological degradation (Korsak, 2008):

$$\text{organic matter} + \text{nutrients} \xrightarrow{\text{Bacteria}} \text{new cells} + CH_4 + CO_2$$

Capareda (2011) reported that during anaerobic digestion it is produced a combustible gaseous fuel comprising primarily of methane and carbon dioxide and traces of other gases such as nitrogen (N_2) and hydrogen sulphide (H_2S). The gaseous mixtures is commonly termed “biogas”. Virtually all nitrogen (N), phosphorus (P) and potassium (K) remain in the digested biomass. The percentage of methane in the final mixture has been reported to vary between 50 to 80%. A typical mixture consists of 65% methane and 35% CO_2 with traces of other gases.

The degradation process can be highly effective. It produces only 5-10% of sludge as compared to the more conventional aerobic processes, and thus saves considerably cost associated with the sludge disposal (Fung & Liu, 2001). Kanimozhi & Vasudevan (2010) reported that 50% of the COD is converted to sludge after aerobic treatment. In contrast, anaerobic treatment converts over half of the effluent COD into biogas (methane). The biogas thus generated can be utilised for steam generation in boilers, thereby meeting the energy demands of the unit. In addition, since it does not require aeration, anaerobic process also saves substantial amount of cost associated with aeration, including equipment, maintenance and energy consumption. The aspects of energy saving/production and minimum maintenance, make anaerobic process particularly attractive to the developing countries (Fung & Liu, 2001). Anaerobic digestion is particularly suitable for winery and distillery wastewater because their COD/N/P ratio is unbalanced for aerobic treatments, which needs phosphorus and nitrogen addition (Kanimozhi & Vasudevan, 2010).

In an assessment for ‘’The Global Methane Initiative’’ (2011) it is reported that because of its high BOD concentration and a favorable BOD:N:P ratio of 100:2.4:1, distillery spent wash is especially suitable for anaerobic treatment prior to land application for disposal, or as a pretreatment process prior to aerobic treatment if surface water discharge is the only wastewater disposal option. Conventional anaerobic lagoons (Figure 11) are the simplest choice for distillery spent wash treatment but are significant sources of methane emissions.

However, despite of the advantages, there are also disadvantages. Anaerobic digestion systems are rather complex processes that unfortunately often suffer from instability. Such instability is usually witnessed as a drop in the methane production rate, a drop in the pH, a rise in the volatile fatty acid (VFA) concentration, causing digester failure. It is caused by (a) feed overload, (b) feed underload, (c) entry of an inhibitor, or (d) inadequate temperature control. The usual remedy, is a rapid increase in the HRT (hydraulic retention time), and when this fails, the digester has to be primed with sludge from a "healthy" digester. This, however, may be quite costly, in view of the fact that anaerobic digestion is a very slow process (Lyberatos & Skiadas, 1999).

Figure 11: Anaerobic Lagoon in a Distillery in India (‘’The Global Methane Initiative’’, 2011)

A list of the disadvantages of anaerobic treatment of wastewaters is presented below:

- Anaerobic bacteria grow slowly resulting in longer HRT, (Mohana et al., 2009), so long periods of time are required for reactor start-up, and recovery from upsets (Chiu, 1990). In order to solve these problems, several high rate configurations have been developed for treating soluble wastewater at relatively shorter HRTs (Jiranuntipon, 2009; Mohana et al., 2009).

- Anaerobic digestion is very sensitive to organic shock loadings (Mohana et al., 2009) and environmental - operating factors such as toxic compounds and pH (Chiu, 1990).
- Anaerobic treatment is only a front-end treatment process; post-treatment of the treated wastewater (further treatment with an aerobic treatment process) is needed to meet disposal requirements (Özgunerge, 2008; Chiu, 1990).
- Treatment of sulfate rich wastewater: The presence of sulfate not only reduces the methane yield but also inhibits the methanogens due to sulfide production (Özgunerge, 2008). Anaerobic digestion is one of the treatment methods that can be applied to baker's yeast wastewater. However, studies have shown that high sulfate levels in the wastewater caused problems in the anaerobic digestion (Lo & Liao, 1990). A high sulphate content can lead to the destabilization of the anaerobic treatment processes due to the hydrogen sulphide formation, especially if $COD/(SO_4)^{2-}$ is below 10 (Krapivina et al., 2007). A variety of approaches for controlling sulfate problems in the anaerobic process have been reported (Lo & Liao, 1990).
- Treatment of high protein & nitrogen containing wastewater: The anaerobic degradation of proteins produces amines, which are no longer, is degraded anaerobically. Similarly, nitrogen remains unchanged during anaerobic treatment (Özgunerge, 2008). The baker's yeast industry represents the main source of residual nitrogen compounds in wastewater effluents (Ifrim et al., 2008).
- Need for additional nutrient requirements (Satyawali et al., 2010; Özgunerge, 2008) / trace metal requirements: Anaerobic microorganisms especially methanogens have specific nutrients (e.g. Fe, Ni and Co) requirement for optimum growth (Özgunerge, 2008).
- More experience needs to be gathered with the application of the process to direct treatment of wastewaters (Chiu, 1990).

Anaerobic digestion is viewed as a complex ecosystem in which physiologically diverse groups of microorganisms operate and interact with

each other in a symbiotic, synergistic, competitive and antagonistic association. In the process methane and carbon dioxide are generated (Mohana et al., 2009). The process takes place in an enclosed reactor in absence of oxygen (Marti, 2008), where degradation of organic materials occurs through 4 stages, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Appels et al., 2008) as it is depicted in Figure 12. Anaerobic digestion requires strict anaerobic conditions (oxidation reduction potential (ORP) < -200 mV) to proceed, and depends on the coordinated activity of a complex microbial association to transform organic material into mostly CO_2 and methane. Despite the successive steps, hydrolysis is generally considered as rate limiting (Appels et al., 2008). This is caused by a low biodegradability of the cell walls and extra cellular biopolymers in sludge. It is important to reduce the amount of sludge produced and to reduce its residual organic content (Farooq et al., 2009).

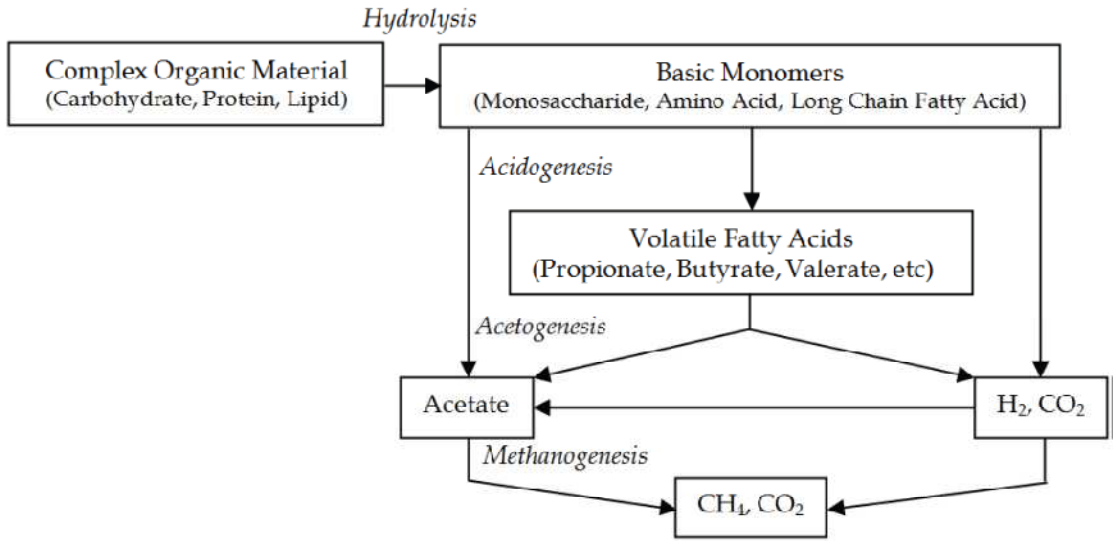

Figure 12: Steps of anaerobic digestion process (Ersahin et al., 2011a)

In the first stage, facultative hydrolytic bacteria using extracellular enzymes hydrolyse particles and complex molecules (proteins, carbohydrates and lipids) to soluble compounds (amino acids, sugars (e.g. glucose) and long chain fatty acids (LCFA) respectively, (Marti, 2008). Chemical oxygen demand (COD) reduction does not occur in this step (Özgunerge, 2008). Hydrolysis step can be merely biological (using hydrolytic microorganisms) or combined: bio-chemical (using extracellular enzymes), chemical (using catalytic reactions) as well as physical (using thermal energy and pressure) in nature

(Zupančič & Grilc, 2011). An approximate chemical formula for the mixture of organic waste is $C_6H_{10}O_4$.

A hydrolysis reaction where organic waste is broken down into a simple sugar, in this case glucose, can be represented by the following reaction (Ostrem, 2004):

$$C_6H_{10}O_4 + 2H_2O \rightarrow C_6H_{12}O_6 + 2H_2$$

The distribution of organic macromolecules like proteins, fats and carbohydrates in the feedstock is of great importance, as their degradation leads to the formation of Volatile Fatty Acids (VFA), the main substrates for bacteria of the last two stages of anaerobic digestion. In particular, high fat contents increase VFA considerably, whereas high protein content leads to large amounts of ammonia (NH_3). VFA and ammonia are not only formed through bacterial metabolism during degradation. They can already be present in considerable amounts in the influent, depending on the type of feedstock. Distillation slops and evaporation condensates may contain very high amounts of VFA (Steffen et al., 1998).

The simpler organics are then fermented to organic acids and hydrogen by the fermenting bacteria (acidogens). The volatile organic acids are transformed into acetate and hydrogen by the acetogenic bacteria (Ersahin et al., 2011a). Biological oxygen demand (BOD) and chemical oxygen demand (COD) are reduced through these pathways (Ostrem, 2004). Archael methanogens use hydrogen and acetic acid produced by obligate hydrogen producing acetogens to convert them into methane. Methane production from acetic acid and from hydrogen and carbon dioxide is carried out by acetoclastic methanogens and hydrogenotrophic methanogens, respectively (Ersahin et al., 2011a). It is estimated that 60 to 70% of methane production in an anaerobic waste reaction is through conversion of acetic acid and the rest through carbon dioxide reduction by hydrogen (Ofoefule et al., 2011). Both carbon dioxide reducing and aceticlastic methanogens play an important role in maintaining the stability of the digester. The failure in an anaerobic digester

can occur if carbon dioxide reducing methanogens fail to keep pace with hydrogen production (Mohana et al., 2009). Thermodynamic conditions play a key role in methane formation. Therefore, appropriate environmental conditions should be provided in order to carry out acetogenesis and methanogenesis, simultaneously (Ersahin et al., 2011a). The remaining, non-digestible organic and mineral material, which the microbes cannot feed upon, along with any dead bacterial residues constitutes the solid digested (Zupančič & Grilc, 2011).

Although anaerobic digestion can be considered to take place in these four stages, all processes occur simultaneously and synergistically, in as much as the first group has to perform its metabolic action before the next can take over, and so forth (Ostrem, 2004).

Wilkinson (2011) reports that ‘’anaerobic digestion systems usually operate either in the mesophilic (35-40ºC) or the thermophilic temperature (50-60ºC) ranges. Operating in the thermophilic temperature range reduces hydraulic retention time (HRT or treatment time) to as low as 3-5 days and more effectively contributes to the sanitisation of the organic waste streams (i.e. improves pathogen and weed-seed destruction). However, greater insulation is necessary to maintain the optimum temperature range, and more energy is consumed in heating thermophilic systems. Larger, centralised systems typically run at thermophilic temperatures. Mesophilic systems need a longer treatment time to achieve good biogas yields but these systems can be more robust than thermophilic systems’’.

3.5.1.1.1 Single-phasic and biphasic anaerobic systems

Anaerobic systems can be operated as single-phase or two-phase systems. Single-phase systems involve only one reactor for the microorganisms to digest the organic matter, whereas two-phase systems separate the acidogenic and methanogenic organisms into two separate reactors (Mohana et al., 2009).

Most anaerobic systems consist of a single-stage digester, which means that all stages take place in the same reactor. In such situation, environmental conditions (i.e. pH, redox potential, temperature, etc.) may favour the development of a certain group of bacteria, but it is important to maintain equilibrium to ensure a balanced degradation process. For this reason, the control of environmental conditions is a key factor, especially regarding methanogenic microorganisms, which are strict anaerobes, with the lowest growth rate and are the most sensitive to sudden changes in environmental conditions (Marti, 2008).

3.5.1.1.2 Types of anaerobic digesters

3.5.1.1.2.1 High rate anaerobic reactors

From the 1970s onwards, the discovery of high rate reactors (which separates the HRT from the solids retention time SRT) increased the popularity of anaerobic systems as a cost effective alternative for wastewater treatments. These high rate reactors include: UASB reactors; contact reactors with bio-physical filters; microbial film expanded bed (MFEB) reactors, biofilm reactors (Gie, 2007), anaerobic fluidized bed reactors, anaerobic batch reactors and anaerobic fixed film reactors (Mohana et al., 2009).

The retention of a considerable amount of active biomass as happens in a number of reactor such as up-flow anaerobic blanket reactor (UASB), anaerobic baffled reactor (ABR) and upflow anaerobic sludge fixed film reactor (UASFF) is known as granulation. These anaerobic granules harbor several metabolic groups of microorganisms, including hydrolytic, fermentative, syntrophic, and methanogenic microorganisms, involved in the anaerobic degradation of complex organic compounds (Tabatabaei et al., 2011). The feasibility and efficiency of anaerobic granules in UASB reactors for removing biodegradable organic matter from municipal and industrial wastewater have been successfully demonstrated since 1980. The success of the UASB depends on the formation of active and settleable granules (Zhang et al., 2011).

3.5.1.2. Aerobic treatment

3.5.1.2.1 Process Description

Aerobic treatment processes take place in the presence of air and utilize those microorganisms (also called aerobes), which use molecular/free oxygen to assimilate organic impurities i.e. convert them into carbon dioxide, water and biomass (Mittal, 2011). In Figure 13 it is depicted the aerobic treatment principle.

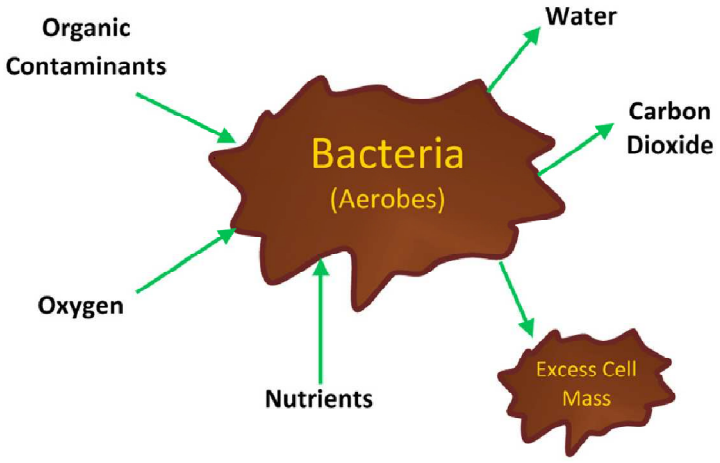

Figure 13: Aerobic Treatment Principle (Mittal, 2011).

In Figure 14 is presented an alternative classification of aerobic reactors, based on the state of biomass fixation. The major difference with relation to

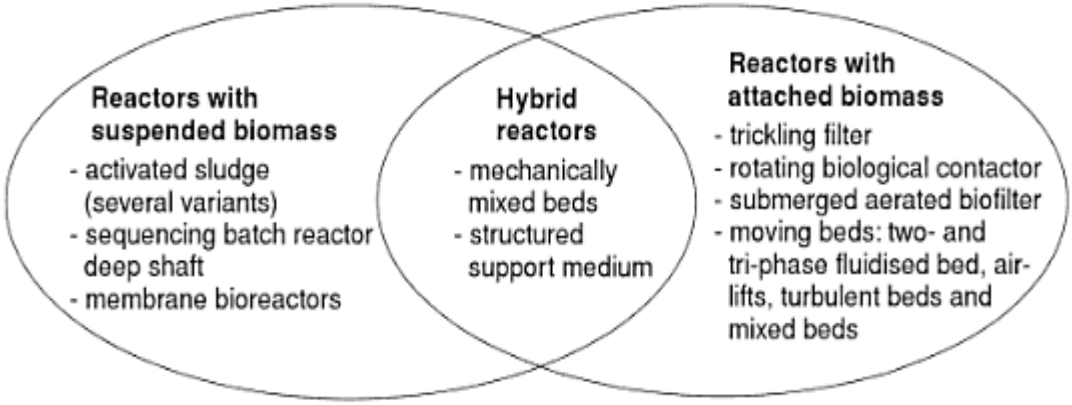

Figure 14: Modern classification of mechanised aerobic treatment processes, with respect to the state of the biomass (von Sperling, 2007).

old similar classifications is the group of hybrid reactors, which incorporate suspended biomass and fixed biomass in the same reaction volume. The processes with suspended biomass involve several variants of activated sludge. Among the hybrid processes, there are those with the support medium mechanically mixed and with structured supports inserted in the aeration tank. Both are variants of the activated sludge systems (von Sperling, 2007). There are multitudes of aerobic biological treatment processes and technologies in literature and practice; however, for the purpose of this thesis, the following five biological treatment technologies are described.

3.5.1.2.2 Suspended Growth Systems

3.5.1.2.2.1 Conventional Activated Sludge Process (ASP) System

This is the most common and oldest biotreatment process used to treat municipal and industrial wastewater. Typically wastewater after primary treatment i.e. suspended impurities removal is treated in an activated sludge process based biological treatment system comprising aeration tank followed by secondary clarifier. The aeration tank is a completely mixed or a plug flow (in some cases) bioreactor where specific concentration of biomass (measured as mixed liquor suspended solids (MLSS) or mixed liquor volatile suspended solids (MLVSS) is maintained along with sufficient dissolved oxygen (DO) concentration (typically 2 mg/l) to effect biodegradation of soluble organic impurities measured as biochemical oxygen demand (BOD_5) or chemical oxygen demand (COD). The aeration tank is provided with fine bubble diffused aeration pipework at the bottom to transfer required oxygen to the biomass and also ensure completely mixed reactor. Roots type air blower is used to supply air to the diffuser pipework. In several older installations, mechanical surface aerators have been used to meet the aeration requirement. The aerated mixed liquor from the aeration tank overflows by gravity to the secondary clarifier unit to separate out the biomass and allow clarified, treated water to the downstream filtration system for finer removal of suspended solids. The separated biomass is returned to the aeration tank by means of return activated sludge (RAS) pump. Excess biomass (produced

during the biodegradation process) is wasted to the sludge handling and dewatering facility (Fig. 15), (Mittal, 2011).

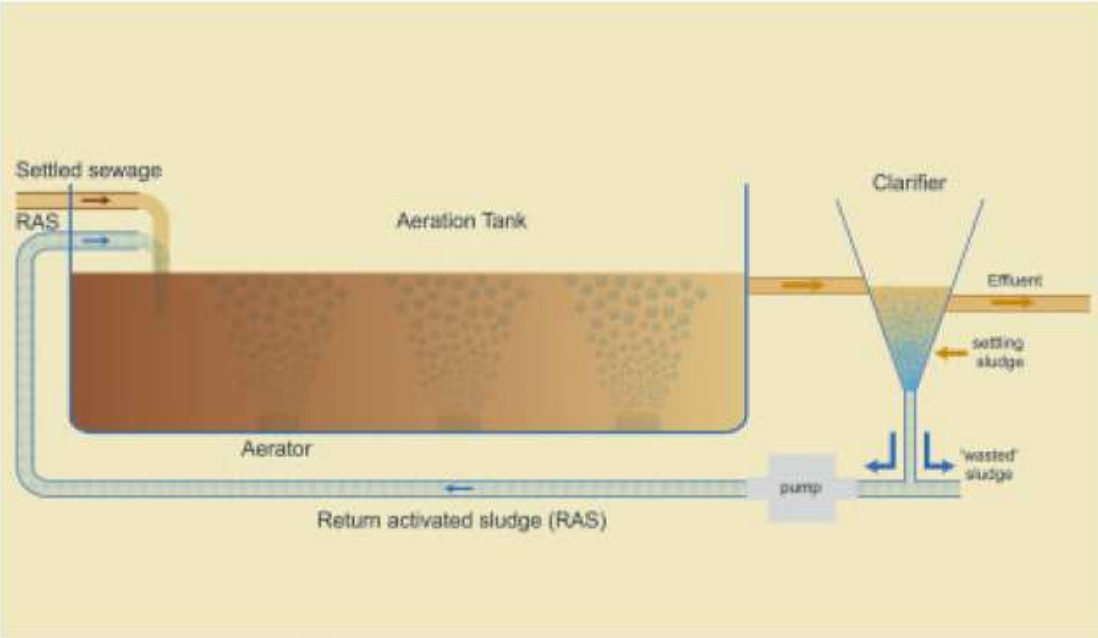

Figure 15: Layout of a plug-flow plant. In its passage through the aeration tank, there is an increase in the activated sludge biomass, as a result of bacterial growth. At the end of the tank, the mixed liquor passes to the clarifier, to allow the sludge to settle. Some sludge is recycled back to the beginning of the tank. The rest, corresponding to the net new biomass, is 'wasted' i.e. dewatered and dried (Davies, 2005).

3.5.1.2.2.2. Sequencing Batch Reactor Systems (SBRs) or Periodic Processes

One cycle of SBR operation has five basic modes; fill, react, settle, decant, and idle.

• ***Fill*** Raw wastewater that has been through primary treatment is added to the reactor. During this phase, aeration may or may not be supplied to provide alternating periods of high or low dissolved oxygen. This mode may occupy 25% of the total cycle time.

• ***React*** Aeration is provided in an effort to obtain rapid biodegradation of organic and nitrogenous compounds. This mode will typically require about 35% of the total cycle time.

• ***Settle*** Aeration is shut off to allow the wastewater to become anoxic (for

denitrification) and to allow for quiescent conditions that allow very effective liquid-solid separation. Clarification will usually take about 20% of the overall cycle time.

• ***Draw*** Clarified supernatant is removed. The decanting is accomplished using adjustable weirs, floating weirs, and submersible pumps. Periodically the excess biosolids must be removed. Decanting generally takes about 15% of the total cycle time.

• ***Idle*** Time is allowed for the first reactor to complete its full cycle, and then switch the flow into the second reactor for parallel operation (Seabloom & Buchanan, 2005).

An important element in the SBR process is that a tank is never completely emptied, rather a portion of settled solids are left to seed the next cycle. This allows the establishment of a population of organisms uniquely suited to treating the wastewater. By subjecting the organisms to periods of high and low oxygen levels, and to high and low food availability - the population of organisms becomes very efficient at treating the particular wastewater (Seabloom & Buchanan, 2005).

3.5.1.2.2.3. Membrane Bioreactor (MBR)

A membrane bioreactor (MBR) combines the activated sludge process with a membrane separation process. The reactor is operated similar to a conventional activated sludge process but without the need for secondary clarification and tertiary steps like sand filtration. Low-pressure membrane filtration, either microfiltration (MF) or ultrafiltration (UF), is used to separate effluent from activated sludge. The two main MBR configurations involve either submerged membranes or external circulation (side-stream configuration) (Fig. 16), (Melin et al., 2006).

3.5.1.2.2.3.1 Membrane Classification

The membrane process is a very important separation process in water and wastewater technology, which becomes increasingly competitive and is superior to the traditional water technology with proven performance and process economics. The most widely applied membrane separation

processes are microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO), electrodialysis (ED) and electro deionization (EDI), whereas the first four processes produce permeate and concentrate. The separation ranges are as follows: 100 to 1000 nm for MF, 5 to 100 nm for UF, 1 to 5 nm for NF, and 0.1 to 1 nm for RO. Membranes are usually made from different plastic and ceramic materials, but metallic membranes also exist. The most widely used materials are celluloses, polyamides, polysulphone, charged polysulphone and other polymeric materials such as polyacrylonitrile (PAN), polyvinylidene difluoride (PVDF), polyethylsulphone (PES), polyethylene (PE), and polypropylene (PP). All of these polymeric materials have a desirable chemical and physical resistance (Radjenovic et al., 2008).

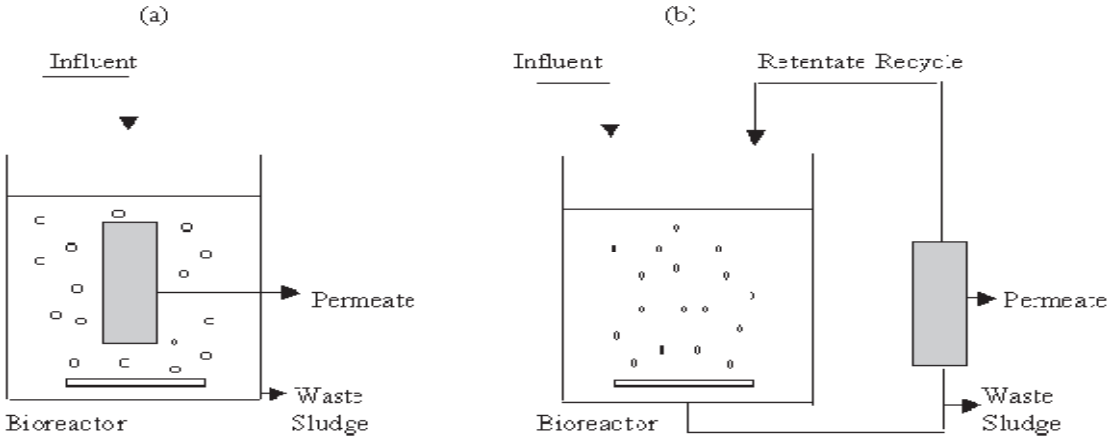

Figure 16: Configuration of MBR systems: (a) submerged MBR, (b) side-stream MBR configuration (Melin et al., 2006).

3.5.1.2.3. Attached Growth and Hybrid Systems

3.5.1.2.3.1 Integrated Fixed-Film Activated Sludge (IFAS)

Integrated fixed-film activated sludge (IFAS) is any suspended growth system that incorporates an attached growth media within the suspended growth reactor in order to increase the amount of biomass in the basin. IFAS systems have higher treatment rates than suspended growth systems and generate sludge with better settling characteristics. Many types of fixed and floating media are available, including:

• **Rope**: also called looped-cord or strand media. Consists of a polyvinyl chloride-based material woven into rope with loops along the length to provide

surface area for the biomass. Proprietary designs include Ringlace, Bioweb, and Biomatrix.

• **Moving Bed with Sponge:** proprietary products include Captor and Linpor.

• **Plastic Media:** several types of free-floating plastic media are available from Kaldnes. Other media types include fabric mesh (e.g., AccuWeb) and textile material (Cleartec), (EPA, 2009).

3.5.1.2.3.2. Moving-Bed Biofilm Reactor (MBBR)

The moving-bed biofilm reactor (MBBR) is similar to the IFAS system in that it uses plastic media with a large surface area to increase biomass within the biological reactor. The MBBR media is submerged in a completely mixed anoxic or aerobic zone. The plastic media are typically shaped like small cylinders to maximize surface area for biomass growth. The difference between MBBR and IFAS is that MBBR does not incorporate return sludge (EPA, 2009). The MBBR process will be described in a separate paragraph for the purposes of this thesis.

3.5.1.3 Plastic biofilm carriers or biocarriers / Moving Bed Biofilm Reactor (MBBR)

Biofilm is a structured community of microorganisms within a self-developed polymeric matrix attached to a surface. In wastewater treatment the biofilm will consist of a mixed culture of biomass dependent upon the substrate supply (Colaco, 2009).

The application of biofilm processes for biological wastewater treatment, as well as other chemical processing, is becoming more popular because of the benefits offered by biofilms. Benefits of biofilm processes occur because the active biomass is built up and maintained in the reactor through attachment to solid surfaces. Hence, the high biomass concentrations which allow large volumetric loadings and good effluent quality are maintained without the need for solids separation and solids recycling (Rittmann, 1982).

Moving bed type reactors are bio-film processes utilizing small, plastic biomass carriers that mix in the water. The use of biomass carriers is

becoming common world-wide, for many applications, some of which are: compact low operation units, industrial wastewater roughing filters, retrofit and upgrade of municipal wastewater treatment plants (Shechter & Stamper, 2012). Wastewater treatment that have biofilm growth on the surface of moving carriers within the reactor tank are called suspended carrier biofilm processes (SCBPs) or moving bed biofilm reactors (MBBRs). These processes are shown to overcome problems such as clogging, which conventional biofilm processes, such as biorotors, biofilters, trickling filters and packed bed reactors are often reported to have (Sankaran, 2012) Design and operation allow manipulation of the nature of the bio-film that develops on the protected surfaces of the bio-mass carriers, in order to achieve the required process performance (Shechter & Stamper, 2012).

Full-scale and lab-scale applications using different types of biocarriers for treating various kinds of wastewater have repeatedly demonstrated enhanced performances in comparison with that of the traditional activated sludge process (Levstek & Plazl, 2009). In the moving-bed biofilm reactor process, the carriers are suspended and moving through out the entire water volume of the reactor either pneumatically or mechanically (Colaco, 2009) and retained by a sieve placed at the reactor outlet (Levstek & Plazl, 2009). The system can be used either for aerobic or anoxic processes. In aerobic processes the biofilm carriers are kept in suspension by the agitation created by air from aeration diffusers, while in anoxic processes a mixer keep the carriers in movement. A schematic of the principle in an implementation of the AnoxKaldnes™ MBBR technology is shown below (Fig. 17). Due to erosion caused by frequent collision between the plastic elements, very little biofilm grows on the outside surface of carrier elements, however, floating microorganisms appear to have some effect on the efficiency of organic removal (Hosseiny & Borghei, 2002). Nicolella et al. (2000) refer that biofilm detachment, the interphase transport of biomass from an attached microbial film to the bulk liquid phase, has generally been attributed to four different processes including grazing (the consuming of bacteria from the outer surface of the biofilm by protozoa), sloughing (the periodic loss of large patches of

biofilm), erosion (the continuous removal of small particles from the surface of the biofilm, primarily caused by liquid shear stress), and abrasion (analogous to erosion, but caused by collisions of particles). Among the mechanisms controlling biofilm reactor performance, biofilm detachment is one of the least studied and understood. The biofilm detachment rate is a complicated function of many variables, including hydrodynamics of the liquid flow, biofilm morphology, and support characteristics (Nicolella et al., 2000).

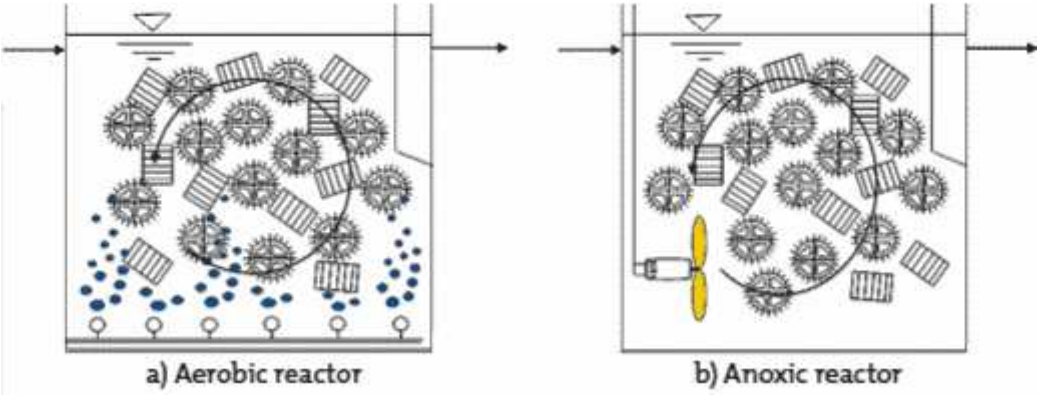

Figure 17: *Schematic showing the principle of the MBBR* (AnoxKaldnes, 2012)

Biomass grows attached to the surfaces of the carriers, while excess sludge detaches from the carrier and is separated from the water downstream of the MBBR without any return of biomass (as in the activated sludge process) to the bioreactor (Levstek & Plazl, 2009). The carriers are designed to provide a large protected surface area for the biofilm and optimal conditions for the bacteria culture when the carriers are suspended in water (AnoxKaldnes, 2012). Carriers can differ from each other in material composition, shape, specific surface area and treatment capabilities (Levstek & Plazl, 2009). Investigations on the shape and size effect of carrier made it clear that the key factor in the design of a moving bed biofllm process for organic matter removal is the effective surface area where biomass may grow. The size and shape of carrier may have an influence on this effective area. The design of process should be based on organic surface area removal rate (Ayati et al., 2007).

AnoxKaldnes has developed several types of carriers with different shape, size and surface area. This gives us the flexibility to use the best suitable carrier depending on wastewater characteristics, pretreatment, discharge standards and available volumes. Currently there are five different types of carriers: K1, K3, BiofilmChip™ M, BiofilmChip™ P and F3 (Fig. 18).

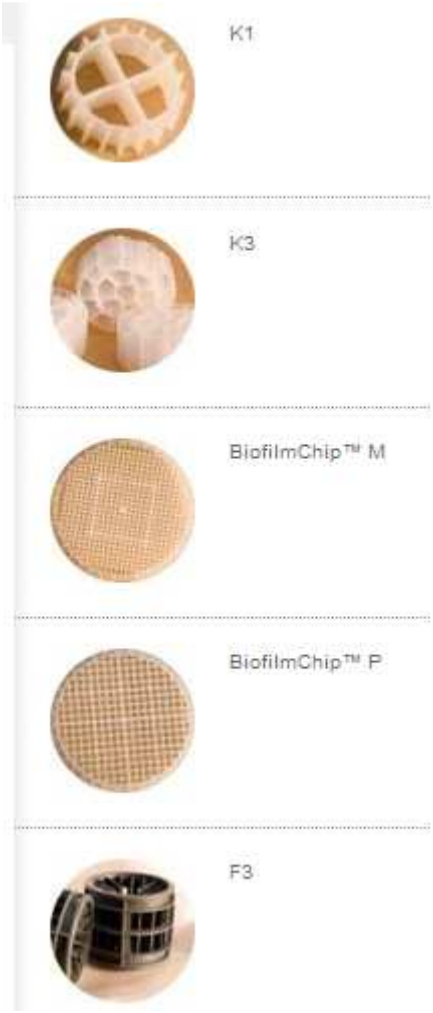

Figure 18: Types of carriers (AnoxKaldnes, 2012)

Biofilm carrier elements (referred to as biocarriers) are made of polyethylene, which has a density slightly less than water. Shaped like a small cylinder each biocarrier is approximately 10 mm in diameter and 7 mm height. Internal cross members installed in each biocarrier increase the available interior surface area for biofilm attachment. Longitudinal fins incorporated on the external surface of the biocarrier increase the external surface area available for biofilm growth (Hewell, 2011).

Bertino (2010) & Colaco (2009) report that the carriers for year 2006 are made of polyethylene (PEHD) with a density of 0.95 g/cm^3, which allow easy movement of the carrier material in the completely mixed tanks. The small difference from the water density avoids negative buoyancy effects (Bertino, 2010). Sheli & Moletta (2009; 2007) refer that the density of the media with biofilm should be similar to the density of water (density of one) in order to spend less energy for agitation. Some of the different carrier models are presented in Table 4 for the year 2006. The total surface area (Table 4) consists of both inner and outer surface while the protected surface area is the effective internal area where the biofilm seems to attach and grow as shown in Figure 3. The performance of a biofilm reactor is primarily dependent upon the biofilm growth surface area ($kg_{substrate}/m^2_{biofilm\ area}/d$) in the reactor and not on the reactor volume (Bertino, 2010).

Table 4. AnoxKaldnes MBBR biocarriers year 2006.

Model	Length (mm)	Diameter (mm)	Protected surface (m^2/m^3)	Total surface (m^2/m^3)
K1	7	9	500	800
K3	12	25	500	600
Natrix C2	30	36	220	265
Natrix M2	50	64	200	230
Biofilm-Chip M	2.2	48	1200	1400
Biofilm-Chip P	3.0	45	900	990

Colaco, 2009

In order to be able to move the carrier suspension freely, it is recommended that filling fractions of biofilm carriers in the reactor should be below 70% of the useful volume (Colaco, 2009). The carriers can occupy up to 70% of the reactor volume on a bulk volume basis but experience has shown that mixing efficiency decreases at higher percentage fills (Bertino, 2010).

The AnoxKaldnes™ MBBR technology is particularly suited for the removal of BOD and biodegradable COD in a very compact environment, whether it is for the treatment of municipal wastewater or of almost any type of industrial effluents.

The heterotrophic bacteria grow as a biofilm on the different types of AnoxKaldnes carriers in a continuously aerated MBBR reactor. Because the carriers are always maintained in suspension due to the intensity of the aeration, and because of the specific design of the carriers themselves, the bacteria are always in contact with the substrate that they need to degrade as well as the oxygen they need to degrade it. BOD and biodegradable COD removal performances of the AnoxKaldnes MBBR technology can be adapted depending on the specific plant requirements and configuration. Anything between 30% and >95% removal can typically be achieved with the choice of the right type of process and the right set-up (AnoxKaldnes, 2012).

The theoretical description of biofilm systems is considerably more complex than that of dispersed cultures, in part due to the reaction processes occurring within the biofilm region where substrate diffusion is of concern. Biofilm thickness on the carriers depends on organic loading, shear forces, temperature and oxygen concentration (Levstek & Plazl, 2009).

As noted above, biofilms are communities of microorganisms growing on surfaces. The biocarriers “carry” the microorganisms throughout the reactors. Figure 19 a & b shows an AnoxKaldnes K1 biocarrier with biofilm growth (Hewell, 2011).

a b

Figure 19: a) Biocarrier with Biofilm, b) Biofilm on a Biocarrier (Hewell, 2011)

The microorganisms in the biofilms are essentially the same as those in suspended activated sludge wastewater treatment systems. Most of the microorganisms in the biofilm are heterotrophic (they use organic carbon to create new biomass), with facultative bacteria predominating. Facultative

bacteria can use the dissolved oxygen in the mixed liquor or, if dissolved oxygen is not available, they will utilize the available nitrate/nitrite as electron acceptors. At the surface of the biofilm is a stagnant liquid layer that separates the biofilm from the moving mixed liquor in the reactor. Nutrients and oxygen diffuse across the stagnant liquid layer from the moving mixed liquor to the biofilm. While nutrients (substrates) and oxygen diffuse through the stagnant layer to the biofilm, biodegradation products diffuse outward from the biofilm to the moving mixed liquor. These “back and forth” diffusion processes are continuous. Figure 20 above shows these diffusion processes (Hewell, 2011).

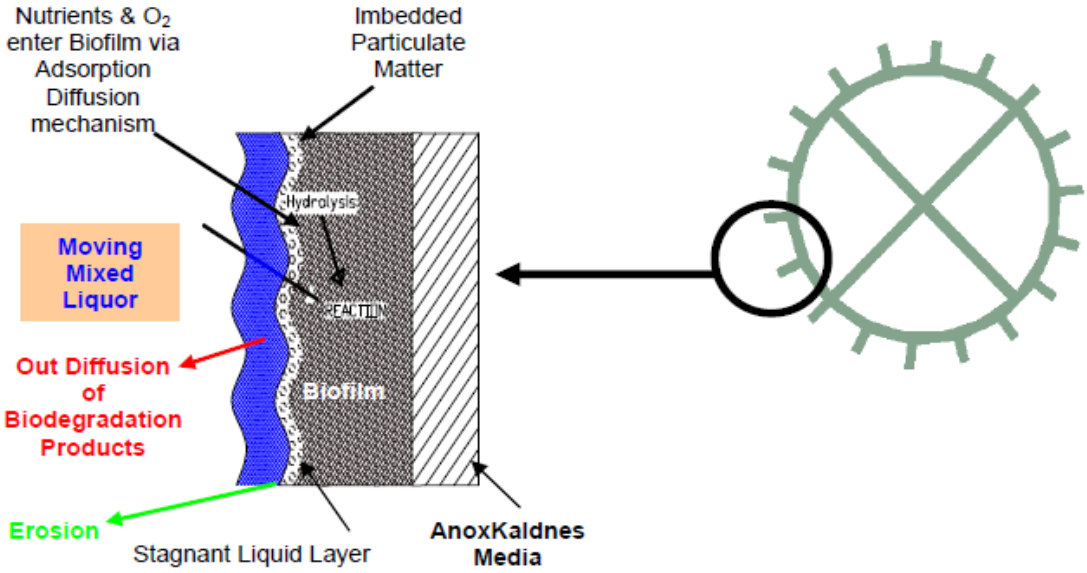

Figure 20: Nutrient Path through a Biofilm (Hewell, 2011)

Several parameters affect how quickly biofilms form and mature, including surface, cellular, and environmental factors. The surface onto which cells will attach has an important impact on biofilm formation. Rough surfaces tend to enhance biofilm formation. Shear forces are lower near a rough surface, and there is a larger surface area to which cells can adhere (Qureshi et al., 2005). Bolton et al. (2006) inform that surface roughness appears to promote biofilm accumulation by providing a larger surface area for attachment and by providing sheltered anchoring points that protect the biofilm from fluid shearing and collision. Bolton et al. (2006) presented a procedure for evaluating and comparing the biological activity of biofilms attached to various biofilrn carriers by measurement of the glucose consumption rate. This

technique allows for the economical design and selection of small particulate biofilm carriers that will maximize substrate removal when used in industrial-scale fluidized bioreactors. Results showed that the accumulation of biofilm depended most strongly on carrier surface properties, such as surface roughness and specific surface area. The biofilm activity as measured by glucose consumption rate correlated well with activity determinations made by COD measurements when a complex carbohydrate was used as substrate in place of glucose. Substrate consumption rates in microreactors were within ±43% of those measured in a 3-L bioreactor.

The pH of the liquid phase may affect the extent of adsorption of bacteria to solid surfaces. The influence of pH on bacterial adsorption depends on the nature of the bacterial surfaces and ionic strength in the solution. pH and ion concentration affect bacterial zeta potential because of the dissociation of carboxylic and amino groups located on the bacterial cell wall. The importance of pH in determining the surface charge increases at low ionic strength. Increased retention of bacteria has been found to occur when pH was decreased from 9.3 to 3.9. For most bacterial species, the isoelectric point lies between pH 1.5 and 4.0, but the surface charge is influenced by growth phase and growth rate. Bacteria in a natural environment (pH about 7) normally have a net negative surface potential (negative zeta potential). For pH values in the solution that are lower than the pzc (point of zero charge), the surface charge is positive, and becomes increasingly positive with decreasing pH-values. At pH-values higher than pzc of the surface, the charge is negative and becomes more negative at higher pH-values. However, the bacterial surfaces may have cellular appendages that are positively charged. There are some conflicting results, with respect to pH. These are probably due to the different iso-electric points and variability in charges around the iso-electric point for different bacterial species (Kristian Stevik et al., 2004).

Biofilm formation also tends to increase with the hydrophobicity of the surface material. Biofilms form much more rapidly on Teflon and other plastics than glass or metal. Possibly this is due to differences in hydrophobicity of the surfaces and ionic charges. The amount of nutrients present in the medium

can affect the rate of biofilm formation. Biofilms tend to form more readily in the presence of ample nutrients. Phosphorus is a particularly important nutrient. Cells saturated with phosphate have a higher tendency to flocculate and adhere due to their increased hydrophobicity, while those cells depleted in phosphate are more hydrophilic and less likely to adhere (Qureshi et al., 2005).

Temperature can have an effect on biofilm formation. Temperatures at the high end of a culture's growth range can enhance biofilm formation. Depending upon the species involved, high temperature increases the rate of cell growth, extracellular polymeric substances (EPS) production, and surface adhesion, all of which enhance biofilm formation (Qureshi et al., 2005). Also these suspended carrier processes can accommodate higher organic loading than conventional mesophilic aerobic processes, such as ASPs (Activated Sludge Processes). The rate of diffusion of organic molecules tends to increase at elevated temperatures. Apparently, an increase in temperature increases the removal rate on the carrier surface area, into a biofilm. Biofilm processes have been shown to be an attractive alternative to upgrade activated sludge processes, owing especially to their high loading capacity and short HRTs (hydraulic retention times), (Sankaran, 2012). The SCBP process is less sensitive to variation in process conditions and has been shown operable at 55^{o}C in laboratory studies (Suvilampi et al., 2003b). Suvilampi et al. (2003a) studied a combined thermophilic (55^{o}C) – mesophilic (35^{o}C) wastewater treatment using a laboratory-scale thermophilic activated sludge process followed by mesophilic ASP or a thermophilic suspended carrier biofilm process (SCBP) followed by mesophilic ASP, both systems treating diluted molasses (dilution factor 1:500 corresponding GF/A-filtered COD (COD_{filt}) of 1900±190 mg/l). With hydraulic retention times of 12–18 h the thermophilic ASP and thermophilic SCBP removed 60±13% and 62±7% of COD_{filt}, respectively, with HRT of 8 h the removals were 48±1% and 69±4%. The sludge volume index (SVI) was notably lower in the thermophilic SCBP (measured from suspended sludge) than in the thermophilic ASP. Under the lowest HRT the mesophilic ASP gave better performance (as SVI, COD_{filt}, and COD_{tot} removals) after the thermophilic SCBP than after the thermophilic

ASP. Measured sludge yields were low (less than 0.1 kg suspended solids (SS) / kg $COD_{filt\ removed}$) in all processes. Both thermophilic treatments removed 80–85% of soluble COD (COD_{sol}) whereas suspended COD (COD_{susp}) and colloidal COD (COD_{col}) were increased. Both mesophilic post-treatments removed all COD_{col} and most of the COD_{susp} from the thermophilic effluents. In conclusion, combined thermophilic–mesophilic treatment appeared to be easily operable and produced high effluent quality. Kristian Stevik et al. (2004) also refer that adsorption of bacteria is substantially greater at higher temperatures. The reduction in attachment with decreasing temperature may have several causes: (a) enhancement in the viscosity of the bacterial surface polymer and of the liquid, (b) reduced chemisorption and certain types of physical adsorption and (c) changes in the physiology of the organisms.

Cellular factors may also affect biofilm formation. A hydrophobic cell will be more able to overcome the initial electrostatic repulsion with the solid surface and adhere more readily. The presence of fimbriae, proteinaceous bacterial appendages high in hydrophobic amino acids, can increase cell surface hydrophobicity. Flagellated cells show increased ability to attach to surfaces. Flagellar motility may serve to overcome initial electrostatic surface repulsion (Qureshi et al., 2005).

Proper design of the biocarriers is critical to successfully ensure efficient mass transfer of both substrate (food) and oxygen to the biofilm microorganisms. Selection of which biocarrier is employed in the MBBR is an engineering design decision. Typical design factors considered in selecting the type of biocarrier include (Hewell, 2011):

- Influent concentrations
  - BOD/COD
  - TSS
  - Nitrogen
  - Phosphorous
  - Alkalinity
- Operating temperature

• MBBR Treatment process selected
• Existing basin configuration (when applicable).

3.5.1.4 Cofermentation – wood chips as agricultural solid wastes

As it already has been mentioned, due to the hard nature of melanoidins, conventional waste water treatment process are unable to remove the color from the molasses wastewater which has then the potential to block out light from contaminated waterways and reduce both photosynthetic activity of aquatic plants and dissolved oxygen level of surface waters. However, microbial and physical de-colorization by agro-industrial wastes were tried; wheat straw, corn cob shred and wood chips or even seeds as Moringa oleifera (Gad & Sayaad, 2010). It has been shown frequently that the performance of digesters could be considerably improved by means of co-substrate addition (Steffen et al., 1998). Wilkinson (2011) reports that a key factor in the economic viability of agricultural anaerobic digestion plants is the biogas yield (often expressed as m^3 biogas produced per kg of volatile solids (VS) added). Co-digestion of sewage sludges with agricultural wastes or municipal solid wastes can improve the methane production of anaerobic digestion processes and has been recently reviewed (Ward et al., 2008). The main issue for co-digestion process lies in balancing several parameters in the co-substrate mixture: macro- and micronutrients, C:N ratio, pH, inhibitors/toxic compounds, biodegradable organic matter and dry matter. Optimum values of C:N and COD:N ratios of 20 and 70, respectively, have been suggested for the stable performance of anaerobic digestion. However, lower values of C:N ratios (between 6 and 9) have been reported as suitable for the anaerobic digestion of nitrogen-rich waste (Alvarez et al., 2010) – the baker's yeast industry represents the main source of residual nitrogen compounds in wastewater effluents (Ifrim et al., 2008) –. Microorganisms generally utilise carbon and nitrogen in the ratio of 25–30:1, but C:N ratios can often be considerably lower than this ideal, for example sewage sludge has a C:N ratio of approximately 9:1. Co-digestion of a low C:N ratio feedstock with a high C:N ratio feedstock such as biomass can adjust the ratio closer to ideality (Ward et al., 2008). Moreover, Monou et al. (2009) refer that the sensitivity of

the anaerobic digestion process may be improved by combining several waste streams. The successful combination of different wastes apart from increasing biogas production, can also improve the nutrient content of the digestate enhancing its value as a fertiliser. Furthermore, potentially negative effects of toxic compounds on the codigestion process may be reduced.

Gad & Sayaad (2010) found that chemically untreated wood chips, corn cob shred and sugarcane bagasse were effective in molasses waste water color removal although Crini (2006) stated that chemical pretreatment of sawdust has been shown to improve the sorption capacity and to enhance the efficiency of sawdust adsorption. Low et al. (2000) inform that various species of local wood chemically modified with *N*-(3-chloro-2-hydroxypropyl)-trimethylammonium chloride (quaternized wood) showed sorption enhancement for Hydrolyzed Reactive Blue 2 (HRB) compared to the untreated samples. Equilibrium studies showed that quaternized Simpoh had a maximum sorption capacity, based on the Langmuir isotherm model, of 250 mg/g for HRB. Under continuous flow conditions HRB could be successfully removed. A sample of textile waste containing a complex mixture of dyes was treated efficiently by quaternized Simpoh under batch conditions. On the other hand, Forss & Welander (2009) studied the decolourization of a mixture of 200 mg/L each of Reactive Black 5 and Reactive Red 2 dye in batch experiments using microorganisms growing on forest residue wood chips in combination with or without added white-rot fungus, Bjerkandera sp. BOL 13. Forest residue wood chips contain a mixture of fungi and bacteria which is an advantage when complex molecules should be degraded. The wood chips furthermore provide the microorganisms with carbon source which make the addition of e.g. glucose unnecessary. The results showed that the microorganisms growing on the forest residue wood chips decolourized the mixture of the two dyes; adding extra nutrients approximately doubled the decolourization rate. Moreover, Bazrafshan et al. (2006) determined the optimum conditions of co-composting of dewatered sewage sludge and sawdust. The results of this study showed that after about 15 days, temperature of the mixture reached up 55^{o}C, and remained stable for fifteen days. Humidity, organic matter, organic carbon and C/N ratio of the mixture

decreased during of the study, due to increasing the temperature. Also organic matter and humidity mainly decreased in thermophilic phase. Organic compounds and pathogenic microorganisms reduced and EPA standards were met during this method.

The sorption mechanisms of sawdust can be explained by the presence of several interactions, such as complexation, ion-exchange due to a surface ionisation, and hydrogen bonds (Crini, 2006). Poots et al. (1976) by investigating the ability of spruce wood to adsorb Telon Blue (Acid Blue 25) proposed a mechanism which probably involves external surface adsorption with a limited amount of adsorption within a small zone in an area confined to the outer shell of the wood particles. However, MacKay (2008) refers that the effectiveness of wood particles to take up dissolved contaminants from the aqueous phase depends on the relative rate of contaminant diffusion to sorption sites inside the wood tissue. Partitioning of organic compounds into wood is proportional to the lignin content of the wood tissue. The uptake capacity of dissolved organic compounds by wood is expected to continue over long times in the field. Wood degradation processes preferentially deplete cellulosic components, leaving lignin structures to undergo very slow transformation. Additionally, colonization of wood surfaces by bacteria populations may facilitate the transformation of entrapped organic contaminants to less toxic compounds. Moreover, the growth of biofilms on the wood surface may provide additional sorption sites for dissolved contaminants (MacKay, 2008). Furthermore, the rate of contaminant removal from the aqueous phase is inversely related to the wood particle size and uptake rates are inversely proportional to compound solubilities (MacKay, 2008). The melanoidin solubility depends on the pH, it is less soluble at acidic pH than at alkaline pH (Miranda et al., 1996). Thus, poor uptake of large molecular weight compounds may be limited by slow diffusion rates (MacKay, 2008). It should be mentioned that depending on their molecular weight, melanoidins can be divided into two classes: low-molecular weight (LMW) below 1000 Da (Wilska-Jeszka, 2007), consisting of two to four linked rings that contain extended double-bond conjugation (Kim & Lee, 2008), and high-molecular weight (HMW) up to 150,000 Da (Wilska-Jeszka, 2007) possessing

discrete chromophore groups (Kim & Lee, 2008). Increased wood particle size is also expected to slow the uptake rate of dissolved metals into wood; however, no studies have yet proposed a mechanistic model that would enable prediction of metal diffusion rates in wood tissues (MacKay, 2008). Poots et al. (1976) investigated the ability of spruce wood to adsorb Telon Blue (Acid Blue 25) as it has already been mentioned. No pretreatment was required and the wood was left to equilibrate to a fixed moisture content prior to sieving and experimental work. The results show that acid dye adsorption on wood is quite successful, although longer contact times are required to reach equilibrium. The authors examined the contact times from the point of view of wood particle size. The smallest particle size appears to have reached equilibrium after 3h while the 710-1000μm particles have not achieved saturation after 6h as it can be seen from Figure 21. Anjaneyulu et al. (2005) also reported that due to hardness of the wood chips, they require longer contact times.

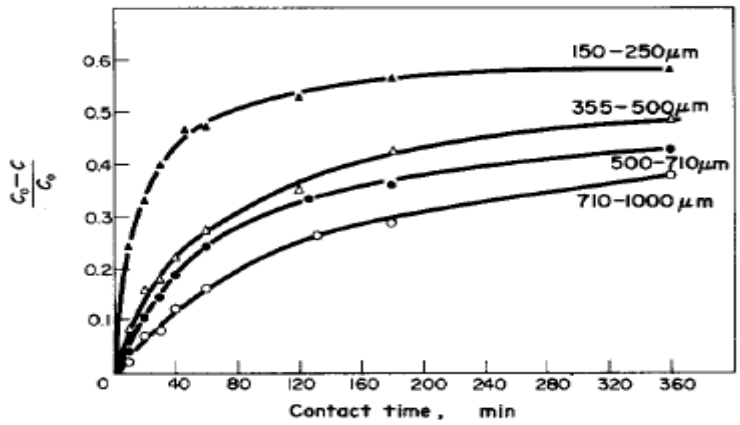

Figure 21: Series of contact time curves at different wood particle sizes for an initial dye concentration of 100ppm (Poots et al., 1976).

Wood chips show a good adsorption capacity for acid dyes like other available sorbents (Anjaneyulu et al., 2005). However, Crini (2006) refers that one problem with sawdust materials is that the sorption results are strongly pH-dependent. There is a neutral pH beyond which the sawdust will be either positively or negatively charged. The same author reports that the sorption capacity of basic dye is much higher than that of acid dye because of the ionic charges on the dyes and the ionic character of sawdust.

Finally, the changing properties of the wood material that occur during exposures to environmental conditions may also influence contaminant retention. Sunlight, moisture content and temperature can promote chemical or microbial alterations of the wood tissue. The ratio of wood components (lignin, cellulose, extractables) could change over time resulting in changes in the availability of sites to sorb dissolved contaminants. The development of small fissures in wood particles could increase accessibility of internal sorption sites for dissolved contaminants, or create more surface roughness to retain particles earned e.g. in storm water (MacKay, 2008).

3.5.2 Ultrasound (sonication)

Research into the use of ultrasound in environmental protection has received a considerable amount of attention with the majority of investigations focusing on the harnessing of cavitational effects for the destruction of biological and chemical pollutants in water (Mason & Lorimer, 2002). The sonochemical degradation of a variety of water contaminants (chlorinated and aromatic hydrocarbons, dyes, surfactants, pesticides, herbicides) have been successfully proven in bench-scale experiments (Destaillats et al., 2001). However, the field is not restricted to water decontamination and it is much broader (Mason & Lorimer, 2002). Ultrasonic irradiation can remove surface contamination and biofilms, enhance soil washing, remove chemical and biological contamination from water, control airborne pollution and accelerate the anaerobic digestion of sewage sludge (Wu et al., 2011; Mason and Lorimer, 2002).

Ultrasound is the term that is used to describe sound energy at frequencies above 20 kHz, i.e., above the range normally audible to human beings (Show et al., 2010), Fig. 22. Ultrasound is usually generated by a transducer, which converts mechanical or electrical energy into high-frequency vibrations. Ultrasound energy can be delivered into a fluid system via a horn or probe (Show et al., 2010). Ultrasonic frequencies in the range 100-1000 kHz have been shown to be more practical than lower frequencies around 20 kHz. At higher frequencies, cavitation is produced in the liquid phase far from the

surface of the transducer, thus protecting it from the mechanical erosion generated by bubble implosion. Transducers, which operate at 20 kHz, must be periodically replaced due to this problem (Destaillats et al., 2001).

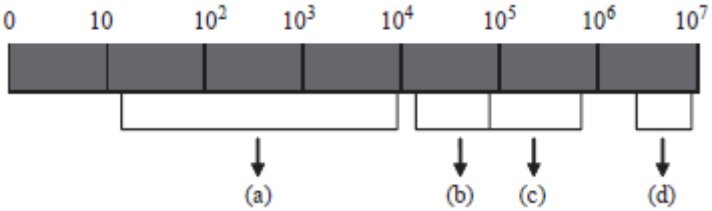

Figure 22: Frequency ranges of sound (a) human hearing: 16 Hz-18 kHz; (b) conventional power ultrasound: 20 kHz – 100 kHz, between (b) and (c) extended for special applications: 20 kHz-1MHz; (d) diagnostic ultrasound: 5 MHz- 10 MHz (Demirdöven and Baysal, 2009).

Acoustic cavitation is the result of pressure variation in a liquid when high-frequency sound waves (ultrasound) pass through it. During the compression cycle the average distance between the molecules decreases, while during the rarefaction cycle it increases. If a sufficiently large negative pressure is applied to the liquid so that the average distance between the molecules exceeds the critical molecular distance required to hold the liquid intact, cavities or voids can be created (Gogate & Pandit, 2000).

Cavitation is defined as the phenomena of the formation, growth and subsequent collapse of microbubbles or cavities occurring in extremely small interval of time (milliseconds), releasing large magnitudes of energy (Gogate & Pandit, 2004; Gogate, 2002) over a very small location (Gogate, 2002). The resultant effects are really spectacular and such events occur at millions of places in the reactor simultaneously (Gogate & Pandit, 2004; Gogate, 2002).

These vaporous cavities subsequently collapse violently causing increase in temperature and pressure locally at several points in a reactor resulting in the formation of reactive hydrogen atoms and hydroxyl radicals (Verma et al., 2011) as it can be seen from the following reaction (Show et al., 2010). Due to these extreme conditions in terms of high temperatures and high pressures

developed within the cavities during their collapse, these cavities are also called "hot spots" or microreactors in an otherwise cold liquid (Shirgaonkar & Pandit, 1998). The principal products from the ultrasonic irradiation of water are H_2O_2 and H_2, and various data support the hypothesis of the intermediacy of hydroxyl radicals and hydrogen atoms (Show et al., 2010).

$$2H_2O \xrightarrow{\text{Ultrasound}} 2OH^{\bullet} + 2H^{\bullet} \rightarrow H_2O_2 + H_2$$

The formed hydrogen peroxide promotes oxidation reactions and is responsible for the destruction of refractory compounds (Verma et al., 2011). Moreover, Show et al. (2010), refer that the wide range of oxidations and reductions that occurs with aqueous sonochemistry is often a consequence of secondary reactions of these high energy intermediates. Hydroxyl radicals are non-selective in nature and they can react without any other additives with a wide range of contaminants whose rate constants are usually in the order of 10^6 to 10^9 $mol.L^{-1}.s^{-1}$ (Mohajerani et al., 2009). These hydroxyl radicals attack organic molecules by either abstracting a hydrogen atom or adding hydrogen atom to the double bonds (Mohajerani et al., 2009; Ryan et al., 2009). It makes new oxidized intermediates with lower molecular weight or carbon dioxide and water in case of complete mineralization (Mohajerani et al., 2009). It has been suggested that the decolourisation of molasses process wastewater is caused by the cleavage of the chromophoric C═C double bonds found in melanoidins and other humic substances. However, molasses process wastewater also contains high levels of alkalinity (~9000 mg/L as $CaCO_3$) due to the presence of bicarbonate ions. These ions are strong inhibitors of reactions between hydroxyl radicals and the organic content (Ryan et al., 2009).

At this point it must be mentioned that chemical pre-treatment should ideally be highly selective towards the least biodegradable fractions of wastewaters, thus leaving the most biodegradable species intact for the subsequent biological step. Unfortunately, the dominant mechanism through which the majority of AOPs and WAO degrade organic pollutants is the formation of

hydroxyl radicals that are highly reactive but poorly selective (Mantzavinos & Psillakis, 2004).

Several factors may affect the degradation/oxidation of pollutants by cavitation, among these the most important ones are: the frequency and intensity of ultrasound, reactor geometry, type and nature of contaminant, bulk temperature and the water matrix (Rizzo, 2011). The production of the hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and other oxidants has been exploited during the application of ultrasonic irradiation as an AOP. Previous investigations of sonolysis have demonstrated its effectiveness; however, optimization with respect to solution conditions and reactor configurations is still necessary. In particular, optimization of $OH^{\bullet}$ and H_2O_2 production is crucial to the successful application of sonolysis to realistic treatment situations (Hua & Hoffmann, 1997).

Cavitation is classified into four types based on the mode of generation viz. Acoustic, Hydrodynamic, Optic and Particle (Gogate & Pandit, 2004; Gogate, 2002). In the case of acoustic cavitation, the pressure variations in the liquid are effected using high frequency sound waves, usually ultrasound, with frequencies in the range of 16 kHz–100 MHz (Gogate & Pandit, 2004; Gogate, 2002). Specifically, when high acoustic intensities are applied, particularly in the low and mid frequency range, gas bubbles are generated that will grow by taking in gas and vapor from the liquid. These bubbles change in size in relation to the acoustic wave and can collapse in the compression cycle (implosion), with the final implosion in microseconds . This is called acoustic cavitation. (Show et al., 2010). The violent collapse produces very powerful hydromechanical shear forces in the bulk liquid surrounding the bubble. It has been shown that macromolecules with a molar mass above 40,000 are disrupted by the hydromechanical shear forces produced by ultrasonic cavitation. The mechanical forces are most effective at frequencies below 100 kHz (Tiehm et al., 2001). At the implosion of the bubbles, extreme temperatures (5,000 K) and high pressures (500 bars) exist in the gaseous phase (Show et al., 2010). These extreme conditions can lead to the thermal destruction of compounds present in the cavitation bubbles and to the

generation of very reactive hydroxyl radicals. In this way sonochemical reactions can degrade volatile pollutants by pyrolytic processes inside the cavitation bubbles and non-volatile pollutants by hydroxyl radical reactions in the bulk liquid (Tiehm et al., 2001).

Pyrolytic decomposition, takes place inside the cavities and affects the vapor from the liquid medium or dissolved organic compounds, which may penetrate into the bubbles. The main property determining the entrance of a compound into the bubble is its hydrophobicity rather than its vapor pressure. Thus, hydrophilous organic compounds such as phenol and chlorophenols may undergo a hydroxyl radical attack in the bulk solution or in the interfacial film (Show et al., 2010). In conclusion, the process is more selective towards hydrophobic and volatile organic compounds that can be degraded easily via pyrolytic reactions, while hydrophilic and less volatile compounds are degraded slowly via hydroxyl radical-induced reactions (Mantzavinos & Psillakis, 2004).

The chemical changes taking place due to the cavitation induced by the passage of sound waves are commonly known as sonochemistry (Gogate, 2002).

Although the ultrasonic irradiation with intensities greater than 10 W/cm^2 is well known to be destructive to biological materials, it is found that low intensity ultrasound can increase the activity of enzymes or improve the metabolism of cells by improving the mass transfer or stimulating physiological activity of cells (Liu et al., 2007). In microbiology there are two zones where the ultrasonic enhancement of mass transfer will be important. The first is at the membrane and / or cellular wall and the second is in the cytosol i.e. the liquid present inside the cell (Mason & Lorimer, 2002). Most ultrasonic experiments are carried out in temperature controlled systems to ensure that isothermal conditions are maintained. Even a small general increase in microbial temperature can influence both the active and passive transport systems of the cell membrane/wall and this in turn may lead to an increased uptake of compounds. If the temperature is not controlled then

sonication could result in a large temperature increase which will lead to the denaturation (deactivation) of enzymes, proteins and other cellular components present within the microorganism (Mason & Lorimer, 2002).

It is a surprising phenomenon that ultrasound activated cultures continue with their higher activity for hours after irradiation stopped. Perhaps changes in the membrane permeability or enzymatic differences of the organisms are generated. Interestingly, discontinuous ultrasonic treatments are more beneficial for activating fermentation than the continuous exposure. It is suggested that only few steps in intracellular metabolisms (e.g. enzymatic biosynthesis) are supported by ultrasound. The use of low power ultrasound in bioreactors may present a significant improvement in cost reduction in the biotechnology industry especially in water treatment plants (Farooq et al., 2009).

Sonication is generally performed as a pre-oxidation step before biological treatment as it is reported to increase biodegradability. However, its effect on decolorization of industrial effluents has not been reported (Verma et al., 2011). Many researchers have found that ultrasonic stimulation (low power ultrasonic exposure) has the function of promoting the activity of enzyme, cell growth and cell membrane permeability (Liu et al., 2009; Xie et al., 2009).

Normally, a significant increase of biological activity could be obtained only in a very narrow range of ultrasonic power. Although the exact mechanism of low power ultrasonic on the biodegradability of sludge is not clear, it is postulated to be related to soluble substances and to the variation of the microbial system of sewage sludge (Liu et al., 2009). The same researchers investigated the effect of low power ultrasonic radiation on anaerobic biodegradability of sewage sludge. According to their results, the well known hydromechanical shear forces and heating effect of low frequency ultrasound plays a major role in the sludge pre treatment process. Besides, the increase of soluble substance may partly result from the destruction of microbial cell by excess ultrasonic pretreatment, which will inhibit the anaerobic process. The increase of soluble substances will induce the modification of microbial

system, which will affect the biodegradability of sludge. So, the optimization of each ultrasonic parameter is essential.

On the other hand, Pham et al. (2009) reported that the hydromechanical shear forces produced by ultrasonic cavitation disrupt the cells in sludge, leading to release of organic substances of sludge into the liquid phase. The sludge disintegration was also enhanced by the increase of temperature in the bulk liquid during ultrasonication. The objective of their work was to study the optimization of ultrasonication process to improve the solubilization and the biodegradability of wastewater sludge using response surface methodology. They observed that the demand in soluble chemical oxygen and biodegradability, by aerobic sludge digestion process, in terms of total solids consumption increased by 45.5% and 56%, respectively under the optimal conditions of ultrasonic pre-treatment (0.75W/cm^2 ultrasonication intensity, 60 min pre-treatment exposure time, and 23 g/L total solids concentration).

Kwiatkowska et al. (2011) stated that ‘’ultrasound has the potential to greatly influence the activities of enzymatic processes provided the energy input is not too great to disrupt the function of the enzyme under study. The application of low-power ultrasound increases growth in microbial cell cultures but high power causes cell disruption and hence can be considered as microbicidal. Ultrasound has also been used to extract and release intracellular enzymes and their subsequent activity can be further enhanced by the application of ultrasound. However, it must be stressed that the influence of sonic radiation on the activity and stability of enzymes depends on the sonication parameters and the specific enzyme preparation’’. The same authors reported that the majority of research has predominantly focused on the destruction of organic matter by ultrasound treatment, thereby enhancing the hydrolysis process. In contrast, very little attention has been dedicated to investigate the effects of ultrasound to enhance activity of microorganisms.

The basic goal of ultrasound technique is to spiflicate bacterial cell walls and to facilitate intracellular matter available for subsequent degradation to CH_4

and CO_2 in anaerobic digestion (Pilli et al., 2011). In fact, ultrasound treatment leads to the breakage of the cell walls and bacteria membranes, so improving the bacterial eso-enzymes release into solution and enhancing the biocatalysis of the hydrolytic reactions (Tomei et al., 2008).

The activated sludge method is under continuous development and improvement (Farooq et al., 2009). The anaerobic digestibility of ultrasound pretreated sludge has been studied by several researchers. However, studies on aerobic digestibility of ultrasonic pretreated sludge have been very limited. The aerobic digestion process consists of two steps (1) the direct oxidation of biodegradable matter and (2) endogenous respiration where cellular material is oxidized. The operating temperature and sludge retention time condition are the main parameters affecting performance of the aerobic digestion process (Chang et al., 2011). The activated sludge is composed of water, microorganisms (mainly bacteria), extra-cellular polymer substances and the entrapped impurities. One approach to increase the sludge microbial activity is to change the micro-organisms via gene alteration or species optimization and another approach is to stimulate the biomass via simple physico-chemical methods (Farooq et al., 2009). Salsabil et al. (2009) reported that the major effects of sonication on physico-chemical characteristics of sludge are well known: solubilization and release of organic components measured as COD, proteins, nucleic acids, polysaccharides, reduction of floc size and biodegradability improvement. So, an ultrasonic pre-treatment of sludge could increase the extent of waste activated sludge biodegradability through enhanced hydrolysis. Chang et al. (2011) reported that the mechanism of sludge destruction by ultrasound is divided into three stages. The first stage is the flocs loosing, where particle size decreases and extracellular materials are escaped from the surface of the flocs. At E_S (E_S: specific energy) > 15.000 kJ/kgTS (TS: Total Solids), an immediately appreciable effect of ultrasonic disintegration is floc size reduction, easily seen under the microscope (Figure 23). It must be stressed that as well as sludge solubilization, bacterial cell damage is expected as a result of sludge disintegration (Foladori et al., 2010). The mechanical phenomena of sludge sonication leads to sludge floc disintegration and microorganisms destruction, according to the treatment

time and power. The energy input for lysis is high, and inactivation of microorganisms was observed prior the occurrence of cell lysis. A threshold for specific energy is often reported for sludge solubilisation. This threshold specific energy ranges from 1000 to 16,000 kJ/kgTS and depends on sludge TS concentration. Indeed, the higher the sludge concentrations, the lower the specific threshold energy (higher efficiency); since cavitation bubbles have higher probabilities of contacting sludge particles. However, the optimal range of solids content for sonication lies between 2.3% and 3.2% TS; if the solids concentration is too high, increased viscosity hinders cavitation bubble formation (Carrere et al., 2010).

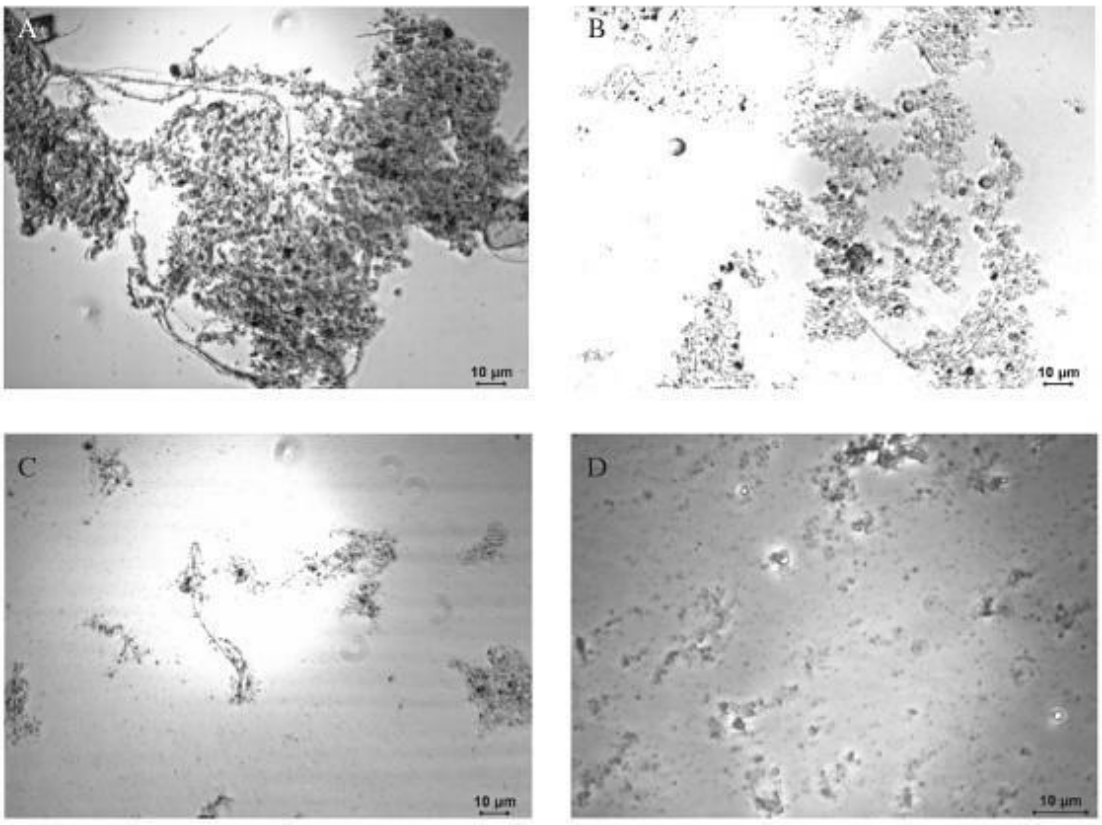

Figure 23: Microscope images of the following sludge samples: (A) untreated sludge; (B) sludge disintecraied at E_s, of 15,000 kJ/kgTS: (C) sludge disintegrated at E_s of 40,000 kJ/kgTS: (D) sludge disintegrated at E_s of 108,000 kJ/kgTS (Foladori et al., 2010).

In order to investigate the effect of ultrasonic disintegration on the integrity or death of bacteria, advanced microbiological techniques were applied. These

are based on the direct detection of cells after the fluorescent staining of their nucleic acids which allows the limits of the conventional cultivation based methods to be overcome. In particular the flow cytometry technique was used (Foladori et al., 2010).

The second stage is the cell breakage, where the intracellular organic materials inside the cells are set free, but most of them were macromolecular compounds. In the last stage, the macromolecular compounds were degraded into short chain organic low molecular compounds. Thus, these low molecular and soluble organic compounds released during this process would eventually enhance the efficiency of the sludge digestion process, by reducing the quantity of the sludge produced (Chang et al., 2011).

Recently, it was observed that ultrasound at low energy can stimulate the growth of bacteria, while only high energy is able to cause their disintegration (Foladori et al., 2010). In fact, ultrasonic waves at low frequency and low energy were found to increase the concentration of microorganisms and to enhance bioreactor performance (Foladori et al., 2010; Farooq et al., 2009). Furthermore, studies observed that low frequency was more effective in stimulating bacteria activity than high frequency, demonstrating that the mechanism is mechanical action rather than radical reactions. Ultrasound waves cause vibration of water molecules, microorganisms and other solutes in water at a frequency equal to the ultrasound frequency. This vibration enhances mixing and favors contact between substances, microorganisms and enzymes. Furthermore, ultrasound also improves cell membrane permeability, facilitating the transport of substances into the cells. The combination of these factors favors microbial growth and biodegradation (Foladori et al., 2010).

On the other hand, the results showed that ultrasonic irradiation at an improper intensity or period was destructive to biological materials, disrupting the cell membranes and deactivating biological molecules such as enzymes and DNA (Liu et al., 2007). Intense ultrasound is known to damage macromolecules such as enzymes probably from unfolding and scrambling

the native protein and breaking the chain into radicals or small peptides (Chisti, 2003). Neis & Blume (2002) studied the impact of ultrasound on E. coli and faecal streptococci. They found that for long sonication times (up to 60 minutes) and maximum ultrasound density applied, a significant reduction of microbial counts could be observed. Figure 24 shows that a maximum reduction of 2.9 log units of E. coli was achieved at a dose of 400 Wh/L (60 min at 400 W/L). In the same figure it is depicted that at the applied dose faecal streptococci are significantly less vulnerable to cavitation effects than coliform bacteria. This is due to the cell wall structure: gram-positive streptococci's cell walls are notably thicker (200 Å) than gram-negative enterobacteria's (100 - 150 Å).

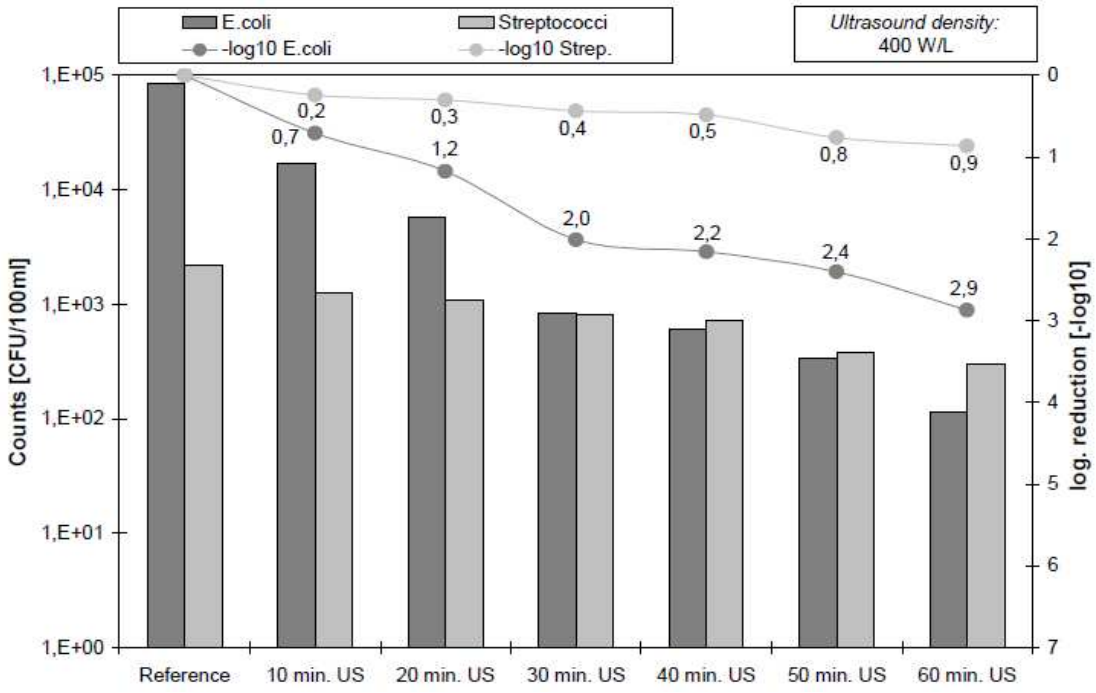

Figure 24: Effect of long sonication on E. coli and faecal streptococci (Neis & Blume, 2002).

Therefore, an optimum ultrasonic intensity and irradiation period should be selected carefully in bio-enhancement technology (Liu et al., 2007).

Sangave et al. (2007b) increased the overall efficiency of the treatment process of distillery spent wash (distillery wastewater, DW) using a combination of different treatment techniques. Initially the effluent samples

were subjected to Thermal Pretreatment (TPT-DW) and anaerobic treatment (ANA-DW). Advanced oxidation techniques, viz., Ultrasound (US) and Ozone were then used for further COD reduction followed by the conventional aerobic oxidation using mixed microbial consortium. Ultrasound pretreatment of TPT-DW as a stand alone technique enhanced the subsequent aerobic oxidation rate. A maximum of 13% COD reduction was attained at the end of 48h of aerobic oxidation. Anaerobically treated effluent sample (ANA-DW) could be successfully treated aerobically. In this case, however, the use of advanced oxidation techniques did not result in any synergistic effects. The ultrasonic irradiation was introduced using ultrasonic bath which had an operating frequency of 22.5 kHz and a rated power output of 120 W with calorimetric energy efficiency of about 35%. The bath had the internal dimensions of 15 cm length, 15 cm breadth and 15 cm height.

Xie et al. (2009) studied the enhancement effect of low-intensity ultrasound on anaerobic sludge activity and the efficiency of anaerobic wastewater treatment. Dehydrogenate activity (DHA) and the content of coenzyme F_{420} were detected to indicate the change of activity of anaerobic sludge induced by ultrasound at 35 kHz. Single-factor and multiple-factor optimization experiments showed that the optimal ultrasonic intensity and irradiation period were 0.2 W/cm^2 and 10 min, respectively, and the biological activity was enhanced dramatically under the optimal condition. The result of exposure with different ultrasonic intensities on DHA showed that at the ultrasonic intensity of 0.2 W/cm^2, DHA increased to the maximum, and above that value DHA decreased drastically. When ultrasonic intensity exceeded 0.6 W/cm^2, the activity of anaerobic sludge was inhibited by sonication, resulting in lower DHA than that of the control (without exposure). The changes of F_{420} showed the same trend with the DHA. The content of F_{420} increased to the maximum at the same ultrasonic intensity of 0.2 W/cm^2 and it was lower than the control when the intensity exceeded 0.4 W/cm^2. Therefore, low-intensity ultrasound can promote the biological activity of anaerobic sludge remarkably. At an irradiation period of 10 min, the biological activity of sludge increased to maximum, but as the irradiation time was prolonged, it decreased gradually. The chemical oxygen demand (COD) removal efficiency was increased by

ultrasonic treatment (3.60% higher) and the COD in the effluent was 30% lower than that of the control (without exposure).

Verma et al. (2011) as it has already been mentioned, developed a novel three-step technology for treatment of four molasses-based raw industrial effluents, varying in their COD, color and turbidity. Sequential steps involved in this treatment are; (1) sonication of the effluents, (2) whole-fungal treatment of these by a ligninolytic marine fungus and (3) biosorption of the residual color with heat-inactivated biomass of the same fungus. Sonication reduced the foul odor and turbidity of the effluents. It increased biodegradability of the effluents in the second stage of treatment. In other words, sonication increased accessibility of the effluents to enzymatic degradation by the ligninolytic fungus in the next step. This was evident from enhanced reduction in color, COD, total phenolics and toxicity. Sonication was carried out using ultrasonic horn with an operational frequency of 30 kHz and calorimetric energy efficiency of 600 M/cm. Sonication of the effluents (40 ml in 100 ml pyrex bottle) was carried out for 30 min at 100% amplitude using a 2 mm titanium probe.

Jiang et al. (2011) characterized the organic matter in sewage sludge before and after microbial fuel cell (MFC) treatment, with or without ultrasound as a pretreatment stage. The 5-d MFC tests with electric load significantly enhanced TCOD removal rate from 11.3% to 19.2% for raw sludge and from 25% to 57% for sludge pretreated with >0.6W/ml ultrasound, using conventional anaerobic digestion test (without electric load) as control.

Chapter 4: Objectives of this study

The objectives of this study are to investigate the factors that enhance the microorganisms ability to aerobically biodegrade the high strength agro-industrial beet-molasses wastewater obtained from the anaerobic outflow of a baker's yeast plant using the following treatment methods:

- Use of poplar woodchips
- Use of low power ultrasound
- Use of plastic carriers

The molasses wastewater is very difficult to be biodegraded giving high COD values. The above treatment methods have as a goal to reduce the COD levels to the appropriate one. Preliminary experiments (data not shown) indicated that the molasses wastewater could not be treated alone. Specifically when the molasses wastewater where treated with returned activated sludge (pH 8.00 – 9.00) from the same baker's yeast plant, it was observed a gradual pH increase above 9.5 leading to the reactors failure. So municipal sewage were selected for their co-treatment. The concentration of the molasses wastewater were 10%v/v.

Chapter 5: Materials and Methods

5.1 BOD Measurement (according to the OxiDirect® manual)

Biochemical Oxygen Demand (BOD)

The biochemical oxygen demand (BOD) of waste water, industrial effluents and surface water is an expression for the amount of oxygen consumed by the decomposition of organic matter in a biochemical process.

Principle of operation

BOD is measured by the pressure difference within a closed system (respirometric BOD). The integrated memory saves a BOD value automaticaly every 24 hours for a test period of more than 3 days.

Principle of Measurement

The BOD system, consisting of the sample bottle and the BOD sensor, represents a closed system. In the bottle, above the sample itself, is a defined volume of air. During the BOD measurement, the bacteria in the sample consume the dissolved oxygen in the sample. This is replaced by oxygen in the bottle above the sample. The carbon dioxide released at the same time react with the potassium hydroxide in the seal gasket. This generates a decrease in pressure within the system. This is measured by the BOD sensor and displayed as a BOD value in mg/l O_2.

Equipment supplied

- 1 x BOD measuring device with integral bottle rack
- 6 x BOD sensor
- 6 x BOD bottles
- 6 x seal cups (Gasket)
- 6 x magnetic stirring rods
- 1 x stirring system drive
- 1 x stirring system controller

- 1 x nitrification inhibitor (ATH)
- 1 x potassium hydroxide solution (KOH solution, 45%)
- 2 x overflow measurement flasks (157 ml and 428 ml)

Procedure

1. Sample Volume

   The sample volume is related to the expected BOD value (Table 5). The OxiDirect® is designed to operate with the following ranges and sample volumes, allowing BOD measurement up to 0 - 4000 mg/l, without any dilution.

   Table 5: Relation between sample volume and the expected BOD value

Range BOD mg/l	Sample volume in ml	Dosage ATH
0-40	428	10 drops
0-80	360	10 drops
0-200	244	5 drops
0-400	157	5 drops
0-800	94	3 drops
0-2000	56	3 drops
0-4000	21.7	1 drops

   Note

   The expected results should be in the upper half of the range.
   For domestic waste it is generally appropriate to consider a BOD_5 value which is approximately 80% of the COD value.

2. Preparing the Water Sample

   Check the pH value of the effluent sample. The optimum pH value for biochemical oxidation is between pH 6.5 and 7.5. If the pH value of the sample is higher or lower, it should be pre-adjusted. Any significant deviation will result in a lower BOD value. If the pH value is too high, it can be reduced by adding dilute hydrochloric acid (1 mol/l) or dilute sulphuric acid (1 mol/l). If the pH value is low, it can be adjusted with a sodium hydroxide solution (1 mol/l).

   Mix the water sample well and allow to settle for a short white. It may also be advisable to filter or homogenise the sample.

Measure the sample volume precisely, using the appropriate overflow measurement flask and pour the sample into the sample bottle fit may be helpful to use a funnel for this). Ensure that the sample in the bottle contains a representative portion of any solids in suspension. It is recommended that each sample should be tested twice or three times.

3. To inhibit nitrification, it is recommended the addition of nitrification inhibitor B (=Allyl Thiourea, or ATH). This is particularly important for the low range 0 - 40 mg/l (for example, when checking discharges from effluent treatment plants). The right amount of nitrification inhibitor B is related to the measurement range.

   Note

   Nitrifying bacteria also consume oxygen. This consumption can occur within the first 5 days and is more likely with samples with low BOD levels. As a general rule, the BOD measurement should not include the oxygen consumption caused by nitrifying bacteria and this can be inhibited by using nitrification inhibitor B. This inhibitor suppresses the activity of the bacteria by enzymatic inhibition, so that only the breakdown of organic substances in the sample will be measured as the BOD value. If it is desired to measure oxygen consumption resulting from nitrification (N-BOD), carry out measurements samples, one with and one without nitrification inhibitor and compare the results. The difference between the two BOD values will represent the oxygen demand of the nitrifying bacteria.

4. Add a clean magnetic stirring rod to each sample bottle and add 3-4 drops of 45% potassium hydroxide solution to the seal gasket (this will absorb the carbon dioxide). Then insert the seal gasket in the neck of the bottle.

   Important

   The sample must never come into contact with the potassium hydroxide solution. Never use grease or any other lubricants as an additional sealing agent, for the BOD sensors or for the seal gasket. Products of this kind may contain solvents which will attack the sensor,

resulting in severe damage to the plastic housing and even to a failure of the sensor.

5. Before measurement begins, the prepared sample must be brought to the desired temperature (e.g. BOD_5 20℃). This can be achieved by placing the sample in a thermostatically controlled cabinet, while stirring the sample continuously with the inductive stirring system.

   The OxiDirect® has an optional "Auto-Start" function, which enables it to start with samples at temperatures from 15° to 20℃. When this "Auto-Start" function is switched on. the system checks in specific intervals whether there has been a decrease of the pressure in the BOD bottle and will not start the timer until a pressure decrease is detected (latest, the timer will start 3 hours after the BOD sensor has been started).

6. Place the BOD sensors on the sample bottles and tighten carefully. This is extremely important - the system must be completely air-tight. Then place the BOD bottle, with the sensor screwed in position, into the bottle rack. This can be done in the thermostatically controlled cabinet itself.

   Alternatively, because of the user-friendly design of the OxiDirect®. you can remove the entire BOD unit, wfth its integral bottle rack, from the thermostatically controlled cabinet, while leaving the inductive stirring system in the cabinet. There is no need to disconnect the cabling. Once the BOD bottles have been placed in the rack, the system is positioned over the inductive stirring system so that the 4 adjustment screws fit in the associated recesses of the stirring system.

7. Start the measurement process.
8. Incubate the sample in accordance with the instruction (e.g. BOD_5 for 5 days at 20 ℃).

5.2 COD measurement (according to Hach-Lange)

Principle

Oxidizable substances react with sulphuric acid–potassium dichromate solution in the presence of silver sulphate as a catalyst. Chloride is masked by

mercury sulphate. The green coloration of Cr^{3+} is evaluated.

Interferences

The method can be used for samples (or diluted samples) with chloride concentrations of up to 1500 mg/l.

The measurements results must be subjected to plausibility checks (dilute and/or spike the sample).

Technical Specifications (Table 6)

Table 6: Technical Specifications

Description	Chemical Oxygen Demand
Digestion required	Yes
Measuring range	100 - 2000 mg/L O_2
Method	Chromsulphoric Acid
Number of tests	25
Parameter	COD

Procedure

1. Bring the sediment into suspension by inverting a few times.
2. ***Carefully*** pipette ***2.0 ml*** sample.
3. Close cuvette, thoroughly clean the outside.
4. Invert.
5. Heat in the thermostat.

HT 200 S: in standard program ***HT*** for ***15 min***

COD classic: 2 h at ***148 ℃***

6. Remove the ***hot*** cuvette.

a. ***HT 200 S:*** After the lock opens, ***carefully*** invert ***twice***.

b. ***COD classic: Carefully*** invert ***twice***.

7. Allow to cool to room temperature.

a. ***HT 200 S:*** in the thermostat

b. ***COD classic:*** in a cooling rack

8. ***HT 200 S:*** Sediment must be completely settled before evaluation is carried out. Clean the outside of the cuvette and evaluate.

COD classic: Clean the outside of the cuvette and evaluate.

The above steps are depicted in Figure 25.

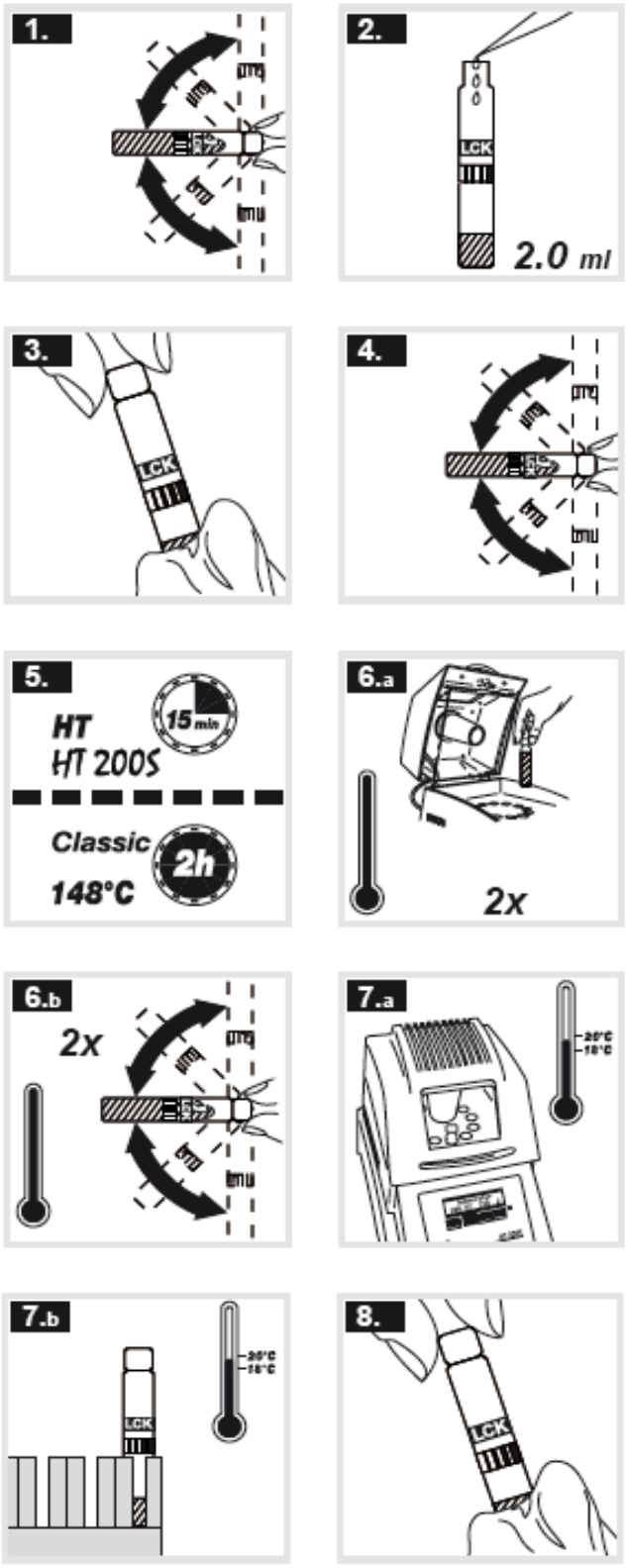

Figure 25: Steps for measuring COD

5.3 Determination of free chlorine (residual chlorine) with iodometric method (Sklari, 2012)

Principle

The iodometric method is based on the titration of iodine released from potassium iodide at a pH value less than 8 with sodium thiosulfate and starch indicator. The reactions that take place are:

$$Cl_2 + 3I^- \rightarrow 2Cl^- + [I_3]^- \quad (A)$$

$$[I_3]^- + 2S_2O_3^{2-} \rightarrow 3I^- + S_4O_6^{2-} \quad (B)$$

Process I:

- In the sample for examination we measure the initial pH value, which should be set between 3 and 4 using acetic acid (CH_3COOH).
- 1g of potassium iodide (KI) is added to the sample and then is dissolved.
- Titrate the sample in a dark place, using sodium thiosulfate solution ($Na_2S_2O_3*5H_2O$) 0.01N to the point where the yellow color change.

What causes the yellow color?

- Add starch.
- Titrate with sodium thiosulfate ($Na_2S_2O_3*5H_2O$) 0.01N to the point where the blue color change.
- Calculate the residual chlorine.

mg Cl as Cl_2/ml = A*N*35.45 / sample ml

Where:

A: ml sodium thiosulfate consumed in the titration

B: normality of sodium thiosulphate solution

Process II:

- In the sample for examination (100 – 500ml) we measure the initial pH value, which should be set between 3 and 4 using acetic acid (CH_3COOH).
- 1g of potassium iodide (KI) is added to the sample and then is dissolved.
- Titrate the sample in a dark place, using sodium thiosulfate solution ($Na_2S_2O_3*5H_2O$) 0.01N to the point where the yellow color change.
- At the same time 'blank determination' is taking place (distilled water instead of sample).
- Calculate the residual chlorine.

$$\text{mg Cl as } Cl_2/l = [(V_1 \pm V_2)*N*35.45 / \text{sample ml}]*1000$$

Where:

V_1: ml sodium thiosulfate consumed in the titration

V_2: ml sodium thiosulfate consumed in the titration of the 'blank determination'

N: normality of sodium thiosulphate solution

5.4 Poplar woodchip

Poplar woodchips were obtained from a processing factory at Thessaloniki. The uniform shape and size distribution of the woodchips enable comparison among different samples. The wood chips were manually mixed on a large plastic sheet to homogenize the batch such that all aliquots used for experiments were assumed to have the same wood source composition and particle size distribution.

5.5 Ultrasound

The following equipment was used:

1. UP100H Ultrasonic Processor
2. Glass beaker capacity 1L
3. Magnetic stirrer Hotplate Gallenkamp
4. Alcohol Thermometer

Functional principle

The ultrasonic processor generates longitudinal mechanical vibrations by means of electric excitation (reversed piezoelectric effect) with a frequency of 30 kHz. The power output of the processor can be steplessly adjusted between 20% and 100% of the maximum output. The vibrations are amplified by the sonotrode fitted to the horn and formed as a $\lambda/2$ vibrator and transferred via its end face to the medium to be sonically irradiated. When using the UP100H ultrasonic processor, the medium to be sonically irradiated is always a liquid. The ultrasound causes cavitation in the liquid, which can be used for various purposes. Solid bodies are placed in a liquid for acoustic irradiation, here the liquid transfers the ultrasound to the surface of the material (e.g. for removing layers of paint). The transferred acoustic power density depends on the form of sonotrode and the size of the sonotrode end face. The various sonotrodes available provide an optimum selection for solving different tasks.

Operating conditions in this experiment:

- Rotary regulator for pulse control mode (Cycle)
    - Setting: 1
    - Significance: Continuously switched on
- Rotary regulator for the amplitude (ultrasonic output)
    - Setting: 100%

5.6 Plastic carriers

The plastic carriers which were used was a donation from AnoxKaldnes. The type of carrier was K3 and its characteristics are described in Table 4.

5.7 Experimental procedure

A certain amount of wastewater was taken from the anaerobic effluent treatment (alkaline wastewater pH 8.00 – 9.00) from a baker's yeast plant located in the industrial zone of Thessaloniki. From the biological treatment plant of Thessaloniki was also obtained a certain amount of activated sludge

(pH 7.50) from the aerobic effluent and sewage from the primary sedimentation effluent in order to reduce the total solids (TS) of the activated sludge. The samples were placed in barrels of 20L and then they were distributed under stirring into plastic bottles of 1,5 L which were placed in the refrigerator at 4^{o}C. Part of the wastewater from the anaerobic effluent treatment of the baker's yeast plant, since it was divided in two glass flasks of 1L, it was sterilized in an autoclave at 121^{o}C for 15min. It was allowed to cool to ambient temperature and then it was stored at 4^{o}C. 6 glass reactors (capacity 5L) were utilized with the following composition: see Table 7. The poplar woodchips were sterilized at Aristotle University of Thessaloniki in an autoclave at 121^{o}C for 15min and stored at 4^{o}C. On the lid of the reactors it was created a 0.5cm hole in order to fit the plastic air supply pipe which could reach the bottom of each reactor. The system turns on. To perform the measurements, the ventilation system is closed for 1h. 200ml are taken from the supernatant and then the pH is measured in each reactor. In these 200ml the parameters COD and BOD measured. For the BOD, if more sample is required for the measurement depending on the expected BOD range (see Table 5), then an equal quantity will be supplemented to the reactor. The measurement of these two parameters is performed once a week. For the system maintenance, 200ml are supplemented daily with 10% wastewater concentration of the baker's yeast plant. The influent to each reactor has the following composition: see Table 8.

Table 7: The composition of the reactors

Reactor / composition	1 (control)	2	3	4	5	6
Activated sludge from the aerobic effluent of the biological treatment plant	2L	0	2L	2L	2L	2L
Wastewater from the anaerobic effluent of the baker's yeast plant	0.2L	2L	0.2L	0.2L	0.2L	0.2L
Woodchip (poplar)	0	200g sterilized	200g sterilized	200g non sterilized	0	0
Plastic carrier	0	0	0	0	150g	0
ultrasound	0	0	0	0	0	40min

Table 8: The influent composition of the reactors

Reactor / composition	1 (control)	2	3	4	5	6
Municipal sewage	200mL	180ml	180ml	180ml	180ml	180ml
Sterilized wastewater from the anaerobic effluent of the baker's yeast plant	0	20ml	20mL	0	0	0
Non sterilized wastewater from the anaerobic effluent of the baker's yeast plant	0	0	0	20ml	20ml	20ml

In the 5th reactor ultrasound are also applied daily. Each time they are transferred to a beaker (capacity 1L), approximately 550ml of the reactor (overall 4 times). The beaker is placed on a magnetic stirrer in agitation value 3 and the application time of ultrasonic is 10min. At its edge, a thermometer is positioned, which is mounted in the holder of a buret so that if 37^{o}C are overcomed, the process will stop (see theoretical part) but also for better stirring of the sample. Moreover, near the edge of the wall and near the thermometer, the sonotrode of the ultrasound device is immersed in the beaker by 3cm. Since the procedure is completed, the 5th reactor is placed back in the same room with the rest. In the 6th reactor, the filler material (plastic carriers) occupies the 50% of the filled volume of the reactor. The humidity in the room of the reactors is that of the environment and the temperature is set at 20^{o}C. The experiment began on the 20/02/2012 (operation day 0) and finished on the 19/6/2012 (operation day 120). On the 13/03/2012 (operation day 22) the temperature was raised to 25^{o}C to improve the microorganisms action. The bottles for the preparation of the influent mixtures in the reactors were placed in the same room with them and not in the refrigerator. To simulate the solar light an Osram Duluxl 36W bulb was used which turned off and on automatically in the hours the sun rises and sets, using a timer. Several kinds of lamps have been used as light sources in solar simulators, such as xenon arc or mercury xenon arc lamps, metal halide lamps, high pressure sodium vapor lamps, mercury vapor lamps and incandescent spotlights. Metal halide lamps, despite a higher energy output in the ultraviolet range and lower in visible range than natural solar light, are

suitable for applications requiring the simulation of the full spectrum (Meng et al., 2011).

In the same room with the reactors, it was also placed a plastic vial (capacity 20ml) with distilled water (18.0ml), sterilized molasses wastewater (10%, 0.2ml), sterilized municipal wastewater (1.8ml) and woodchips (2g) so that we can determine if the woodchips burden the organic load in the reactors.

Because of the fact that the reactors 5 and 6 did not give satisfactory results their operation was stopped earlier than that of reactors 1 (control), 2, 3 and 4.

Chapter 6: Results and Discussion

6.1 Control reactor results

In Figures 26 and 27 the control reactor results are presented.

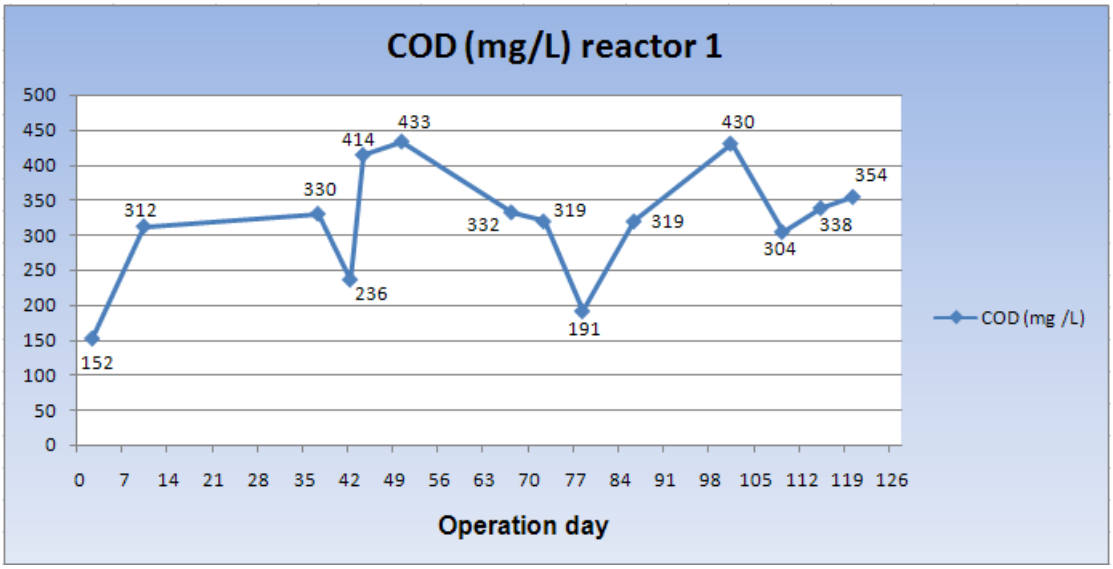

Figure 26: COD for reactor control

It has been observed that in a heterogeneous system as encountered in bio-oxidation processes involving a whole sequence of enzymatic reactions a mean pH range will be established. In many waste oxidation systems, the pH will tend to approach pH 8.0. In alkaline wastes, reaction between CO_2 produced from the respiration and the carbonates and hydroxides will be formed from bicarbonate while in wastes containing organic acids, the CO_2 from the oxidation of the acids is stripped out by aeration resulting in a pH rise. The bicarbonate will buffer the system at a pH of about 8.0 (Sangave & Pandit, 2004). Moreover Sangave et al. (2007a) reported that the increase in the pH can be attributed to the accumulation of bicarbonate (i.e., mineralization of organic matter with the formation of CO_2 leading to the shifting of the acid–base equilibrium to HCO_3^-). Cunha-Santino & Bianchini-Junior (2004) studied the humic substances mineralization. The predominance of a neutral to alkaline pH suggests that the humic substances (HS) established a butter system, which is characteristic of the fulvic acid (FA) and humic acid (HA) molecules. HS exhibit buttering capacity over a wide range of pH and the maximum buffering capacity for HA, as well as FA,

depends upon ions concentration of the solution. As the degradation proceeds it was also proposed that a second butter system, the carbonate system (CO_2, HCO_3^-, CO_3^{2-}), coming from the CO_2 released by the mineralization, is also important. Such system is responsible for the stabilization of pH during the experiment. The relative proportion of CO_2, HCO_3^- and CO_3^{2-}, is pH-dependent and according to the pH values in the mineralization chambers that ranged between 7 and 8, the HCO_3^- was the predominant chemical ionized compound.

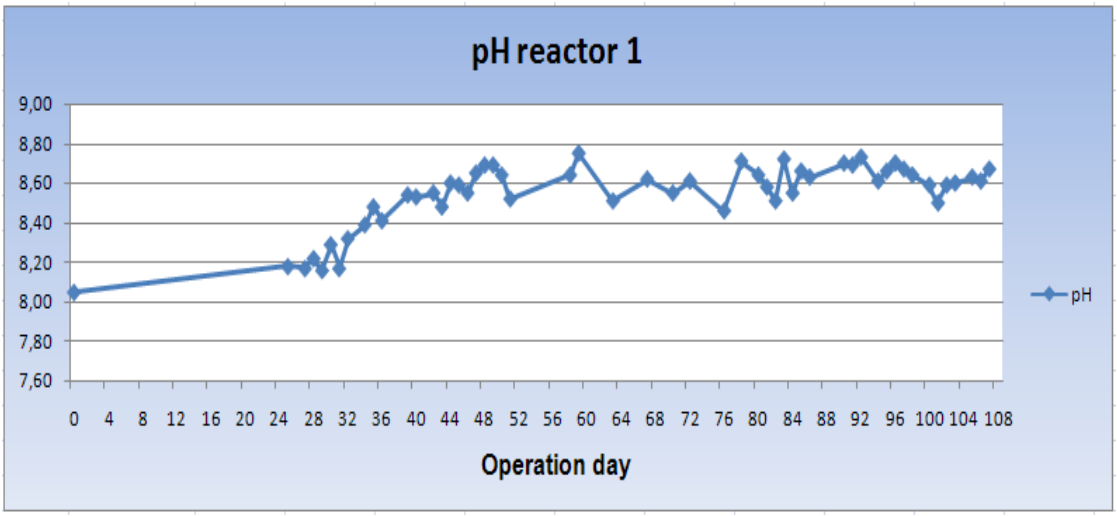

Figure 27: pH for reactor control

At this point it is important to be stressed out that on the basis of the similarities of the physical and chemical properties of the melanoidins and humic acids, melanoidins could be precursors of nitrogen-containing humic acids (Kim et al., 2004). Organic compounds present in vinasse are of humic in nature, similar to those in soil, except that fulvic acid predominates over humic acid (Naik et al., 2008, Thakur, 2006). Philp et al. (1992); Suzuki & Philp (1990) reported that this complex polymeric material, called melanoidin, is considered by many to be an important precursor of geopolymers such as fulvic acids, humic acids and proto-kerogens (melanoidin-like geopolymers).

In reactors 1, 5 and 6 a gradual increase in pH values is observed (Figures 27, 35 and 37) between the range 7.80 – 8.90. This can be attributed to conversion of the carbon source to organic acids and their subsequent

consumption (Sangave & Pandit, 2004). Moreover, due to the low biological activity in reactors 5 and 6, and the increased pH, accordingly, a repolymerization of the coloured compounds took place (Miranda et al., 1996). Veronica et al. (1993) reported that at higher pH values, polymerization or colorization of melanoidin might occur. The molecular weight patterns of melanoidin at two different pH values showed a slight shift toward high molecular weight distribution at higher pH values (data not shown). The same researchers also stated that polymerization of melanoidin in model systems increases with increasing pH due to the occurrence of a strong-base catalysis in the range of pH 6.5 – 8.5 where the rate of formation of ketosamines is favored. In addition, some intermediates in melanoidin formation are considered to polymerize through aldol-type condensation, which is likely to be base-catalyzed. Furthermore, as it has already been mentioned in the theoretical part, due to the nature of melanoidins which are resilient in nature to the biological treatment and resistant to biodegradation, the brown color in these effluents is imparted by this pigment and does not disappear and it can even increase due to repolymerization of colorants (Gengec et al., 2011; Zhou et al., 2008). So, the repolymerization of melanoidins due to low biological activity in reactors 5 and 6 could explain the COD increase (Figures 34 & 36). COD results from the ultrasound and plastic carrier treatment show in the beginning of the experiment a COD decrease. Then the pH increases indicating a low biological activity.

The molasses wastewater in reactors 2 and 3 is sterilized in contrast to reactor 4 where the molasses wastewater is not sterilized. At this point it must be stressed out that two sample independent t-test shows that sterilization does not influence COD while it affects the pH in the influent ($p = 0.802 > 0.05$ and $p < 0.001$ respectively). In fact the pH of influent 1 (sterilized molasses wastewater) is greater than the pH of the influent 2 (non sterilized molasses wastewater) by 1.20 units ($p < 0.001$). The initial pH value of the molasses wastewater lies between 8.00 – 9.00 while the pH value of the sterilized molasses wastewater lies between 9.95 – 10.12 indicating that due to sterilization intermediate products are formed or as Miranda et al. (1996) postulated, structural changes in compounds take place when the wastewater

is treated at high temperature. Agarwal et al. (2010) reported that at higher temperature during sterilization melanoidin-pigments decompose to low molecular weight compounds. Thus, the pH rises and sterilization explains why the pH of influent 1 is greater than that of influent 2. The mean value of the municipal sewage is 7.59 (normal distribution).

6.2 Woodchips

Wastewater features input to the reactors: COD (Table 9) and pH (Table 10).
Note: Influent 1: For reactors 2 and 3. Influent 2: For reactors 4, 5 and 6.

Table 9: Influent COD

Influent	Operation day	COD (mg/L)
1	24	1302
2	24	1434
1	72	1380
2	72	1259
1	109	1025
2	109	882
1	115	1066
2	115	1171
1	120	1021
2	120	1200

The experiment with the woodchips (reactors 2, 3, 4 equals to experiment 2, 3, 4 respectively) is conducted in order to examine the following:

The experiment 2 (Figures 28 & 29) works in a sterile way, with sterilized wood chips and sterilized molasses wastewater, in order to evaluate adsorption caused by the woodchips. On the other hand experiment 3 (Figures 30 & 31) contains 2L of activated sludge (AS) for the evaluation of adsorption and biodegradable COD decrease due to the activated sludge. Experiment 4 (Figures 32 & 33) operates with non sterilized wood chips (as a microbiological support), non sterilized molasses wastewater, so as the effect of the non sterilization of the wastewater to be investigated, plus AS.

Experiment 2 gave very good results showing a great constant decrease (57.4%) in the COD values, comparing to those of the influent COD, which are very close to those of the control (reactor 1). In experiment 3 we can also observe a decrease but this is not of the same degree of the experiment 2 (9.6% higher COD values or 47.8% decrease compared to the influent COD), which lasts for approximately 3 months (2 – 86 operation days). Then, an increase is watched indicating that the woodchips are saturated.

Table 10: Influent pH

Operation day	Influent 1	Influent 2	Municipal sewage
42	9.34	8.34	7.59
44	9.47	8.00	7.46
46	9.21	8.29	7.46
49	9.12	8.01	
50	8.91	7.85	7.55
51	9.12	7.93	7.40
58	8.90	8.11	7.50
63	9.01	7.80	7.28
70	9.04	7.80	7.62
72	9.15	7.86	
76	8.99	7.66	7.50
82	9.39	7.92	7.93
85	9.32	8.27	7.40
90	9.40	7.73	7.62
96	9.39	8.16	7.60
97	9.41	7.81	7.71
100	9.19	8.00	7.51
101	9.25	7.95	7.66
103	9.35	7.93	7.94
105	9.38	7.88	7.77
106	9.24	7.80	7.75
107	9.24	7.82	7.78
111	9.20	7.75	7.59
112	8.92	7.76	7.30
113	9.20	7.83	7.52
117	9.20	7.72	7.62

This means that only the woodchips degrade the molasses wastewater through sorption mechanisms. In fact activated sludge hinders the wastewater sorption to the woodchips leading to their saturation and no biodegradation

due to the activated sludge microorganisms is observed. On the contrary, it can be said that activated sludge creates a system that promotes the woodchips sorption from the beginning of the experiment. In experiment 2 a gradual decrease in COD is noticed.

The pH is an important physiological factor for development of the heterotrophic microbial communities. In general, microorganisms cannot thrive at extremes pH values, in such conditions exposed microbial cells components can be hydrolyzed or proteins denatured (Cunha-Santino & Bianchini-Junior, 2004). The neutral to alkaline pH of the mineralization chambers could not have strongly affected the progress of the mineralization process to the study of Cunha-Santino & Bianchini-Junior (2004) but in this experiment the higher pH range 8.5 – 9.1, (Figures 29, 31), caused trivial biological activity. Another explanation could be the suitability / type of the activated sludge. Gad & Sayaad (2010) indicated the efficiency of treating molasses waste water by fungal sludge before its disposal into the environment, by decreasing BOD to 522, COD to 5557 also, NO_3 to 1.11 and NH_4 to 0.42(mg/l). Moreover, melanoidins are recognized as being acidic compounds with a net negative charge (see theoretical part). Therefore, a

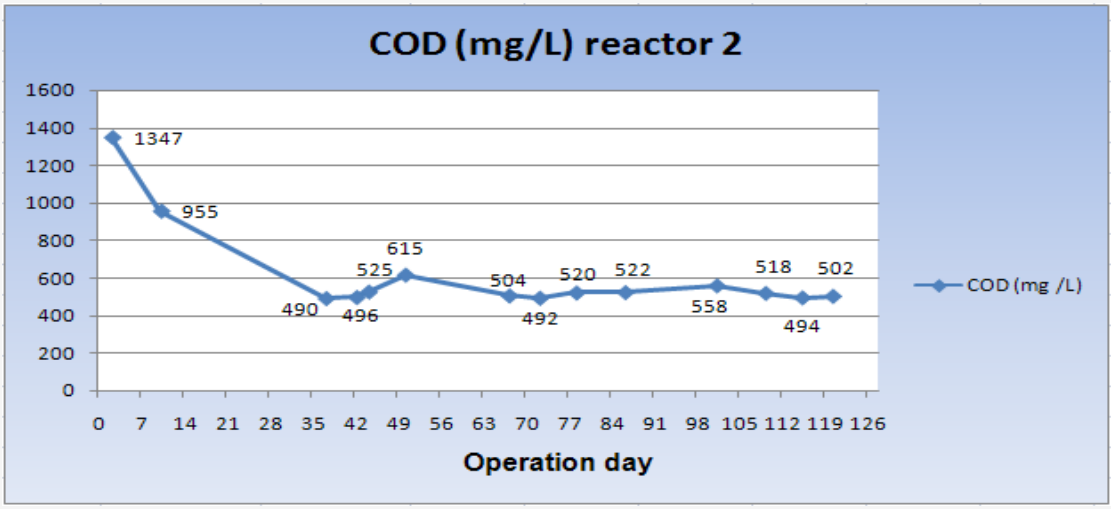

Figure 28: COD for reactor 2 (sterilized woodchips and sterilized molasses wastewater).

repulsion has to exist between the negative electrical charge of activated sludge and the negatively charged melanoidins. Basibuyuk & Forster (2003) studied the biosorption of one acid dye (Acid Yellow 17) and one basic dye (Maxilon Red BL-N) onto live activated sludge. The results showed that binding of Acid Yellow 17 where the colouring group is anionic onto activated sludge was not promising while Maxilon Red BL-N where the colouring group is cationic was adsorbed well by activated sludge. They explained the main reasons for the poor adsorption capacity of acid dye as the negative electrical charge of activated sludge under normal pH conditions so repulsion between negatively charged sorbate ions and negatively charged sorbent surface, and the number of sulpho groups in acid dye reducing dye adsorption.

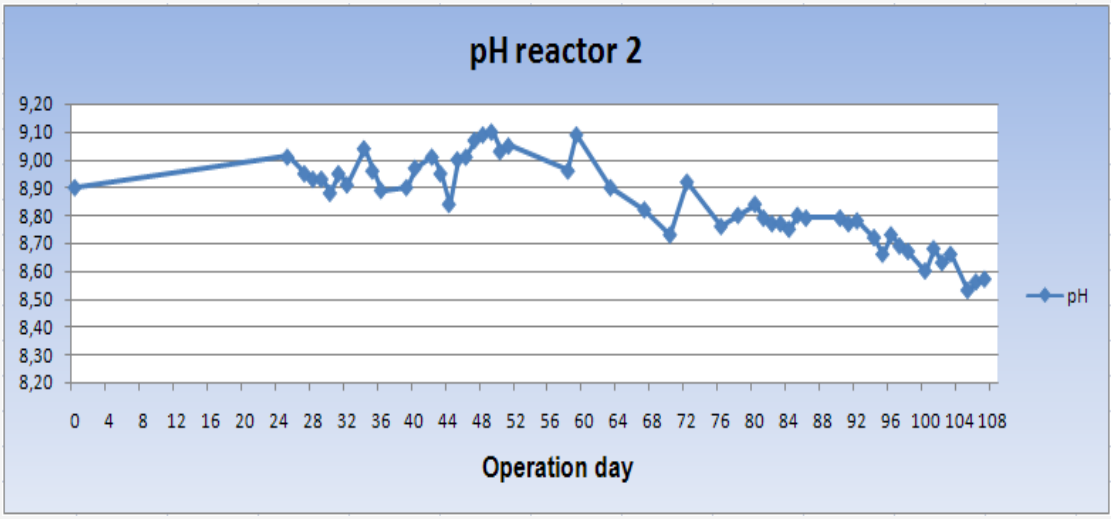

Figure 29: pH for reactor 2 (sterilized woodchips and sterilized molasses wastewater).

Some organic substances, which are resistant to biooxidation because of their chemical structure, can be adsorbed by activated sludge. Sorption is considered to be the primary removal mechanism for most dyes in aerobic biological treatment systems. However, not all dyes are adsorbed by activated sludge. Adsorption of dyes by activated sludge depends on dye structure (molecular structure and the type and number of the position of the substituents in the dye molecule), coloring group and solubility. For example, in basic dyes, the coloring group is cationic and shows good adsorption, while

acid dyes (like melanoidins) where the coloring group is anionic, do not (Basibuyuk & Forster, 2003).

Chu & Chen (2002a) utilized activated sludge biomass for adsorption of various dyes from wastewater in a batch process at constant temperature. The adsorption test showed that the biomass had no affinity for selected anionic dyes, such as Direct Orange 39 and Direct Red 83, or for selected non-ionic dyes, such as Disperse Violet 8 and Disperse Yellow 54 at contact time of 6 h. However, the biomass could integrate with the cationic dyes (basic dyes) under the same adsorption condition. The experimental result of COD removal (%) indicates that activated sludge biomass is a suitable adsorbent for various basic dyes. Thus, these results show that the biomass provides a passive uptake of basic dyes in a manner of surface adsorption. Chemical structure, basicity and molecular weight of basic dye molecules have an influence on the adsorption capacity in film-diffusion-controlled processes. The curves of temperature effects are smooth and continuous, suggesting that this adsorption is chemisorption; that is, the formation of a unimolecular layer on the surface of the adsorbent.

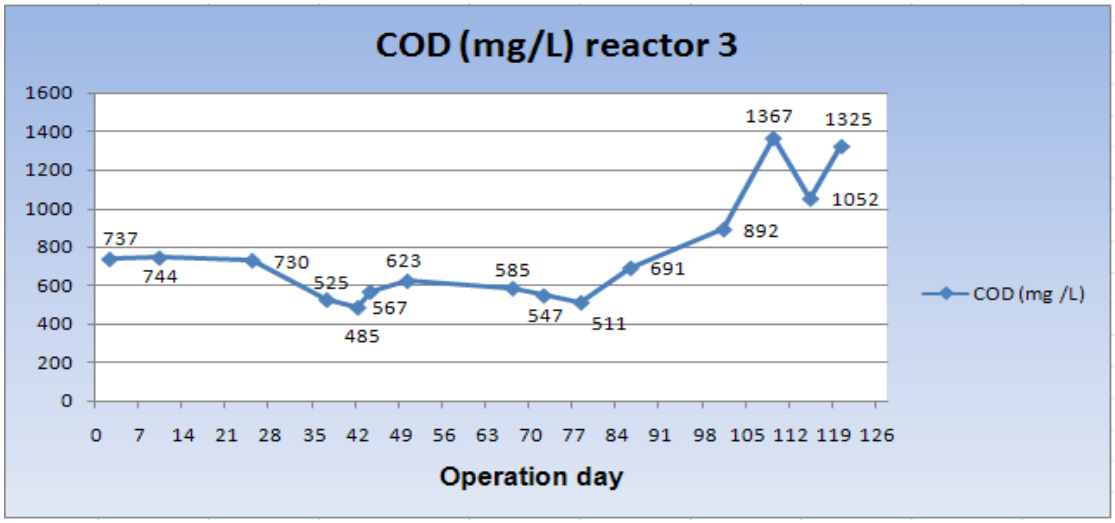

Figure 30: COD for reactor 3 (sterilized woodchips, sterilized molasses wastewater and activated sludge).

Esparza-Soto & Westerhoff (2003) studied the biosorption of humic and fulvic acids (HA, FA) to live activated sludge (AS) biomass. Their biosorption

increased under acidic conditions. The higher HA/FA biosorption under acidic conditions may be attributed to hydrophobic interactions between HA/FA and AS biomass extracellular polymers (EPS). Humic acid was removed more efficiently than fulvic acid because humic acid was more hydrophobic.

An explanation for the pH decrease in reactors 2 and 3 (Figures 29, 31) would be the interaction of the charge between the woodchips with that of the melanoidins. If such interaction exists, then the pH decrease in reactors 2 and 3, can be attributed to this interaction. The sorption mechanisms of woodchips can be explained by the presence of several interactions, such as complexation, ion-exchange due to a surface ionisation, and hydrogen bonding. One problem with sawdust materials is that the sorption results are strongly pH-dependent. There is a neutral pH beyond which the sawdust will be either positively or negatively charged (Crini, 2006). The same author reported that the sorption capacity of basic dye is much higher than that of acid dye because of the ionic charges on the dyes and the ionic character of sawdust. So, their sorption can be explained by the interaction between the negative charge of the melanoidins and the positive charge of the woodchips. In reactor 3, because of the woodchips saturation, it is possible that pH will start to stabilize.

Poots et al. (1976) by investigating the ability of spruce wood to adsorb Telon Blue (Acid Blue 25) proposed a mechanism which probably involves external surface adsorption with a limited amount of adsorption within a small zone in an area confined to the outer shell of the wood particles. However, MacKay (2008) reported that the effectiveness of wood particles to take up dissolved contaminants from the aqueous phase depends on the relative rate of contaminant diffusion to sorption sites inside the wood tissue. Partitioning of organic compounds inside wood is proportional to the lignin content of the wood tissue. The uptake capacity of dissolved organic compounds by wood is expected to continue over long times in the field. Wood degradation processes preferentially deplete cellulosic components, leaving lignin structures to undergo very slow transformation. The ratio of wood components (lignin, cellulose, extractables) could change over time

resulting in changes in the availability of sites to sorb dissolved contaminants (see theoretical part). Furthermore, the rate of contaminant removal from the aqueous phase is inversely related to the wood particle size and uptake rates are inversely proportional to compound solubilities (MacKay, 2008). The melanoidin solubility depends on the pH, it is less soluble at acidic pH than at alkaline pH (Miranda et al., 1996). Asfour et al. (1985) studied the colour removal from textile effluents using hardwood sawdust as an absorbent. As the wood particle size decreased, the amount of dye adsorbed increased.

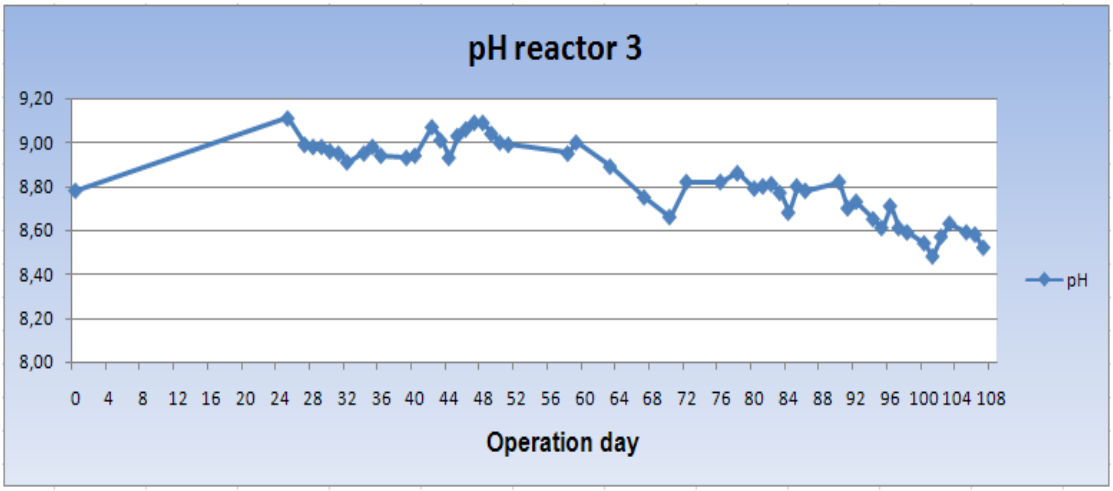

Figure 31: pH for reactor 3 (sterilized woodchips, sterilized molasses wastewater and activated sludge).

It could be deduced that the mechanism of adsorption of dye from the solution by the hardwood sawdust is not controlled solely by intraparticle diffusion. However, the dependence of k_{dm} (rate parameter), on d_m (mean particle diameter), shows that intraparticle diffusion is the predominant rate-controlling step during the adsorption process although a small boundary layer resistance was experienced in the early stages of adsorption.

Kinetics studies and diffusion process for colour removal with wood as adsorbent has shown to be an effective post biological treatment methodology. However due to hardness of the wood chips, they require longer contact times (Anjaneyulu et al., 2005).

Summing up there are four major steps in the adsorption of dye molecules onto different adsorbents (Chu & Chen, 2002b):

1. the movement of the dye from the bulk solution to the liquid film or boundary layer surrounding the adsorbent solid,
2. the diffusion of the dye through the liquid film to the external sites where adsorption occurs (film diffusion),
3. the diffusion of the dye inward through the capillaries or pores within the adsorbent solid (intraparticle diffusion), and
4. the adsorption of the dye onto the available sites of the capillary walls or surfaces.

In practice, the adsorption rate may be limited by one or more of the previous steps (Chu & Chen, 2002b).

Experiment 4 gives 12.7% higher COD values compared to experiment 2 or 44.7% COD decrease in comparison to influent COD and 3.1% higher COD values versus experiment 3. Moreover, after three months of operation (2 – 86 operation days), again an increase is watched.

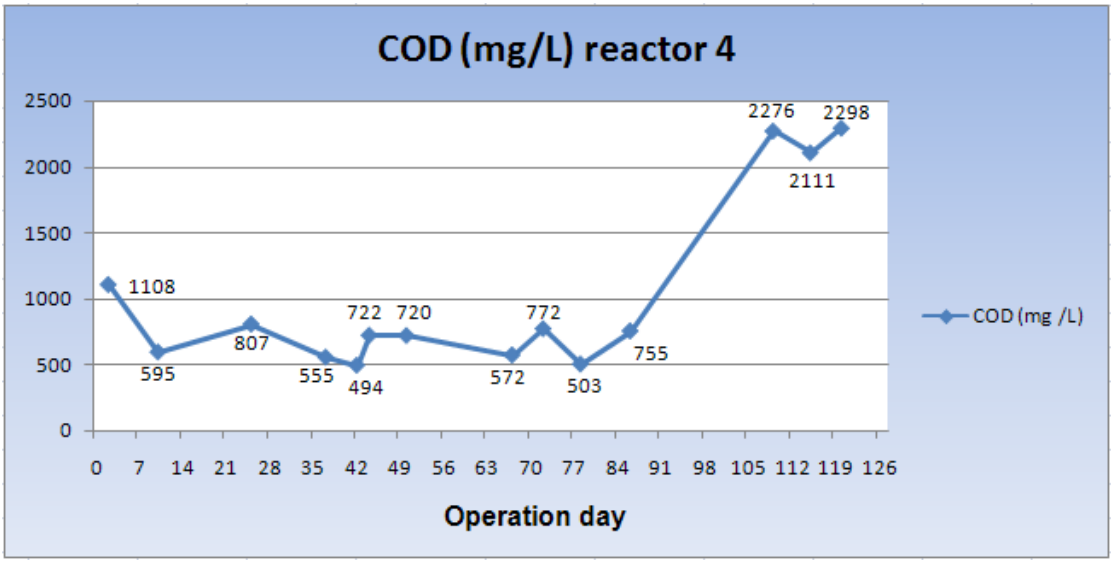

Figure 32: COD for reactor 4 (non sterilized woodchips, non sterilized molasses wastewater and activated sludge).

The COD variation is similar to that of the experiment 3 with the exception of observing two differences. First, a gradual COD decrease is watched in the beginning. Secondly, a fluctuation in the COD values is also observed. It must be stressed out that are noticed low COD values comparable to those of experiment 2. The role of the activated sludge and the woodchips sorption mechanisms were stated before. Emphasis must be given to the non sterilized woodchips and molasses wastewater. The fluctuation of the COD can be attributed to the competition between the microorganisms living on the woodchips and those of the molasses wastewater. Despite the fact that a stable trend in the pH value is observed (pH 8.70 – 9.10), the low COD values between 494 – 595 ppm, indicate that the poplar woodchips might contain microorganisms that can tolerate the high pH values. The higher COD values indicate that the microorganisms present to the wastewater also stand the alkaline pH. So, due to this competition and the depending on the growth phase of the woodchips microorganisms, a fluctuation in the COD is observed. The start-up of the experiment is a period when the microorganisms start to become adapted to the environment and start to produce necessary enzymes for degradation (Palacios, 2009). This could explain why in the experiment 4 it is noticed a slow rhythm in the COD decrease, while in the experiment 3 it is observed a decrease from the first day of operation of the system, showing that adsorption takes place from the beginning.

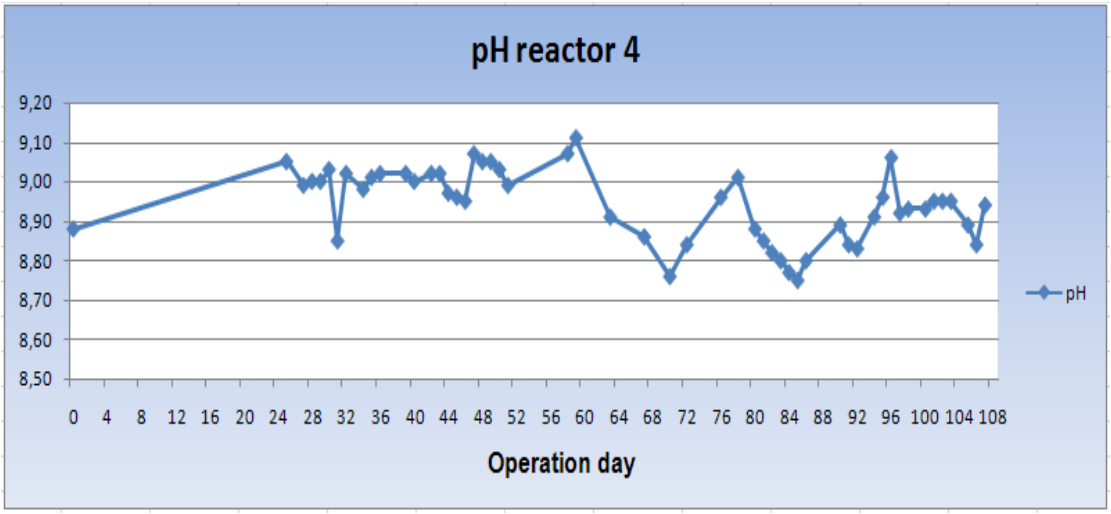

Figure 33: pH for reactor 4 (non sterilized woodchips, non sterilized molasses wastewater and activated sludge).

Sterilization is essential for color removal so as not to leave space for contaminating microorganisms that can inhibit the growth or compete for the nutrients in the media (Gad & Sayaad, 2010). Moreover, preliminary experiments (data not shown) from Miranda et al. (1996) proved that color removal yield was higher when the culture medium (molasses wastewater plus added nutrients) was sterilized. They claimed that this better color elimination could have been due to structural changes in compounds when the wastewater was treated at high temperature. These, in combination with the role of the activated sludge, can explain the increase in COD after three months of operation. Additionally, wood chips provide the microorganisms with carbon source which make the addition of e.g. glucose unnecessary. It is possible that, this carbon source was consumed faster due to the contaminating microorganisms.

Forest residue wood chips contain a mixture of fungi and bacteria which is an advantage when complex molecules should be degraded (Forss & Welander, 2009). Wood chips often contain bacteria as well as fungi which might be an advantage when molecules with complex structures should be degraded. The fungi might degrade structures which are difficult for bacteria to handle while the bacteria might degrade intermediates formed by the fungi (Palacios, 2009). Lignocellulose-containing materials consist partly of complex molecules with similar structures as textile dyes which might make the microorganisms growing on these materials adapted to refractory organic compounds (Forss & Welander, 2009), like melanoidins. It would be important to make more analyses for instance by high performance liquid chromatography (HPLC) in order to evaluate if any intermediates are formed or not. Tiwari et al. (2012) studied the decolorization of melanoidin by a novel thermotolerant yeast, *Candida tropicalis* RG-9. The HPLC analysis data, before and after the treatment of spentwash confirms the biodegradation of melanoidin. A major peak appeared at a retention time of 2.60 min in treated sample which was less compared to untreated and clearly indicates the ability of the yeast to decolorize/degrade the spentwash. The reduction in physico-chemical characteristics may be due to degradation of melanoidin in the

presence of carbon and nitrogen sources through metabolism. Therefore, these intermediates could explain the stabilizing trend in the pH values to reactor 4, because the woodchips are non sterilized in contrast to reactors 2 and 3 where due to the sterilization of woodchips this microbiological support is absent.

MacKay (2008) reported another advantage of the non sterilized woodchips use. Colonization of wood surfaces by bacteria populations may facilitate the transformation of entrapped organic contaminants to less toxic compounds. Moreover, the growth of biofilms on the wood surface may provide additional sorption sites for dissolved contaminants. Obviously, due to the presence of activated sludge, colonization of wood surfaces by bacteria populations will be difficult.

Forss & Welander (2009) studied the decolourization of reactive azo dyes with microorganisms growing on soft wood chips. From their results it can be seen that the decolourization of the dyes was more efficient in the series 2:3 (Bjerkandera sp. + native microflora from the forest residue chips) than in the series 2:4 (Bjerkandera sp.). Both these series contain Bjerkandera sp. and 1 g/L of yeast extract. This result indicates that the natural microflora is more important than addition of Bjerkandera for the decolourization. Series 2:2 (native microflora from the forest residue wood chips) without the nutrients or Bjerkandera sp. did not display as good degradation as did series 2:3, which indicates that nutrients (1 g/L yeast extract) increase the degradation rate. From the sterile 2:1 series, we can see that adsorption already takes place on the first day, but that after several days the chips are saturated. The results indicate that the native microflora on the forest residue wood chips together with the nutrition give the best degradation. In experiment series three, they wanted to further study whether the basis of the degradation in this study was the native microflora or Bjerkandera sp. The two series 3:6 and 3:7, using nonsterile forest residue wood chips inhabited by native microflora, displayed better degradation than did series 3:5 inhabited by only Bjerkandera sp.. These results suggest that the native microflora inhabiting Swedish forest residue wood chips are better than Bjerkandera sp. at degrading the Reactive

Black 5 and Reactive Red 2 azo dyes in a competitive environment, i.e., forest residue wood chips in water. It is also clear that adding a crude nutrient, in this case, 1 g/L of yeast extract, increases the degradation speed.

Therefore, it would be interesting the woodchips experiment to be repeated with the following conditions:

- Non sterilized woodchips
- Sterilized and non sterilized molasses wastewater to examine the nessecity of wastewater sterilization
- Absence of activated sludge

Finally, as it has already been mentioned, the melanoidin solubility depends on the pH, it is less soluble at acidic pH than at alkaline pH (Miranda et al., 1996). The results from all the experiments (2, 3 and 4) show that the woodchips can successfully work between the pH range 8.5 – 9.0 and they probably contain microorganisms that can biodegrade the molasses wastewater in this pH range.

From the results in Table 11 a decrease in COD is noticed and it can be concluded that the woodchips do not burden the organic load in the reactors.

Table 11: Plastic vial with distilled water, sterilized molasses wastewater (10%), sterilized municipal wastewater and woodchips.

plastic vial	COD (mg /L)	Operation day
1	1038	86
2	1088	101
3	736	109
4	733	115

Domingos et al. (2009) studied the COD release from woodchips in water. The COD released from woodchips was measured by placing 100g woodchips in 1L of tap water, COD was measured and the 1L water batch changed weekly. After 65 days the 100g of woodchips released a total of 2262mg COD. At 65 days a plateau is evident at the cumulative graph (see

Domingos et al., 2009) with no significant increase of COD taking place. This may be the time to replace the woodchips. This result however according to them, should be used just as guidance as COD release will vary depending on the type, coarseness, and age of the woodchips. The experiment showed that woodchips can be used as a slow release COD substrate. A great proportion of COD is released in the first two weeks and then slowly released over the next two months.

6.3 Ultrasounds

In the theoretical part it is described the action mechanism of ultrasound in wastewater decontamination. Here, it will only presented why ultrasound were not successfully implemented.

It has been suggested that the decolourisation of molasses process wastewater is caused by the cleavage of the chromophoric C=C double bonds found in melanoidins and other humic substances. However, molasses process wastewater also contains high levels of alkalinity (~9000 mg/L as $CaCO_3$) due to the presence of bicarbonate ions. These ions are strong inhibitors of reactions between hydroxyl radicals and the organic content (Ryan et al., 2009). As it can be noticed from Figure 35 the pH range lies between 8.20 – 8.91. This fact can explain why the application of ultrasound failed as the COD values increase (Figure 34).

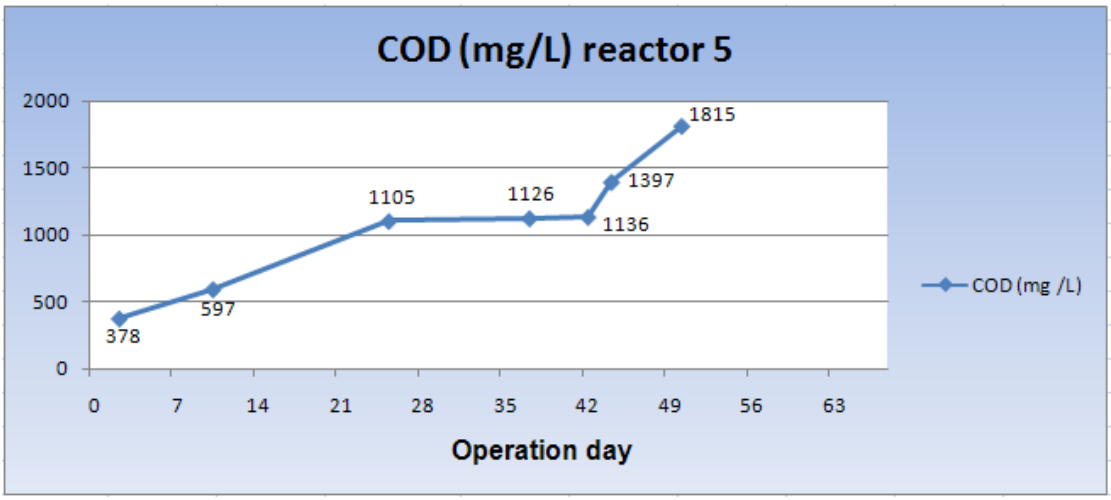

Figure 34: COD for reactor 5 (implementation of ultrasound with ≈0.20W/cm^2).

Miranda et al. (1996) noted that the buffer capacity of molasses wastewater is very high and, accordingly, a large amount of hydrochloric acid would be needed to decrease the pH of the medium in a practical process. As a result of this addition of acid a precipitate will be formed. Our preliminary experiments (data not shown) come with agreement to Miranda et al. (1996).

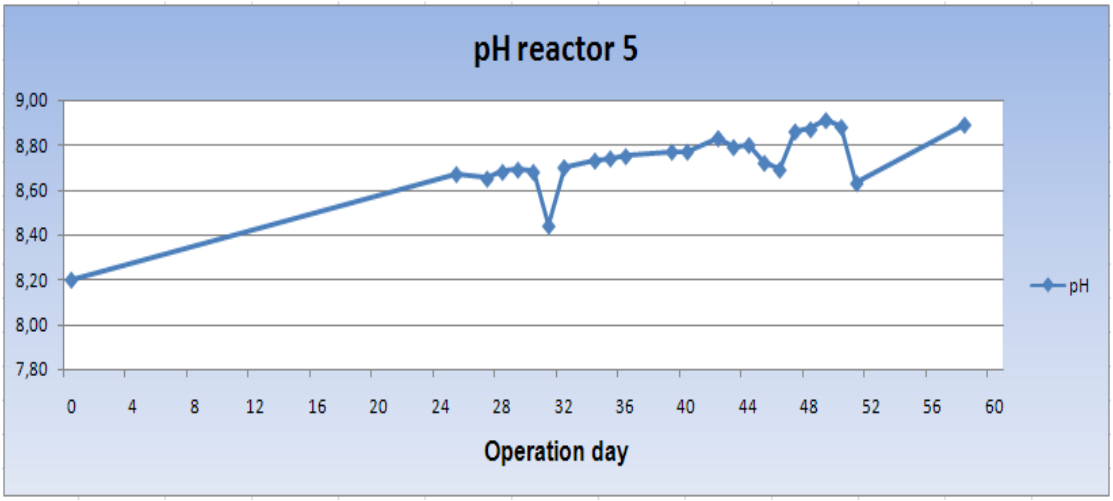

Figure 35: pH for reactor 5 (implementation of ultrasound with ≈0.20W/cm^2).

Moreover, although the ultrasonic irradiation with intensities greater than 10 W/cm^2 is well known to be destructive to biological materials, it is found that low intensity ultrasound can increase the activity of enzymes or improve the metabolism of cells by improving the mass transfer or stimulating physiological activity of cells (Liu et al., 2007). In our experiment the ultrasound intensity was approximately 0.20 W/cm^2 (90W/550cm^2) which could possibly be very low. In other words, it was probably insufficient to enhance the biological activity. Pham et al. (2009) observed that the increase in soluble chemical oxygen demand and biodegradability, by aerobic sludge digestion process, in terms of total solids consumption increased by 45.5% and 56%, respectively under the optimal conditions of ultrasonic pre-treatment (0.75W/cm^2 ultrasonication intensity, 60 min pre-treatment exposure time, and 23 g/L total solids concentration). Xie et al. (2009) studied the enhancement effect of low-intensity ultrasound on anaerobic sludge activity and the efficiency of anaerobic wastewater treatment. Dehydrogenate activity (DHA) and the content of coenzyme F_{420} were detected to indicate the change of

activity of anaerobic sludge induced by ultrasound at 35 kHz. Single-factor and multiple-factor optimization experiments showed that the optimal ultrasonic intensity and irradiation period were 0.2 W/cm^2 and 10 min, respectively, and the biological activity was enhanced dramatically under the optimal condition. At an irradiation period of 10 min, the biological activity of sludge increased to the maximum, but as the irradiation time was prolonged, it decreased gradually. The irradiation period in our study was also 10min but it was not investigated if it exerts any influence.

Normally, a significant increase of biological activity could be obtained only in a very narrow range of ultrasonic power. Although the exact mechanism of low power ultrasonic on the biodegradability of sludge is not clear, it is postulated to be related to soluble substances and to the variation of the microbial system of sewage sludge (Liu et al., 2009). Here, it must be stressed again that the melanoidin solubility depends on the pH, it is less soluble at acidic pH than at alkaline pH (Miranda et al., 1996). Benito et al. (1997) also noted that some compounds responsible for the molasses wastewater color were soluble over a certain pH range (basic) and insoluble at acid pH.

Carrere et al. (2010) reported that if the solids concentration is too high, increased viscosity hinders cavitation bubble formation and by extension the formation of reactive hydrogen atoms and hydroxyl radicals. The optimal range of solids content for sonication lies between 2.3% and 3.2% TS. The activated sludge used, was probably out of this range influencing in this way the treatment of the molasses wastewater.

6.4 Plastic carrier

Similarly with the woodchips experiment, it is observed in the beginning a decrease in the COD values (Figure 36). The start-up of the experiment is a period when the microorganisms start to become adapted to the environment and start to produce necessary enzymes for degradation (Palacios, 2009). Then, an increasing trend is observed, probably due the alkaline pH values (Figure 37).

As it was stated in the theoretical part, the pH of the liquid phase may affect the extent of adsorption of bacteria to solid surfaces. The influence of pH on bacterial adsorption depends on the nature of the bacterial surfaces and ionic strength in the solution. The importance of pH in determining the surface charge increases at low ionic strength. Increased retention of bacteria has been found to occur when pH was decreased from 9.3 to 3.9. There are some conflicting results, with respect to pH. These are probably due to the different iso-electric points and variability in charges around the iso-electric point for different bacterial species (Kristian Stevik et al., 2004).

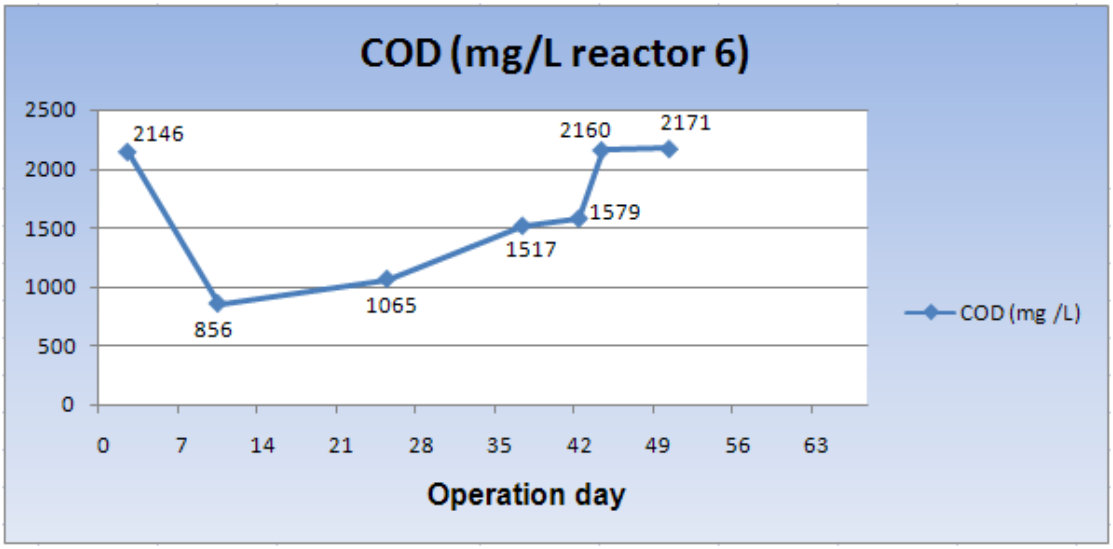

Figure 36: COD for reactor 6 (with plastic carriers).

Moreover, temperature can have an effect on biofilm formation. Temperatures at the high end of a culture's growth range can enhance biofilm formation. Depending upon the species involved, high temperature increases the rate of cell growth, extracellular polymeric substances (EPS) production, and surface adhesion, all of which enhance biofilm formation (Qureshi et al., 2005).

Additionally, the rate of diffusion of organic molecules tends to increase at elevated temperatures (Sankaran, 2012). The SCBP process is less sensitive to variation in process conditions and has been shown operable at 55^{o}C in laboratory studies (Suvilampi et al., 2003b). Suvilampi et al. (2003a) studied a combined thermophilic (55^{o}C) – mesophilic (35^{o}C) wastewater treatment and concluded that a combined thermophilic–mesophilic treatment appeared to be

easily operable and produced high effluent quality. Kristian Stevik et al. (2004) also reported that adsorption of bacteria is substantially greater at higher temperatures. The reduction in attachment with decreasing temperature may have several causes: (a) enhancement in the viscosity of the bacterial surface polymer and of the liquid, (b) reduced chemisorption and certain types of physical adsorption and (c) changes in the physiology of the organisms. So, the low temperature (25^{o}C), could be another reason for the increasing trend in the COD values.

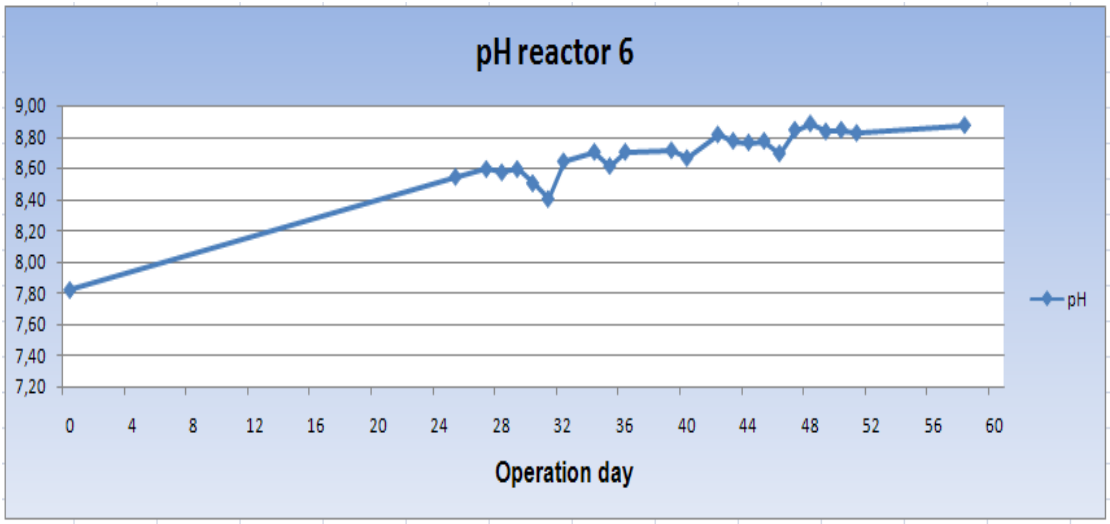

Figure 37: pH for reactor 6 (with plastic carriers).

Finally, carriers can differ from each other in material composition, shape, specific surface area and treatment capabilities (Levstek & Plazl, 2009). Investigations on the shape and size effect of carrier made it clear that the key factor in the design of a moving bed biofllm process for organic matter removal is the effective surface area where biomass may grow. The size and shape of carrier may have an influence on this effective area. The design of process should be based on organic surface area removal rate (Ayati et al., 2007). Therefore, it must be examined if the selected carrier is the appropriate one.

6.5 Summary results

In Figures 38, 39 and Table 12 the summary results (COD and pH respectivelly) are presented for a direct comparison of all the treatment methods.

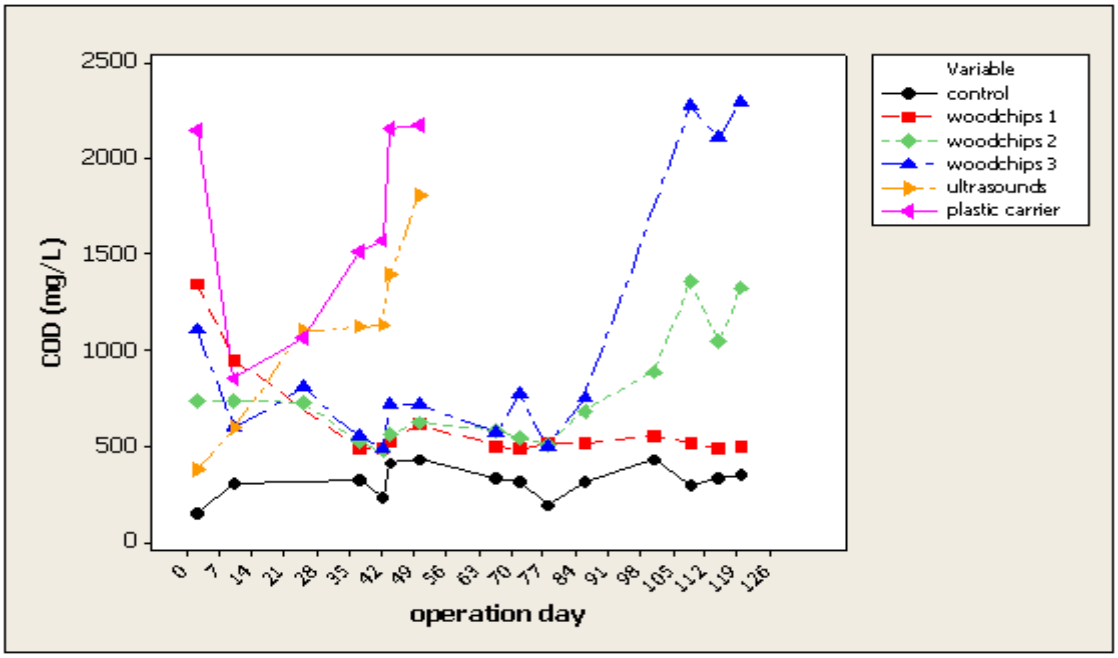

Figure 38: COD variation for all of the treatment methods

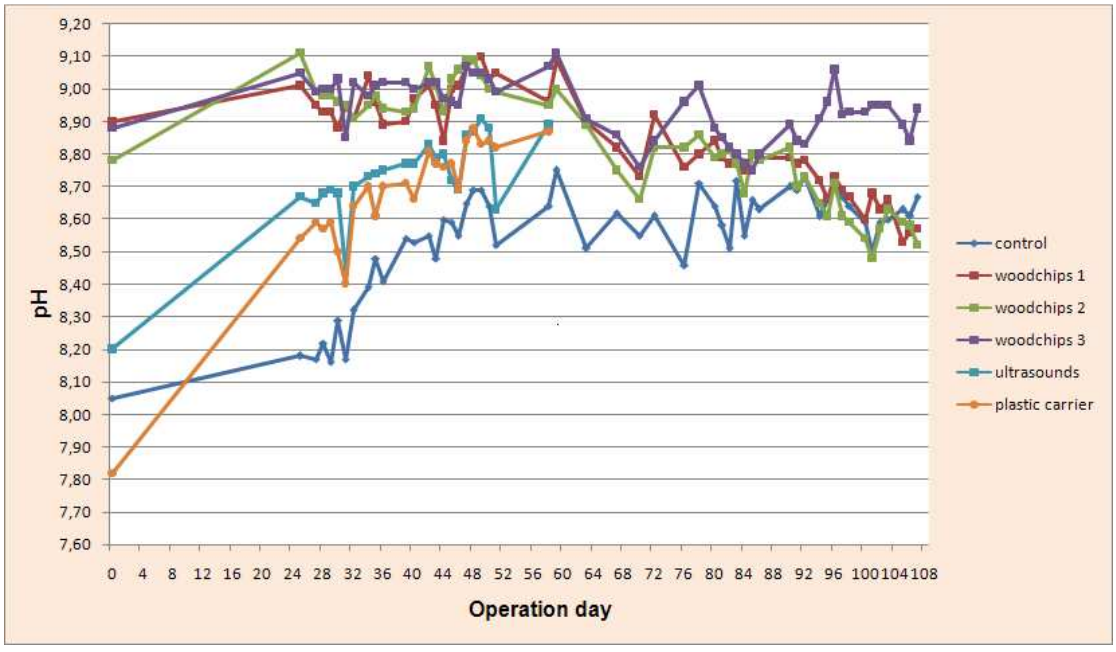

Figure 39: pH variation for all of the reactors

Table 12: Removal percentages for all of the treatment methods

Reactor	COD removal (%)
2 vs influent	57.4
3 vs influent	47.8
4 vs influent	44.7
3 vs 2	9.6 higher COD
4 vs 2	12.7 higher COD
4 vs 3	3.1 higher COD
5 vs influent	reactor failure
6 vs influent	reactor failure

6.6 BOD

Because of the fact that the molasses wastewater was difficult to be biodegraded the obtained BOD values was not satisfactory and they are presented separately in Table 13.

Table 13: BOD values (mg/L) for all of the reactors

reactor	day 1	day 2	day 3	day 4	day 5	day 6	measurement on:
1				44	126		2/4/2012
2			12	64	157		
3			10	48	127		
4			23	76	166		
Influent 2	129	185	231	OFL			
6							
1	UFL	UFL	UFL	UFL	UFL	8	9/4/2012
2	UFL	OFL	17	11	20	81	
3	UFL	OFL	88	54	39	133	
4	UFL	OFL	164	117	55	124	
5	14	F	226	179	88	151	
6							
1	UFL	UFL	UFL	37	141		27/4/2012
2	UFL	UFL	UFL	5	15		
3	UFL	UFL	UFL	UFL	UFL		
4	UFL	UFL	UFL	5	14		
5	8	69	101	129	156		
6	UFL	16	34	52	76		
1	UFL	UFL	UFL	F	F		2/5/2012
2	UFL	UFL	UFL	F	F		
3	UFL	UFL	UFL	F	F		
4	UFL	UFL	UFL	F	F		
1	UFL	UFL	UFL	UFL	UFL		7/5/2012
2	UFL	UFL	UFL	UFL	UFL		
3	UFL	UFL	UFL	UFL	UFL		
4	UFL	UFL	UFL	3	9		

Chapter 7: Conclusions

The treatment methods which were implemented were the utilization of:

- woodchips
- low power ultrasounds and
- plastic carries

The best results were obtained from woodchips experiment 2 (sterilized woodchips, sterilized molasses wastewater, activated sludge absence) showing a great gradual stable decrease in the COD valeus (55.7%), comparing to those of the influent COD, which are very close to those of the control (reactor 1). Comparing experiment 3 (sterilized woodchips, sterilized molasses wastewater, activated sludge presence) with woodchips experiment 2, we can conclude that:

- Only the woodchips degrade the molasses wastewater through sorption mechanisms.
- Activated sludge hinders the wastewater sorption to the woodchips leading to their saturation and no biodegradation due to the activated sludge microorganisms is observed.
- Activated sludge creates a system that promotes the woodchips sorption from the beginning of the experiment.

The application of activated sludge failed for the following reasons:

- The alkaline pH range 8.5 – 9.1, caused trivial biological activity
- The type of the activated sludge used might not be suitable, i.e. the microorganisms present in this activated sludge might not be able to biodegrade the molasses wastewater
- The repulsion between the negative electrical charge of the activated sludge and the negatively charged melanoidins hindered their sorption by the activated sludge
- The biosorption of melanoidins to live activated sludge biomass might increase under acidic conditions due to hydrophobic interactions

between melanoidins and activated sludge biomass extracellular polymers (EPS).

The sorption mechanisms of woodchips can be explained by the presence of several interactions, such as:

- complexation,
- ion-exchange due to a surface ionisation,
- and hydrogen bonds.

Moreover, the adsorption of the molasses wastewater dye molecules onto woodchips can be summarized in four major steps:

1. The movement of the dye from the bulk solution to the liquid film or boundary layer surrounding the adsorbent solid,
2. The diffusion of the dye through the liquid film to the external sites where adsorption occurs (film diffusion),
3. The diffusion of the dye inward through the capillaries or pores within the adsorbent solid (intraparticle diffusion), and
4. The adsorption of the dye onto the available sites of the capillary walls or surfaces.

Comparing experiment 4 (non sterilized woodchips, non sterilized molasses wastewater, activated sludge presence) with woodchips experiment 2 and 3, we can deduce that:

- The fluctuation of the COD (494 – 807ppm) can be attributed to the competition between the microorganisms living on the woodchips and the molasses wastewater who can tolerate the high pH values.
- Molasses wastewater sterilization probably exerts an influence on their treatment. Sterilization is essential for color removal so as not to leave space for contaminating microorganisms that can inhibit the growth or compete for the nutrients in the media (Gad & Sayaad, 2010). Forest residue wood chips contain a mixture of fungi and bacteria which is an

advantage when complex molecules should be degraded (Forss & Welander, 2009).

- The start-up of the experiment is a period when the microorganisms start to become adapted to the environment and start to produce necessary enzymes for degradation (Palacios, 2009). This could explain why in the experiment 4 it is noticed a slow rhythm in the COD decrease, while in the experiment 3 it is observed a decrease from the first day of operation of the system, showing that adsorption takes place from the beginning.
- The presence of activated sludge probably hinders the colonization of wood surfaces by bacteria populations and by extension, the growth of biofilms on the wood surface, which may provide additional sorption sites for dissolved contaminants.

The results from all the experiments (2, 3 and 4) show that the woodchips can successfully work between the pH range 8.5 – 9.0.

Finally, the data from the plastic vial show a decrease in COD indicating that the woodchips do not burden the organic load in the reactors.

Ultrasound implementation failed for different reasons:

- Bicarbonate ions are strong inhibitors of reactions between hydroxyl radicals and the organic content. The pH range observed lies between 8.20 – 8.91 (high alkalinity).
- A large amount of hydrochloric acid would be needed to decrease the pH of the medium in a practical process.
- The activated sludge used, was probably out of the optimal range of solids content. If the solids concentration is too high, increased viscosity hinders cavitation bubble formation and by extension the formation of reactive hydrogen atoms and hydroxyl radicals.
- The ultrasound intensity was probably insufficient to enhance the biological activity (≈0.20 W/cm^2).

- The irradiation period was 10min, as it was found in a case study in the literature. However, it was not investigated if it exerts any influence to the ultrasound implementation.
- The low COD value in the beginning of the experiment, indicates a low biological activity.
- Due to the low biological activity and the increased pH, accordingly, a repolymerization of the coloured compounds took place leading to a COD increase.

Plastic carrier implementation failed for different reasons:

- Due to the alkaline pH values. Increased retention of bacteria has been found to occur when pH was decreased from 9.3 to 3.9.
- Adsorption of bacteria is substantially greater at higher temperatures. The SCBP process has been shown operable at 55^{o}C in laboratory studies.
- The selected carrier might not be the appropriate one.
- The low COD value in the beginning of the experiment, indicates a low biological activity.
- Due to the low biological activity and the increased pH, accordingly, a repolymerization of the coloured compounds took place leading to a COD increase.
- The filling percentage might be lower or higher than the appropriate one

The slow rhythm in the COD decrease, like woodchips experiment 4, can also be attributed to the start-up period when the microorganisms start to become adapted and adsorb onto the plastic carriers.

The pH in reactors 1, 5 and 6 rises due to the:

- accumulation of bicarbonate
- conversion of the carbon source to organic acids and their subsequent consumption

- fact that the CO_2 from the oxidation of the acids is stripped out by aeration

An explanation for the pH decrease in reactors 2 and 3 would be the interaction of the charge between the woodchips with that of the melanoidins. The intermediates which are probably formed by the microorganisms living on the woodchips, could explain the stabilizing trend in the pH values to reactor 4.

It must be stressed out that the pH is an important physiological factor for development of the heterotrophic microbial communities. In general, microorganisms cannot thrive at extremes pH values, in such conditions exposed microbial cells components can be hydrolyzed or proteins denatured (Cunha-Santino & Bianchini-Junior, 2004) influencing in this way the effectiveness of the tested treatment methods.

For the molasses wastewater treatment from the above methods tested, the woodchips one is proposed, as long as the activated sludge is absent. It is required that this wastewater is co-treated with municipal sewage at a maximum proportion of 10%.

Chapter 8: Suggestions for future work

1. Repetition of the woodchips experiment with the following conditions:
   - Non sterilized woodchips
   - Sterilized and non sterilized molasses wastewater to examine the necessity of wastewater sterilization
   - Absence of activated sludge
2. Testing with higher concentration of the molasses wastewater e.g. 40% and co-treatment with other wastewater.
3. Treatment with chemically pretreated woodchips.
4. Study of the effect of wood particle size, quantity and kind of wood chips.
5. Investigation in reactor 2 the wood chips duration.
6. Conduction of HPLC analysis in order to be found the nature of the intermediate products due to action of the wood chips microorganisms.
7. Different ultrasonic conditions: Rotary regulator for pulse control mode (cycle) < 1 (not continuously switched on), ultrasound intensity (≠ 0.20W/cm^2), irradiation period (≠ 10min) and use of diluted activated sludge.
8. Conducting the experiment with the plastic carrier at lower pH if it is practical, at higher temperature (55^oC) and different filling percentages.
9. Testing of different carriers.
10. Examine the effect of phosphorous, nitrogen and TSS on microorganisms adsorption to the plastic carrier.
11. Investigation of melanoidins decolorization due to biological activity and adsorption onto the microorganisms.
12. Treatment with enzymes e.g. manganese-independent peroxidase (MIP), manganese peroxidase (MnP), cellulase, laccase, sorbose-oxidase or glucose-oxidase and others.
13. Treatment with other AOP's such as ozonation.
14. Treatment with inorganic compounds such as Ferric chloride ($FeCl_3 \cdot 6H_2O$), ACH (aluminium chlorohydrate), aluminum chloride ($AlCl_3$) and polyaluminium chloride (PAC).
15. Treatment with microorganisms or a consortium of microorganisms (bioaugmentation). Moreover a pretreatment of microorganisms can be

applied. Researchers have shown that some physical or chemical pretreatment processes can increase the adsorption capacity of biomass. These pretreatment methods mainly included drying, autoclaving, contacting with organic chemicals, such as formaldehyde, or inorganic chemicals, such as NaOH, H_2SO_4, $NaHCO_3$, and $CaCl_2$ (Aksu, 2005).

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APPENDIX: Statistical analysis results

Two-Sample T-Test and CI: Influent COD 1; COD 2

```
Two-sample T for COD 1 vs COD 2

                          SE
       N  Mean  StDev  Mean
COD 1  5  1159    170    76
COD 2  5  1189    200    89


Difference = mu (COD 1) - mu (COD 2)
Estimate for difference:  -30
95% CI for difference:  (-301; 240)
T-Test of difference = 0 (vs not =): T-Value = -0,26  P-Value = 0,802  DF =
8
Both use Pooled StDev = 185,2558
```

Two-Sample T-Test and CI: 1 / Influent 1; 1 / Influent 2 for pH

```
Two-sample T for 1 / Influent 1 vs 1 / Influent 2

                  N     Mean    StDev  SE Mean
1 / Influent 1  26  0,10867  0,00198  0,00039
1 / Influent 2  26  0,12629  0,00285  0,00056


Difference = mu (1 / Entrance 1) - mu (1 / Entrance 2)
Estimate for difference:  -0,017622
95% CI for difference:  (-0,018992; -0,016253)
T-Test of difference = 0 (vs not =): T-Value = -25,85  P-Value = 0,000  DF =
50
Both use Pooled StDev = 0,0025
```

Two-Sample T-Test and CI: 1 / Influent 1; 1 / Influent 2 for pH (0.833 = 1/1.20)

```
Two-sample T for 1 / Entrance 1 vs 1 / Entrance 2

                  N     Mean    StDev  SE Mean
1 / Influent 1  26  0,10867  0,00198  0,00039
1 / Influent 2  26  0,12629  0,00285  0,00056


Difference = mu (1 / Influent 1) - mu (1 / Influent 2)
Estimate for difference:  -0,017622
95% upper bound for difference:  -0,016480
T-Test of difference = 0,833 (vs <): T-Value = -1247,60  P-Value = 0,000  DF
=
     50
Both use Pooled StDev = 0,0025
```

In Tables 14 and 15 they are presented the values used for estimating COD removal and the weighted mean used as influent COD respectively. The Kolmogorov-Smirnov Normality test was selected (p > 0.05 normal distribution).

Table 14: Values used for estimating COD removal

Reactors:	COD 2	COD 3	COD 4	1 / COD 2
	490	737	595	0.00204
	496	744	807	0.00202
	525	730	555	0.00190
	615	525	494	0.00163
	504	485	722	0.00198
	492	567	720	0.00203
	520	623	572	0.00192
	522	585	772	0.00191
	558	547	503	0.00179
	518	511	755	0.00193
	494	691		0.00202
	502			0.00199
mean:	519.66	613.18	649.50	0.002
			Derived mean:	500.0

COD 2: p = 0.018 < 0.05 (non normal distribution)

COD 3: p > 0.150

COD 4: p > 0.150

1 / COD 2: p = 0.048 (marginally normal distribution)

Table 15: Estimation of the weighted mean used as influent COD

Influent:	COD 1	COD 2
	1302	1434
	1380	1259
	1025	882
	1066	1171
	1021	1200
mean:	1158,8	1189,2
	weighted mean:	1174

Influent COD 1 p = 0.119 > 0.05

Influent COD 2 p > 0.150

COD variation (rise / decrease): Estimated from the following type:

$$\frac{\text{Final number} - \text{Initial number}}{\text{Initial number}} * 100$$

Reactor 2 compared to influent

$$\frac{500.0 - 1174}{1174} * 100 = -57.4\%$$

Reactor 3 compared to influent

$$\frac{613.18 - 1174}{1174} * 100 = -47.8\%$$

Reactor 4 compared to influent

$$\frac{649.50 - 1174}{1174} * 100 = -44.7\%$$

Reactor 3 compared to reactor 2

57.4 – 47.8 = 9.6 % units higher COD

Reactor 4 compared to reactor 2

57.4 – 44.7 = 12.7 % units higher COD

Reactor 4 compared to reactor 3

47.8 – 44.7 = 3.1% units higher COD