APPLICATION OF IONIZING RADIATION ON THE CORK WASTEWATER TREATMENT

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Abstract

In the framework of the CRP on "Radiation treatment of wastewater for reuse with particular focus on wastewaters containing organic pollutants" Portuguese team is been developed studies on the implementation of ionizing radiation technology as a complementary treatment for industrial effluents and increase the added value of these wastewaters. Based on these assumptions, preliminary studies of the gamma radiation effects on the antioxidant compounds present in cork cooking water were carried out. Radiation studies were performed by using radiation between 20 and 50 kGy at 0.4 kGy/h and 2.4 kGy/h. The radiation effects on organic matter content were evaluated by Chemical Oxygen Demand (COD). The antioxidant activity was measured by Ferric Reducing Power (FRAP) assay. The total phenolic content was studied by Folin-Ciocalteau method. Results point out that gamma radiation increases both the amount of phenolic compounds and antioxidant capacity of cork cooking water. By the other hand, the radiolytic degradation by ionizing radiation of gallic acid and esculetin as models for recalcitrants were studied. The objective of this study was to find out if radiolytic degradation, followed by microbial degradation could increase the treatment efficiency. A natural cork wastewater bacterium was selected from the irradiated wastewater at 9 kGy. The applied methodology was based on the evaluation of growth kinetics of the selected bacteria by turbidimetry and colony forming units, in minimal salt medium with non-irradiated and irradiated phenolic as substrate. The overall obtained results highlights the potential of this technology for increase the add value of cork waters and raised some issues to explain by new methodological setup on biodegradation studies.

1. Objective of the research

The main goal of the research to be developed by the Portuguese team is the implementation of ionizing radiation technology as a complementary treatment for industrial effluents. The current research studies are based on two fields: 1) to find out if radiolytic degradation, followed by microbial degradation could increase the wastewater treatment efficiency and 2) to study the potentialities of ionizing radiation to increase cork wastewaters added value.

2. Introduction

Cork production and transformation process are important sectors of activity in Portugal. This is the raw material that feeds an industry of great importance to the national economy [1]. In cork processing, after the stabilization period of the cork planks (3-12 months), the planks are immersed in water at 100ºC to cook. The water is reused (3 labor days) and, at the end of each cycle, there is a filtration step to minimize the organic load and solid waste of the cooking water.

The cork cooking waters are complex mixtures of plant extracts turning water to dark brown liquor. These waters have a high organic load and considerable toxicity due to hot extraction that occurs in cooking process. Among other natural compounds, these waters have a high concentration of polyphenols such as phenolic acids, tannins, 2,4,6-trichloroanisole, benzoic acid and cinnamic acids[2]. Some of these polyphenols are known for their high antioxidant activity. According to Minhalma and Pinho [3], the main phenolic acids in these waters are gallic, protocatechuic, vanillic, ferulic and ellagic acids.

Advanced oxidation processes are being studied as potential technologies to reduce the organic matter content as phenolic compounds, increasing the biodegradability of wastewaters [4, 5, 6]. The Fenton oxidation process is one of the most recognized technology where the generation of highly reactive hydroxyl radicals (HO•) through catalytic decomposition of hydrogen peroxide reduces the amounts of organic matter [7]. However, the reagent and catalyst (ferrous iron) added to generate the HO• radicals are expensive and potential pollutants and it would be desirable to reduce its use. Ionizing radiation has the same mechanism of Fenton oxidation, i.e., the generation of free radicals to promote compounds degradation but without adding any chemical. Therefore, ionizing radiation seems to be a potential technology to be applied in order to increase the biodegradability [8, 9, 10]. In addition, the use of natural bacteria from wastewater as degraders could be a promising approach to remediation of wastewaters [11].

3. Materials and Methods

3.1. Gamma irradiation of cork cooking wastewater, gallic acid and esculetin solution

Cork boiling wastewater was collected from Fabricor, S.A., the cork industry used as case study in this project. The sampling procedure had three purposes:1) irradiation of raw cork wastewater collected in the end of cooking process before the discharge to the wastewater treatment plant in order to assess the gamma radiation effects on organic matter and antioxidant activity; 2) the characterization of natural cork wastewater microbiota and 3) the isolation of the survivor population after irradiation. Therefore, the cork wastewater sample was irradiated at 2 kGy up to 50 kGy and for the radiolytic degradation of tested phenolic compounds, pure solutions of 1 mmol/dm^3 gallic acid (>99%; Sigma-Aldrich) solution and a 0.1 mmol/dm^3 esculetin solution (>99%; Sigma-Aldrich) were also irradiated at 9 kGy absorbed dose. The gamma irradiations were carried out at the Co-60 facility in the Radiation Technology Unit located at Nuclear and Technological Institute [12]. The dose rate used was 2.5 kGy/h and the measured absorbed doses by routine dosimeters [13].

3.2. Isolation and morphological characterization of cork boiling wastewater microbiota

The characterization of cork wastewater microbiota was made using conventional microbiological techniques (e.g. microscopic observation; gram staining; catalase and oxidase tests) and, after microrganims growth, colonies with distinct macroscopic morphologies were isolated by sub-culturing on the same medium. The most frequent isolate from irradiated sample was identified by API system (Biomérieux, France) according to manufacturer instructions. This microorganism was selected to be used in the growth evaluation studies.

3.3. Growth evaluation on synthetic medium

A minimal mineral medium [1.28% Na2HPO4.7H2O, 0.3% KH2PO4, 0.05% NaCl and 0.1% NH4Cl] was supplemented separately with three carbon sources for gallic acid: i) 200 mg/L of gallic acid solution (GA); ii) 200 mg/L of irradiated gallic acid solution at 9 kGy (GA9) and iii) 200 mg/L of irradiated gallic acid solution at 38 kGy (GA38); and with two carbon sources for esculetin: i) 200 mg/L of esculetin solution (E) and ii) 200 mg/L of irradiated esculetin solution at 9 kGy (E9). Addition of each of the tested compounds to the growth medium caused no change in pH. For both studied compounds, Tryptic Soy Broth (TSB) was used as growth positive control (C) and minimum mineral medium without carbon source as negative control (B). The selected isolate was inoculated in all culture medium at an initial concentration approximately 107 cfu/mL (initial Optical Density (O.D.610nm) approximately 0.06 for gallic acid and 0.2 for esculetin) and incubated at 30 ± 2 ^{o}C in an orbital shaker (100 rpm). The bacterial growth for each culture assay was monitored at regular time intervals by: 1) turbidity measurement at 610 nm (Shimadzu spectrometer UV-1800) and 2) CFU counts by direct plating into Tryptic Soy Agar of aliquots of serial decimal dilutions.

3.4. Chemical Oxygen Demand (COD) and Total Phenolic Content (TP)

COD was measured by Titrimetric method accordingly to the Standard Methods for the Examination of Water and Wastewater [14]. Samples were diluted 10-fold. The TP was determined based on Folin-Ciocalteau method (Singleton at al., 1999) using gallic acid as standard. Samples were diluted 5-folds and 0.5 mL of the diluted sample was added to 35 mL of water and 2.5 mL of Folin-Ciocalteau reagent (FC). After shaking and incubating 5 minutes at room temperature, it was added 7.5 mL of sodium carbonate solution and water to the 50 mL line. The solution was incubated at room temperature for 2 hours and after that the absorbance of the reaction mixture was measured at 765 nm using a Shimadzu UV 1800 spectrophotometer. Results were expressed as mg of gallic acid equivalents per liter of sample.

3.5. Evaluation of antioxidant activity by Ferric Reducing/Antioxidant Power (FRAP)

The assay was carried out according to the method described by Benzie and Strain[15]. FRAP reagent was freshly prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and 20 mM FeCl3.6H2O in a ratio of 10:1:1 at 37°C. One hundred microliters of sample diluted 10-folds in ultrapure water were added to FRAP reagent (3 mL) in a test tube. After 15 minutes of incubation at 37°C, the absorbance was measured at 593 nm. The antioxidant potential of the sample was determined from a standard curve using FeSO4.7H2O solution between 0 and 1.0 mM.

4. Results and Discussion

The natural cork wastewater mesophilic bacterial population was isolated before and after irradiation. A total of 27 isolates in non-irradiated and 11 in irradiated samples at 9 kGy were phenotyped according to microscopic morphology and biochemical characteristics. The relative frequency of each phenotype is presented in Figure 1.

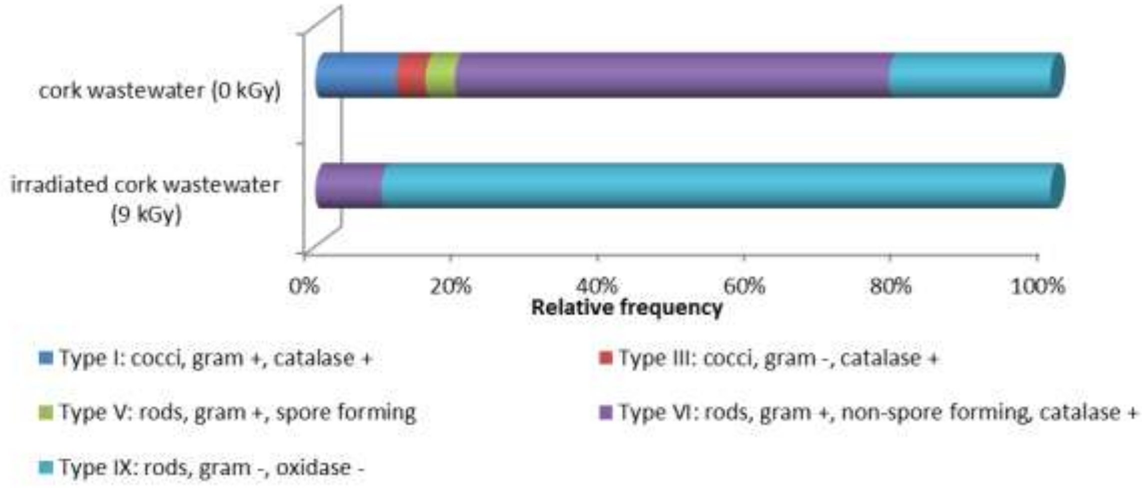

Fig. 1. Relative frequency (in percentage) of the phenotypes of the isolates from non-irradiated cork wastewater and irradiated cork wastewater at 9 kGy.

The isolates from non-irradiated wastewater sample were grouped into five morphological types, being the most frequent the gram positive rods non-spore forming (59%). After irradiation at 9 kGy the diversity decreased, only two of the initial morphological types had persisted. The most frequent was the gram negative oxidase negative rods (91%). The major isolate from this group was identified as *Stenotrophomonas malthophilia* (%id = 92.4). Although it was an environmental isolate, strains from this microorganism are known to metabolize a broad range of aromatic compounds [16, 17]. Based on that assumption, this isolate was used as test strain to evaluate the degradation of target compounds before and after irradiation. This evaluation was made by inoculation of the microorganism into a mineral medium with 200 mg/L of non-irradiated or irradiated gallic acid (GA) and esculetin (E) solution, separately, as sole carbon and energy source. As growth positive control TSB medium was used and as growth negative control, mineral medium without carbon source was used.

4.1. Standard phenolic compounds

The bacterial growth monitoring by turbidity at 610 nm for both gallic acid and esculetin is represented in Figure 2.

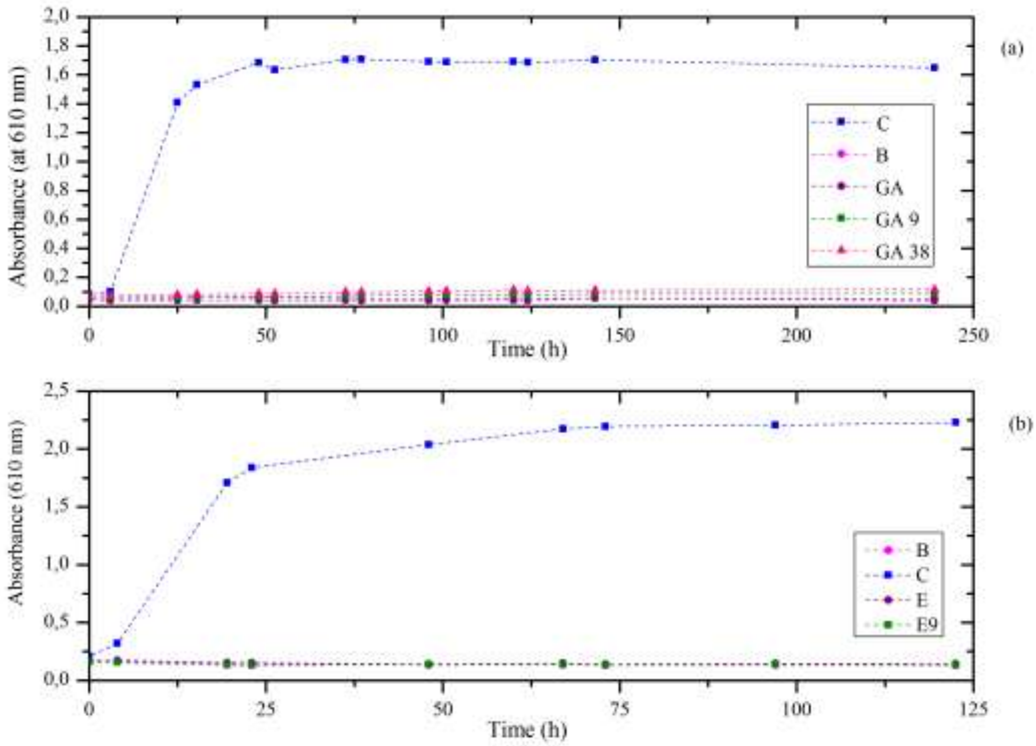

Fig. 2. Turbidity results monitored as Optical Density (O.D.) at 610 nm for each different carbon source culture medium for both studied compounds: (a) (C) positive control (TSB medium); (B) negative control (mineral medium without carbon source); (GA) GA sol., 0 kGy; (GA9) GA sol. 9 kGy and (GA38) GA sol., 38 kGy; (b) C) positive control (TSB medium); (B) negative control (mineral medium without carbon source); (E) E sol., 0 kGy and (E9) E sol. 9 kGy.

The presented turbidity growth results suggested that the test strain was not able to metabolize as sole carbon source either gallic acid solution (irradiated and non-irradiated) and esculetin solution (irradiated and non-irradiated) at the used concentration. The positive growth control indicated the viability of the inoculum denoted by the obtained well defined growth curve and negative control indicated that selected bacteria was not capable to growth in the minimal mineral medium.

The other method used to monitored growth, the cfu counts, also failed to detect bacterial growth in gallic acid cultures (see Figure 3).

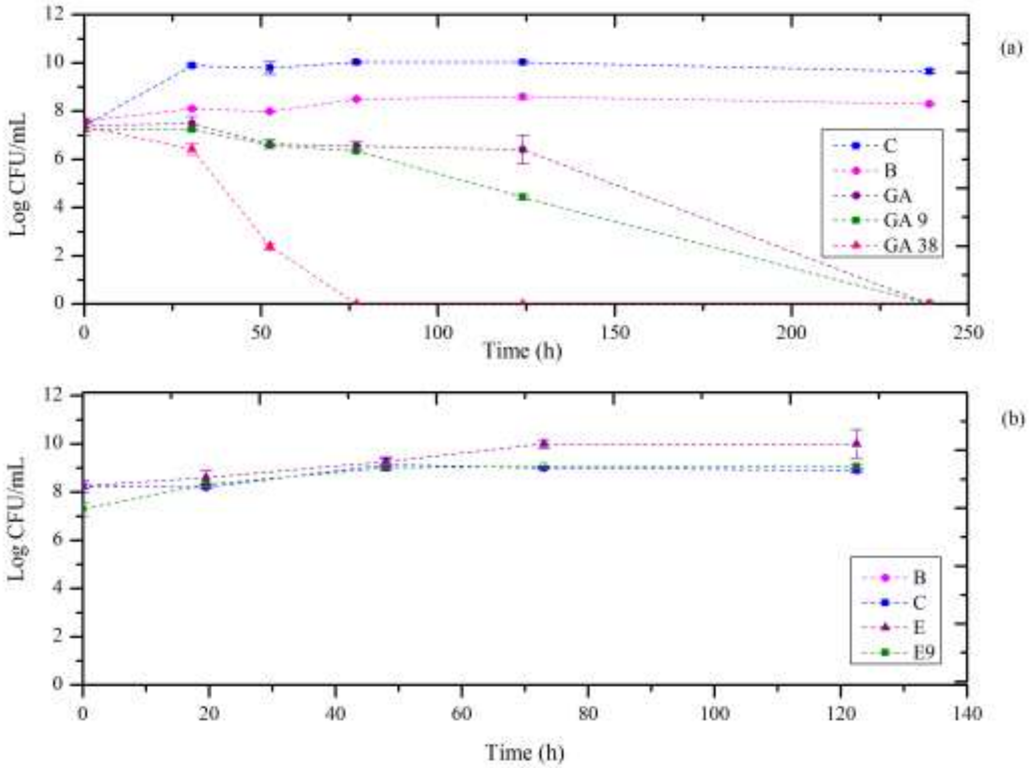

Fig. 3. Average CFU counts of Stenotrophomonas maltophilia *strain in both gallic acid and esculetin different cultures: (a) (C) positive control (TSB medium); (B) negative control (mineral medium without carbon source); (GA) GA sol., 0 kGy; (GA9) GA sol. 9 kGy and (GA38) GA sol., 38 kGy; (b) (C) positive control (TSB medium); (B) negative control (mineral medium without carbon source); (E) E sol., 0 kGy and (E9) E sol. 9 kGy. Error bars correspond to 95% confidence intervals about mean values (n= 6; α = 0.05).*

In agreement with turbidity measurements, the CFU counts of the positive control (C) increased during the initial incubation hours, indicating the adequacy of methods to evaluate the growth of the test strain either gallic acid and esculetin medium cultures. As expected, for the negative control (B) it was not observed substantial variations in growth during incubation time.

As presented in Figure 3(a), the results point out to a decrease in CFU counts in GA solution with absorbed dose. Therefore, the radiolytic by-products seem to have higher negative effect on *S. malthophilia* growth then the parent compound. The antimicrobial activity of phenolic acids, namely gallic acid, have been demonstrated against gram negative bacteria in planktonic state [18, 19]. Phytochemical products (e.g. gallic acid) are routinely classified as antimicrobials on the basis of susceptibility tests that produce growth inhibitory effects in the range of 100 to 1000 µg/mL [20]. Since the GA concentrations used (non-irradiated and irradiated) in cultures medium were 200 µg/ml it is reasonable to assume an antimicrobial action of gallic acid against the tested strain.

Melo et al. [21] reported that at 9 kGy the gallic acid radiolytic byproducts are mainly fragments from gallic acid degradation with aromatic ring opening, connected to some still resilient gallic acid structures. This fact could explain the low biodegradability of irradiated gallic acid solution. However, irradiated gallic acid solution at 38 kGy showed that not only gallic acid, but also the aromatic intermediate products, tend to disappear [21] meaning that even non-aromatic byproducts present an antimicrobial action against the tested strain. Besides the bacterial growth assessment, it was also important to determine the quantity of gallic acid present in the cultures supernatant.

Concerning esculetin solution, Figure 3(b) shows that there is no gamma radiation effect on esculetin solution. The radiolytic products of esculetin were identified in our lab (submitted) and are mainly aliphatic compounds since esculetin structure degrades at low absorbed doses. As for gallic acid biodegradation assay, the tested strain point out to be not able to use either irradiated or non-irradiated esculetin as carbon source, denoting a growth inhibition. Although, must be noticed that the test strain was maintained viable in esculetin supplemented cultures with unchangeable counts during almost 6 days. This fact could suggest a bacteriostatic effect of esculetin against *S. maltophilia* strain. A study performed by Dürig et al. [22] demonstrated that esculetin abolish biofilm formation but have no effect on planktonic growth of a *S. aureus* strain at the concentration of 128 µg/ml. Other authors [23] have stated the antimicrobial activity of esculetin against several microorganisms.

To our knowledge, this is the first study that tries to associate the metabolic capacities of cork natural microbiota along with a radiolytic process, in order to promote the recalcitrant compounds degradation. Besides the obtained inconclusive results and not comparable with the literature, this preliminary bench scale approach have raised some issues to explain by a new methodological setup. Briefly, a detailed organic content characterization will be performed in order to select the main recalcitrant compounds for the studied cork wastewater and a mixed natural wastewater microbial population will be tested. The well-known potentialities of gamma radiation as complementary wastewater treatment technology will be carried on to a future scale up on a Portuguese cork Industry.

4.2. Chemical Oxygen Demand (COD)

COD is a routine parameter used to reveal the total organics in wastewaters as well as the extent of wastewater treatment [24, 25]. This parameter reflects a global oxygen capability uptake that can be due to a large variety of compounds including the phenolic compounds. The COD results are presented in the Figure 4.

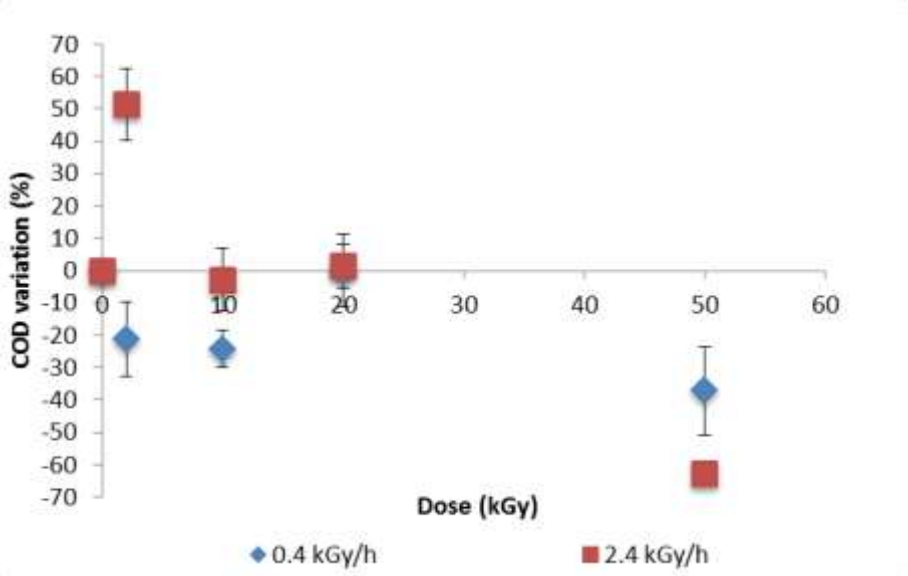

Fig. 4. COD vs. Absorbed dose (n=5; α=0.05).

Figure 4 shows that for a 50 kGy absorbed dose the % COD variation decreases (average 37±14% at 0.4 kGy/h and average 63±0.01% at 24 kGy/h) for both dose rates. With the exception of 2 kGy absorbed dose at high dose rate (2.4 kGy/h) the gamma radiation effect is associated with a decrease of COD and therefore indicates that there is a chemical degradation of organic matter [26] . Other authors [27, 28] also reported that the COD decreases with gamma irradiation in industrial wastewaters. At low absorbed doses (2 kGy) and high rate (2.4 kGy/h) the momentary increase of COD value can, probably, be done to the degradation of some heavy mass compounds in a large number of lower mass compounds that actively contribute to that increase. However, for larger doses, also these smaller compounds are degraded leading to a final decrease of COD value.

4.3. Correlation between antioxidant activity by Ferric Reducing/Antioxidant Power (FRAP assay) and Total phenolic compounds (TP)

Figure 5 shows the correlation found between FRAP and TP and showed that the main responsible for the antioxidant activity are the phenolic compounds, as expected. The Pearson coefficient (r) for the both doses rates is strong and positive (r=0.902 for 0.4 kGy/h and r=0.998 for 2.4 kGy/h) that indicates a strong correlation between TP and the antioxidant activity. The higher antioxidant activity increase at 2.4 kGy/h dose rate could be related to a greater amount of radical species generated that tend to for a higher number of antioxidant active compounds.

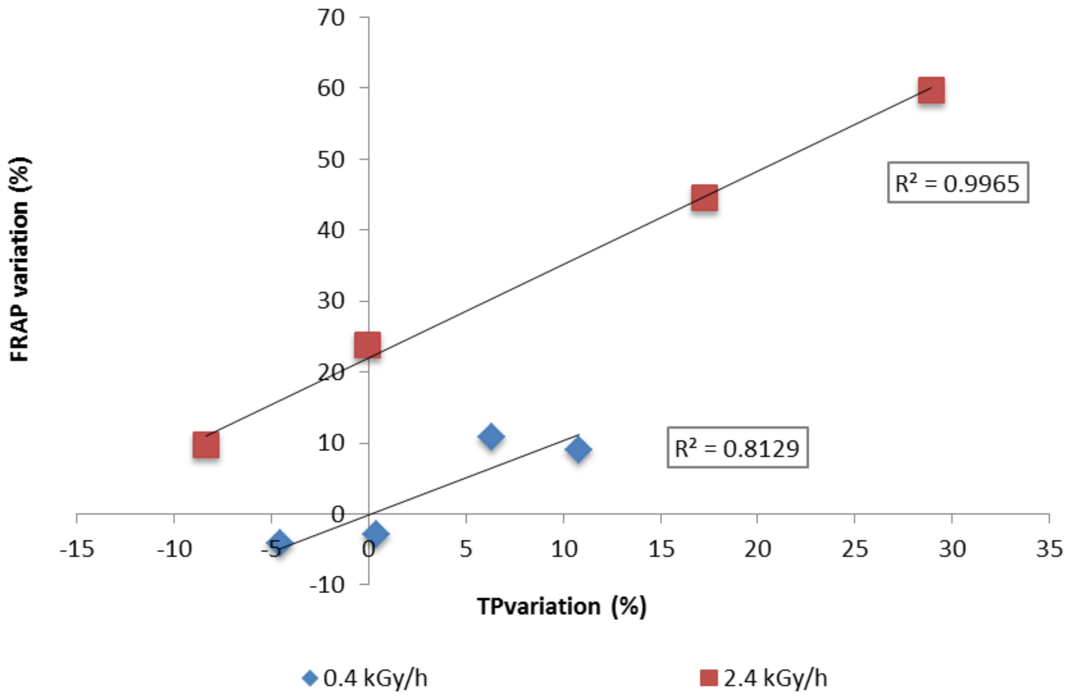

Fig. 5. Antioxidant activity vs. total phenolic compounds.

These results could be explained by hydroxyl radical attack to large molecules (eg. tannins and other complex phenolic compounds) but without breaking most of the phenyl groups. The breaking of those compounds leads to an increase of the concentration of smaller phenolic structures with a global higher antioxidant activity.

In conclusion, gamma radiation was applied as a pretreatment for cork cooking water. High dose rate (2.4 kGy/h) and low dose rate (0.4 kGy/h) were applied and the obtained results point out that there are significant differences between dose rates. COD results, together with FRAP and TP results, show us a picture where larger molecules are degraded producing a lot of small phenol compounds. This is a very important result to cork industry since a treatment like this one increases dramatically the added-value compounds (antioxidants) in the cooking water while decreases other organic matter leading to a much more valuable solution as raw material for other industries. To our knowledge, there are no studies about gamma irradiation effects on antioxidant activity of cork cooking water. Further studies have to be performed to access the technical viability of extraction, identification, purification and reuse of now detected antioxidants.

5. Ongoing activities and further work

Ongoing studies are focusing on two main fields:

1) extraction of potential antioxidants: activated carbon (olive seeds); the economic viability of these techniques; toxicity tests and repetition of experiments with electron beam

2) As mentioned above, the application of a new methodological setup for biodegradation will be performed and a new microbial population will be selected in order to optimize the biological treatment as a complement with ionizing radiation.

3) The understanding of the mechanisms of different antioxidant activity methodologies in cork wastewater irradiation. These studies could be helpful to better understand the oxidation mechanisms in radiolytic reactions. Analytical techniques (e.g.: High Performance Liquid Chromatography (HPLC) and ionization by electrospray (ESI-MS)) will be used to characterize and quantify the radiolytic products produced in irradiated wastewaters. In addition, modulation tools will be used to develop kinetic models and calculate reaction rate constants to understand the reaction mechanism of these compounds after irradiation that will be applicable to several free-radical based technologies.

The synergy between both studies will allow the optimization of the irradiation parameters (e.g. type of energy, geometry, absorbed doses and dose rates) to be implemented in a pilot plant on cork industry.

Acknowledgments

We are grateful to the International Atomic Energy Agency (IAEA, Austria) by the supporting the CRP 1539 – F23029 “Radiation Treatment of Wastewater for Reuse with Particular Focus on Wastewaters Containing Organic Pollutants” project. We are also grateful to the cork industry (Fabricor S.A) for allowing us to collect all the samples necessary for the accomplishment of this work. This work was supported by grants from the Institute of Nuclear Technology (ITN) and the Foundation for Science and Technology (FCT) of the Ministry of Science, Technology and Higher Education (MCTES).

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