Bulgarian Journal of Agricultural Science, 20 (Supplement 1) 2014, 31–36
Agricultural Academy

PIGMENTED YEASTS SURVIVED 20 KGY GAMMA IRRADIATION

I. A. IVANOVA and D. Y. POPOVA
University of Sofia "St. Kliment Ohridski", Faculty of Biology, BG – 1164 Sofia, Bulgaria

Abstract

IVANOVA, I. A. and D. Y. POPOVA, 2014. Pigmented yeasts survived 20 kGy gamma irradiation. *Bulg. J. Agric. Sci.,* Supplement 1: 31–36

The cultural heritage deposited in museums, galleries, castles, libraries and private collections is subject to devastation caused by insects, bacteria and fungi. It is beyond the capacity of the small number of restorers, conservation workers and private collectors to combat those hazards using the classic methods. For that reason a radiation laboratory at the Sopharma JSC, Bulgaria, was used to study the biocidic effects of ionising radiation emitted by radioactive isotope of cobalt. That technique is particularly useful for books, as it is highly effective for the entire volume of paper under attack, and as it does not use any chemicals, it does not harm the finishes of the objects treated – binding, book cover, leather, etc. The biocide effects of the applied irradiation on the microorganisms populations on paper were estimated with classic method using cultivation in different nutrient media before and after the irradiation. The obtained results showed significant decrease of the total number of microorganisms in all the paper samples after the lowest applied irradiation dose – 4 kGy. Only several heterotrophic bacteria, fungi and oligotrophic microorganisms survived this dose. Similar data were obtained after 10 kGy. Irradiation with 20 kGy led to highest degree of disinfectation of the papers, only single mixed culture of bacilli and yeast survived such dose. This is the first report about the such kind of irradiated isolate presenting an association of yeasts and bacilli.

The present study showed that the gamma-irradiation of 4 kGy can be successfully applied for efficient disinfection of the library archives, if higher dose is to be avoided to prevent structural changes of the paper like depolymerization of the cellulose, decrease of their mechanical strength etc. Further studies on the identification of the microbial isolates have to be performed.

Key words: gamma-irradiation resistance, bacteria, fungi, paper conservation, disinfection, biocide effect

Introduction

The biocide effect of the ionizing radiation is known since the beginning of 20th century and it is widely used for disinfection and sterilization of different materials, like medical devices, pharmaceutical products and cosmetics, foodstuff, archives, as well as cultural heritage artifacts. Gamma-irradiation is considered as an especially efficient method for disinfestation due to its high penetration, reliability, lack of harmful residues and no need to use toxic chemical fumigants (Butterfield, 1987; Brokerhof, 1989; Kunstadt, 1998). As currently, classical treatment methods using toxic gases like methyl bromide or ethylene oxide, are forbidden, alternative methods must be carefully considered (Ramiere, 1982; Unger et al., 2001; Rizzo et al., 2002; Negut et al., 2007). The comparative advantages are certain and accepted: speediness (the treatment duration takes hours), penetrability (large objects of any form may be treated), simplicity (the object may be treated in its transport package); possibility to treat composite objects could be efficiently at reasonable cost (Ramiere,1982; Urban and Justa, 1986; McCall, 2007). In Bulgaria as is the case in other countries, the treatment applies rarely and only as salvation treatment. Ionizing radiation treatment is still exotic from both subjective

*E-mail: ilivanova@abv.bg

and objective reasons. Librarian archives often are susceptible to insect, fungal, or bacterial attack, which may cause both damage of the material and provoke allergic reactions or other illnesses in people.

Although the advantages of the gamma-irradiation for the archives disinfestation, this technique is not popular and routinely used. There are no such investigations in the last 35–40 years in Bulgaria. The present study aims to evaluate the bactericidal effects of the gamma-irradiation on different microbial populations, found on biodeteriorated books, taken from the Sofia University library.

Materials and Methods

Paper samples collection and treatment

The paper samples were taken from nine different issues, published in Germany, Russia and USA in the period from 1896 to 1965 years as follow: sample 1 – Physics library,1962,USSR; sample 2 – monography, 1965, USSR; sample 3 – 1962, monography, USSR; sample 4 – jornal since 1962, USA; sample 5 – Beilstain, reference book, 1942, Germany; sample 6 – Referativen journal, 1952, USSR; sample 7 – 1923, Germany; sample 8 – monograpgy since 1962, USSR; sample 9 – Chemische Berichte journal since 1896, Germany. The pages of the deteriorated books are cut from the issues in the library and put in a sterile plastic sachets. They were transported in this way to the microbiological lab and total microbial number was determined on different media. After that the paper samples were transported to the radiation laboratory. Each sample was separated on 3, put in plastic pockets again and irradiated with 4 kGy, or 10 kGy or 20 kGy, usually cited in the literature (Ramiere,1982; Urban and Justa, 1986; McCall, 2007). The gamma-irradiation was performed at the radiation facility BULGAMMA based on JS-850 60Co type gamma irradiator of Sopharma JSC, Bulgaria. The cobalt source emits only gamma radiation, which like *radioisotope thermoelectric generator* or UV radiation cannot activate the objects treated, nor leave behind any residues that are harmful to health. The absorbed dose distributions were measured with Ethanol Chlorobenzene routing dosimeters, and was calculated from a calibration curve connecting it with the electric conductivity of the dosimetric solution measured with oscilotitrator.

Microbiological analysis

The total number of microorganisms on the tested paper was counted using classical cultivation method before irradiation and on the 20th day after the irradiation. A sterile cotton swab is used to collect microorganisms from each of the paper surfaces at the edges and deteriorated parts of the pages. The cotton swab is put in 1 ml sterile physiological solution and leached on a shaker for 30 minutes at 120 RPM in order to equalize the microbial quantity on the swab and in solution. After that 3 decimal dilutions were prepared in 0.9% NaCl and aliquots were plated in nutrient agar, Gause and Sabouroud agar, oligotrophic medium (10 mg peptone, 20 g agar, 1L dH_2O); Imshenetzki and Hatchinson media for cellulose degrading microorganisms (Gousterov et al., 1977). Two types of starch were used – for Gauze No1 was used soluble starch, and for Gauze 2 – unsoluble starch. Sporeforming microorganisms were counted after pasteurization at 80°C for 10 min and sowed in nutrient agar (Conda, Spain). Bacterial colonies were counted on nutrient agar at the second day after inoculation, the fungal and streptomycetes colonies – after the first and second week; oligotrophs – at the second and 4th week. All the inoculations were proceeded in minimum 3 replications. The obtained pure bacterial cultures were Gram stained, malachite green stained and pasteurized to prove formation of spores. The fungal colonies were observed using magnifying glass by 10 and 20 time magnification and fotos were prepared at larger magnification.

Results

The conservation irradiation could sterilize objects made of all kinds of organic materials for treatment: wood, leather, textiles, paper, etc.

The plastic bags with paper samples were opened in the laminar box and processed aseptically. Fungi, actinomycetes, oligotrophic, heterotrophic and sporeforming bacteria were identified on the non-irradiated papers. The results obtained are presented at Figure 1.

The highest number of heterotrophic bacteria were counted on nutrient agar for the 4th sample from 1962, USA (figure1). In the 5th sample from 1942, Germany the quantity of fungi growing on Sabouraud agar was prevalent. In all other samples the quantity of oligotrophic microorganisms prevailed. The first sample (1962, USSR) was the most deteriorated and contaminated with the most various and comparatively abundant microorganisms. Those growing on starch were the most colorful suggesting pollution by the air. The microbial count in all paper samples did not exceed a 110 CFU/ml, as the most numerous were the heterotrophic bacteria at 4th sample from journal paper,1962, USA (Figure 1), followed by the oligotrophic bacteria and fungi in the other samples. Seventh sample from 1923, Germany has the least number of various microorganisms.

After irradiation with the 4 and 10 kGy the microbial quantity drops by one order of magnitude. The results are presented at Figures 2 and 3.

In all samples irradiated with 4 kGy only the single colonies were detected. The highest number of colonies counted on nutrient media is 7 oligotrophic microorganisms and 6 heterotrophic found at 4th (journal paper, 1962, USA) and 5th sample – Beilstain,1942, Germany. On the other media at four of the samples only 1 fungal colony has grown, at one of the samples no survived microorganism was detected. As expected, the most contaminated sample (No4 – journal paper, 1962, USA) before irradiation kept the highest microbial number. Again the most abundant were the fungi growing on all kind of media – oligotrophic and heterotrophic.

In all samples treated with 10 kGy the quantity of microorganisms is comparable with those in samples irradiated with 4 kGy. Obviously the higher irradiation of 10 kGy did not affect the oligotrophic fungi – they are in the same quantity in the same samples. The quantity of fungi

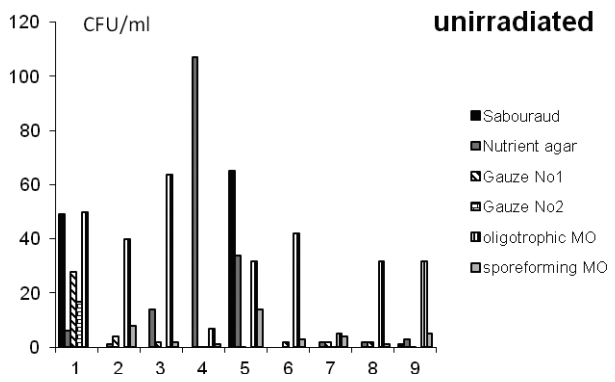

Fig. 1. Quantity of microbial populations growing on unirradiated paper in different media: Sabouraud – fungi, Nutrient agar – heterotrophs; Gauze 1 and 2 – starch-degrading microorganisms; oligotrophic and sporeforming bacteria. The number of samples is under the abscissa axis

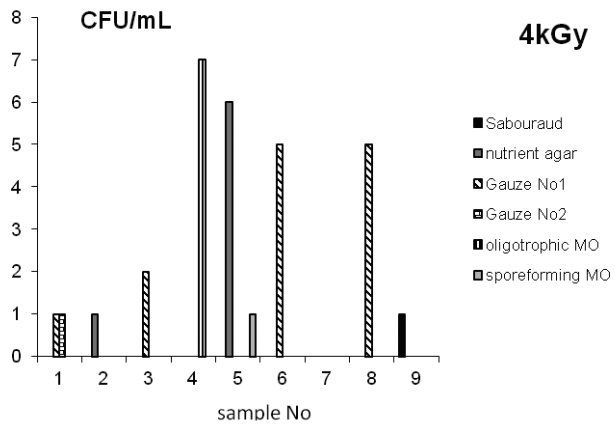

Fig. 2. Quantity of microbial populations growing on different media after 4 kGy irradiation: Sabouraud – fungi, Nutrient agar – heterotrophs; Gauze 1 and 2 – starch-litic microorganisms

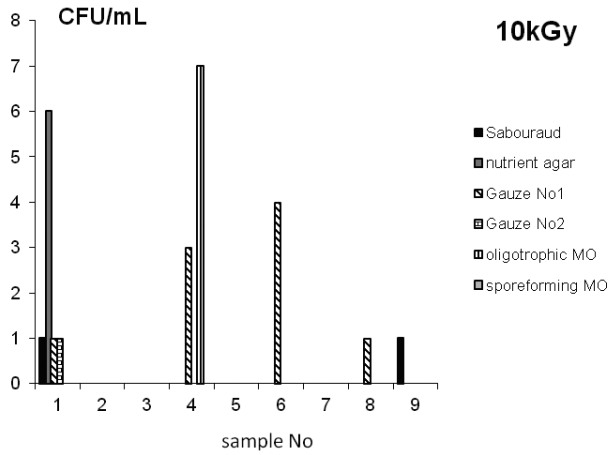

Fig. 3. Quantity of microbial populations growing on different media after 10 kGy irradiation: Sabouraud – fungi, Nutrient agar – heterotrophs; Gauze 1 and 2 – starch-litic microorganisms; oligotrophic and sporeforming bacteria

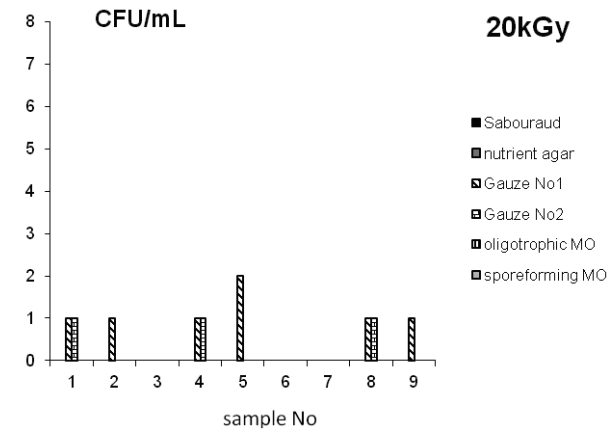

Fig. 4. Quantity of microbial populations growing on different media after 10 kGy irradiation: Sabouraud – fungi, Nutrient agar – heterotrophs; Gauze 1 and 2 – starch assimilating microorganisms; no oligotrophic and sporeforming bacteria.

on Gauze starch media decreased in sample No6 (Referativnii journal, 1952, USSR) and 8 (monography paper, 1962, USSR), but appeared on nutrient agar in sample No1 (journal paper, 1962, USSR). This is possible because of different part of the sample papers was intended for irradiation with doses of 4 and 10 kGy.

The less number of microorganisms is preserved on the paper, irradiated with 20 kGy. The results are presented in the Figure 4. Three of the paper samples after such a treatment were entirely sterile.

As only single colonies were obtained in irradiated with 20 kGy paper samples, was accepted that the result is due to high fungal resistance. The taxonomic investigation of isolate revealed that the single colony survived the highest irradiation is not a pure, but a mixed culture of filamentos fungus, bacillus and yeast in close symbiosis. Even after 5–6 monts subcultivation and maintaining in different media with and without the antibacterial antibiotic Chloramphenicol was impossible to separate them. They grow as a homogenous culture with white colonies at first two days (Figure 5-1), but at the 5th day the color changed in a dark blue with a white center (Figure 5-2). The color and size of 1–2 mm mislead at first, that this is a streptomyces colony. Bacteria start to grow first, after that appeares the yeast and the size of colony grow to 5–6 mm, after the 7th day some white filaments are formed as substrate mycelium around the colony and are 2–3 mm larger than the colored air mycelium (Figure 5). The pigment changes to black in the old cultures after 2 weeks at the centrum and perifery of the colony. After 2 months all the medium is colored black. The microscopic analisys revealed the association between 2 type of yeasts and bacilli (Figure 5–3). One kind of yeast has a little bit larger and prolonged cells than the other. The larger cells are colored darker with methylene-blue than the others (second type). The fact that isolated culture is mixed suggest, that is very possible the culture was in the paper during irradiation, but survived the highest dose because of the natural pigmentation. This is the first report, as far as we know, about the such kind of irradiated isolate presenting a symbiosis of fungi and bacilli.

The microscopic investigation prove the hypothesis, that the fungi and bacteria live in very close symbiosys and could protect each other under gamma irradiation. Several subcultivations on selective yeast malt agar give us the possibility to obtain pure yeast culture. Colonies are creamy, opaque, smooth central part of the colony with a peak, flat, highly developed pseudomicelia. Yast cells are of various shape and size, short and extended-elliptic, singles and in pairs. Further investigation with API 20 C AUX has shown identification No 6773377 suggesting *Candida* spp. *(*possible C. *ciferrii*). Further investigations are needed for final determination.

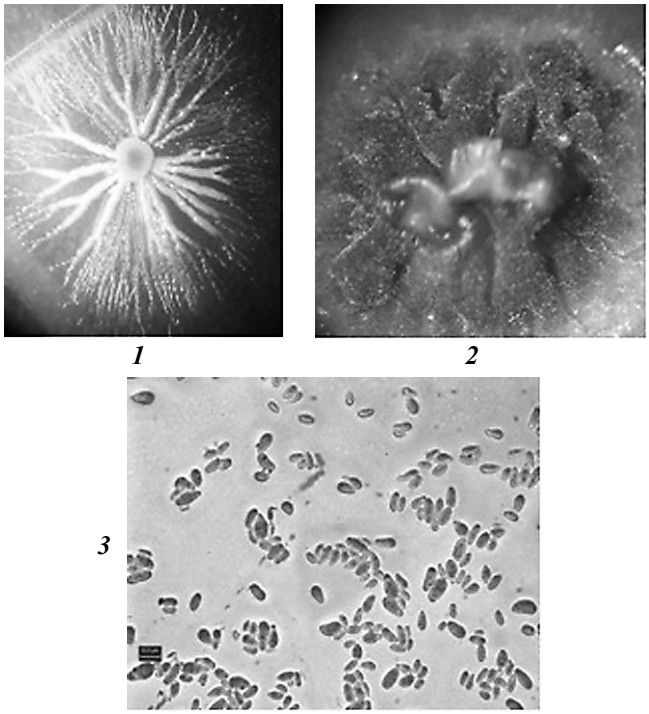

Fig. 5. Micrographs of the symbiotic culture: *1* – at first 2–3 days; *2* – at a week; *3* – one week culture stained with methylene-blue (magnification 630x)

Discussion

The present study showed that the gamma-irradiation with 4 kGy can be successfully applied for efficient disinfection of library archives, if higher dose is avoided to prevent structural changes of the paper like depolymerization of the cellulose, decrease of their mechanical strength etc. Even some single microorganisms survived (about 1–2%) the higher irradiation doses, the most of them vere killed.

Most of isolated bacterial cultures are Gram-positive, sporeforming bacilli – generally from 21 investigated pure bacterial cultures 14 are sporeforming. This is about 67% of isolated pure cultures. Formation of spores is proved by staining with malachite green and pasteurization. The result is not a surprise, as spores can survive during a long time without nutrient medium and without humidity. A lot of isolates survived irradiation are pigmented, which suggest the transportation by air. Most of the isolated bacterial strains (about 90%) are rodshaped, some of them forming chains. Most of them have amylase activity. It was mentioned, that at the first days of cultivation bacteria appeared on oligotrophic and heterotrophic medium, after

that followed by fungal growth on the bacterial colonies. This suggest the mutual assistance between the bacteria and fungi.

The research presented here has additional limitations because samples are not acquired in situ, directly from the objects. The paper samples were carefully packaged and sealed for shipping, thereby minimizing the potential for contamination. However, the abundance of fungal spores everywhere means maintaining a sterile environment between collection and determination of microbial quantity. As a sterile paper envelope was used over the plastic bags, to obtain some contamination is hardly possible. The only reason for detecting a few single colonies at the most paper samples after the highest radiation dose is the fungal resistance to irradiation. Additional air samples of the storage spaces in Sofia University library would provide a more accurate analysis of the type of spores within the room environment. Also, monitoring the relative humidity and temperature fluctuations during the incubation period of the samples of agar would provide more valuable research as to the influence of those conditions on growth. Finally, the observations recorded here were taken at the classic microbiological methods. Further studies exploring direct PCR techniques and sequence analysis would be beneficial, especially since a specialist for mold identification may not always be available.

Scientific studies on the applicability of irradiation for the control of invasive pests appear to be a few. In a classic paper by Kunstadt (1998), he states that of the organisms of concern in wood (insects, nematodes and fungi), insects succumb to 0.7 to 1.3 kGy, while eliminating fungi, the most resistant species, requires significantly higher doses. In a paper by K. Csupor et al. (2000) they demonstrated that the effects of a 2 kGy dose on fungi is significant and over 12 kGy none survived. Other authors (Moise et al., 2012) show that an effective treatment can be performed with doses lower than 10 kGy. In our case single fungal colonies were detected on the paper samples after the 10 kGy irradiation. The most abundant in different samples after this dose of irradiation were fungi as *Aspergillus niger* and *Penicillium* sp. At 20 kGy the strange colony appeared. It attracts our attention because of high radiation resistance, morphology and mixed colors. It appeared on paper irradiated with lower doses too, which means it was disseminated on deteriorated paper. Possibly only the mixed culture of yeasts and bacilli is colored, as pure *Candida* sp. culture is not. Different genera pigmented fungi were studied for melanin production and structure investigation. *Cryptococcus neoformans, Aspergillus niger, Wangiella dermatitides and Coprinus comatus* were used as a model in the study of Casadevall et al. (2012). They report that analysis of melanin in melanized *C. neoformans* encapsulated cells was precluded by the fortuitous finding that the capsular polysaccharide had a diffraction spectrum that was similar to that of isolated melanin. Shrishailnath et al. (2012) discovered melanin pigment in bacteria as *Klebsiella* spp*.,* which gives the possibility of such pigment to exist in genus *Bacillus* too, as our isolate. The fact, that some fungi survived the highest irradiation dose of 20 kGy is in contrast with other reports on the sterilization dose of paper and other masterpieces. At the other hand, the dose of 10 kGy is possibly sufficient to destroy the main microflora, deteriorated the paper and maintenance at low temperature and humidity will be enough to preserve the books and journals in library stores.

Conclusion

As final remarks on the paper treatment with ionizing radiation it should be mentioned:

– The irradiation treatment can be designed such a way that the desired effect (biological decontamination) is achieved and undesired effects (degradation) are minimized – at 10 kGy irradiation;

– The fungal and *Bacillus sp.* spores resist the 20 kGy irradiation doses;

– The mixed *Candida-Bacillus* culture forms dark blue pigment possible melanin.

Acknowledgements

The authors are grateful to the librarians from the Faculty of Chemistry and Pharmacy, and Faculty of Biology, Sofia University, for the provided paper samples. The author I.I. is thankful to Petja Kovacheva and Nelly Boshnakova for the courtesy to transport and process samples, and to Liubomira Yocheva for the helpful comments.

References

Brokerhof, A. W., 1989. Control of Fungi and Insects in Objects and Collections of Cultural Value: A State of the Art; Book. OCLC553976959 Central Research Laboratory for Objects of Art and Science; Amsterdam; Netherlands.

Butterfield, F. J., 1987. The Potential Long-term Effects of Gamma Radiation on Paper. *Studies in Conservation*, **32** (4): 181–191.

Casadevall, A., A. Nakouzi, P. R. Crippa and M. Eisner, 2012. Fungal melanins differ in planar stacking distances. PLoS ONE. **7** (2): e30299. doi:10.1371/journal. pone. 0030299.

Csupor, K., F. Divos and E. Guncz, 2000. Radiation Induced Effects on Wood Materials and Fungi. Proceedings of the 12th International Symposium on Nondestructive Testing of Wood, University of Western Hungary, Soporon, 13–15 September, ISBN 963 71080 88 5

Gousterov, G. K., P. **Andonov, Tz. Todorov, L. Kominkov and M. Gincheva-Starcheva,** 1977. Prakticum on microbiology and virusology. *Nauka I izkustvo*, Sofia, p. 395 (Bg).

Kunstadt, P., 1998. Radiation Disinfestation of Wood Products. *Radiat. Phys. Chem.,* **52** (1–6): 617–623.

Moise, I. V., M. Virgolici, C. D. Negut, M Manea, M. Alexandru[a], L. Trandafir[a], F. L. Zorila, C. M. Talasman, D. Manea, S. Nisipeanu, M. Haiducu and Z. Balan, 2012. Establishing the irradiation dose for paper decontamination. *Radiation Physics and Chemistry,* **81** (8): 1045–1050.

McCall, N., 1985. Ionizing Radiation as an Exterminant: A Study Case; *Conservation Administration News*, **23** (1–2): 20–23.

Negut, D. C., C. C. Ponta, R. M. Georgescu, I. V. Moise, Gh. Niculescu and A. I. M. Lupu, 2007. Effects of gamma irradiation on the colour of pigments. *Proc. SPIE Int. Soc. Opt. Eng*. 6618, 66180R.

Ramiere, R., 1982. Protection de l'environement culturel par les techniques nucleaires. *Proceed. Series IAEA Vienna*, STI/PUB/598, 255–270.

Rizzo, M. M. et al., 2002. Effects of gamma rays on a restored painting from the XVII century. *Radiat. Phys .Chem*., **63** (3–6): 259–262.

Unger, A., A. P. Schniewind and W. Unger, 2001. Conservation of Wood Artifacts. *Natural Science in Archaeology, Biological Methods*, pp. 355–361.

Urban, J. and P. Justa, 1986. Conservation by Gamma Radiation: The Museum of Central Bohemia in Roztoky; *Museum*, **151:** 165–167.

Urban, J., I. Santar, J. Sedlackova and J. Pipota, 1978. Use of Gamma Radiation for Conservation Purposes in Czechoslovakia. ICOM Commitee of Conservation: 5th Trienal Meeting, Zagreb, proceedings.

Shrishailnath, S. S., O. Anjaneya, B. Guruprasad, A. N. Kulkarni, S. Nayak, B. Suresh, T. B. Mashetty and T. B. Karegoudar, 2012. Properties and functions of melanin pigment from *Klebsiella* sp. *GSK Korean Journal of Microbiology and Biotechnology,* (12). www.researchgate.net/publication/235735211_Properties_and_functions_of_melanin_pigment_from_Klebsiella_sp._GSK