AGRICULTURAL UNIVERSITY OF ATHENS

DEPARTMENT OF FOOD SCIENCE AND HUMAN NUTRITION
LABORATORY OF FOOD ENGINEERING

A RESEARCH STUDY FOR THE MSc. POST-GRADUATE DEGREE: FOOD SCIENCE AND TECHNOLOGY & HUMAN NUTRITION

Isolation, quantification and incorporation of violacein into lipid bilayers.

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Athens, 2017

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Acknowledgements

Firstly, I would like to thank my supervisor professor Dr. Kanavouras Antonios Lecturer of the Department of Food Science and Human Nutrition, Agricultural University of Athens for his trust by assigning to me to carry out the present postgraduate study, and for his guidance during that period.

Also, I would like to thank the other two members of my tripartite committee, Dr. Mantala Ioanna Assistant Professor in Physical Properties of the Department of Food Science and Human Nutrition, Agricultural University of Athens and Dr. Skandamis Panagiotis Assistant Professor of Food Microbiology and Food Hygiene of the Department of Food Science and Human Nutrition, Agricultural University of Athens, whose also laboratory had supplied us with the samples of the substance that was used in the present study.

Moreover, I would like to thank Dr. Demetzos Constantinos Professor of Pharmaceutical Nanotechnology of the National and Kapodistrian University of Athens, and also Natassa Pippa Postdoctoral Researcher of the Department of Pharmaceutical Technology, Faculty of Pharmacy, of the National and Kapodistrian University of Athens, for their significant help with the nano encapsulations that were studied in the present study.

I would like also to thank for her crucial help which was a major contributor to my effort and supported in every phase of the research study Dr. Georgiadou Maria of the Department of Food Science and Human Nutrition, Agricultural University of Athens. As also and every member of the Laboratory of Food Engineering for their assistance and their support in all phases of the development of this postgraduate study.

Finally, I would like to thank all these people that where beside me, my parents Thanasis and Anastasia, my brother Stelios, and also my girlfriend Liliana for their unconditional support throughout the course of my studies.

Loukopoulos Panagiotis

Abstract

Violacein, is a pigment that constitutes a product of the secondary metabolism of the bacteria. It is still unclear the reason of its production from the bacteria, but it is a common belief that the main suspect is the stress conditions that the bacteria are facing.

The aims of our subject, were the quantification of violacein produced by Janthinobacteium livindum under various caltivation conditions and the encapsulation of the products in lipid bilayers with the further ambition to estimate the potential of their incorporation in foods. In this later stage we studied the stability of nanostructures with violacein in different pH conditions.

In our research violacein was produced from the bacterium Janthinobacteium livindum. This bacterium was stated that produces not only violacein but also deoxyviolacein, with the analogy as stated 85% violacein and 15% deoxyviolacein (Choi et. al., 2015a).

The substance was dissolved in ethanol, then the samples were divided by the day that where taken, but with no consistent pattern.

Then the samples were characterized by the HPLC according to the Rodrigues' (2012) method.

Following that, the substance samples were encapsulated into phospholipids' nanostructures. Two types of lipids were used: L-α-phosphatidylcholine, hydrogenated soy, (HSPC) and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, (DPPC). Their thermal stability was studied with Differential Scanning Calorimetry (DSC).

The purpose of this work was to map the behavior of nanostructures in different acidity. Because, it is crucial to have the knowledge if the acidity is an obstacle in order to apply these nanostructures in the food systems. Something that does not apply in this case, by the results of the DSC which showed that the structures were stable regardless the modulation of acidity.

Keywords: violacein, deoxyviolacein, acidity, HPLC, DSC, nanostructures, HSPC, DPPC

Περίληψη

Αντικείμενο της συγκεκριμένης μελέτης αποτελεί η βιολασεΐνη. Μια ουσία που αποτελεί προϊόν του δευτερογενή μεταβολισμού των βακτηρίων. Ακόμη δεν έχει αποσαφηνιστεί ο λόγος που παράγεται αυτή η ουσία από τα συγκεκριμένα βακτήρια, παρόλα αυτά ως κύριος υπεύθυνος μπορούν να θεωρηθούν οι συνθήκες stress στις οποίες υπόκεινται τα βακτήρια.

Σκοπός της συγκεκριμένης έρευνας είναι η ποσοτικοποίηση της βιολασεΐνης που παράχθηκε από το βακτήριο Janthinobacteium livindum κάτω από διαφορετικές συνθήκες καλλιέργειας και η ενσωμάτωσή τους σε λιπιδικές διπλοστοιβάδες με την φιλοδοξία να δούμε την πιθανή τοποθέτησή τους σε τρόφιμα. Σε επόμενο στάδιο της έρευνας έπρεπε να μελετηθεί η σταθερότητα των νανο-δομών με τη συγκεκριμένη ουσία σε διαφορετικές συνθήκες οξύτητας (pH).

Στην ερευνά μας η ουσία παράχθηκε από το βακτήριο Janthinobacteium livindum. Για το συγκεκριμένο βακτήριο παρατηρήθηκε ότι η μωβ ουσία δεν περιέχει μόνο την ουσία βιολασεΐνη αλλά την συνοδεύει και η ουσία δεόξυ-βιολασεΐνη με αναλογία όπως αναφέρεται στη βιβλιογραφία 85% βιολασεΐνη και 15% δεόξυ-βιολασεΐνη (Choi et. al., 2015a).

Η ουσία ήταν διαλυμένη σε διάλυμα αιθανόλης τα δείγματα διαχωρίζονταν και κατά μέρα που είχαν ληφθεί, χωρίς όμως να υπάρχει κάποιο σταθερό μοτίβο.

Έπειτα τα δείγματα χαρακτηρίστηκαν με τη χρήση της HPLC σύμφωνα με τη μέθοδο Rodrigues (2012).

Στην συνέχεια ακολούθησε η ενσωμάτωση δειγμάτων της ουσίας με φωσφολιπίδια προκείμενου να προκύψουν οι νανο-δομές. Χρησιμοποιήθηκαν δύο είδη λιπιδίων τα: L-α-phosphatidylcholine, hydrogenated soy, (HSPC) και 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, (DPPC), τα οποία και μελετήθηκαν με τη βοήθεια της Διαφορική Θερμιδομετρία Σάρωσης (DSC), για τη θερμική τους συμπεριφορά.

Αυτό έγινε με σκοπό να γίνει μια χαρτογράφηση της συμπεριφοράς των νανο- δομών σε διαφορετικές τιμές οξύτητας, επειδή είναι βασικό να φανεί εάν είναι εμπόδιο η οξύτητα στο να εφαρμοστούν οι νανο- δομές αυτές στα τρόφιμα. Κάτι που όπως αποδείχθηκε δεν ισχύει μιας και τα αποτελέσματα της DSC έδειξαν ότι οι δομές συγκρατούνται άσχετα από τη διαφοροποίηση της οξύτητας.

Λέξεις κλειδιά: βιολασεΐνη, δεόξυβιολασεΐνη οξύτητα, HPLC, DSC, νανο- δομές, λιπίδια, HSPC, DPPC

Continents

1. INTRODUCTION

1.1. VIOLACEIN SUBSTANCE PRODUCTION (MICROORGANISMS, PATHWAY)

Violacein, a bisindole, is a secondary metabolite, associated with the biofilm production. It is produced by several bacteria species, but those that have been studied more excessively are of the Chromobacterium sp. (Venil et al., 2015, Hoshino et al., 2011, Sasidharan et al., 2015). Violacein has a characteristic purple color which is the reason of appellation. Janthinobacteium livindum produces not only the violacein but also the deoxyviolacein compound, in a ratio of 85% violacein to 15% deoxyviolacein (Choi et. al., 2015a). Deoxyviolacein is the most well-known derivative produced by the violacein's biosynthetic pathway. The difference being that deoxyviolacein lacks a hydroxyl group, as it is showed and in the Figure 21 in the appendices.

There are many researches about violacein and its production by several microorganisms, in the literature, including Chromobacterium violaceum (Moss et. al., 1978, Ahmad et. al., 2012), Collimonas sp. (Hakvag et. al., 2009), Duganella sp. (Aranda et. al., 2011), D. violaceinigra (Li et. al., 2004), Janthinobacterium lividum (Lu et. al., 2009), J. svalbardensis (Avgustin et. al., 2013), Pseudoalteromonas sp. (Yada et. al., 2008), P. luteoviolacea. (Yang et. al., 2007). Some examples are showed in the Table 1 below.

Strain	Comments	Reference
Psychotropic bacterium RT102	Close to J. lividum	Nakamura et al. (2002, 2003)
J. lividum strain DSM1522		Pantanella et al. (2007)
Janthinobacterium lividum S9601		Shirata et al. (1998)
Marine sediment bacterium pseudoalteromonas		Tan et al. (2002)
Psychrotrophic bacterium, XT1	Close to J. lividum	Lu et al. (2009)
C. violaceum	Bacillus violaceum	Tobie (1934)
Alteromonas luteoviolacea	Marine bacteria	Laatsch and Thomson (1984)
C. violaceum CCT 3496		Rettori and Dura´n (1997), Rettori et al. (1998)
C. violaceum B78	From Amazon River, Manaus, Brazil	Riveros et al. (1989)
C. violaceum ATCC 553		De Moss and Happel (1959)
Pseudoalteromonas luteoviolacea	From sponge A. cavernosa	Yada et al. (2008)
Duganella sp B2	From China	Wang et al. (2009)

Table 1. Some Strains that have been cultivated to produce violacein

Violacein is believed either to be the product of a defense mechanism against the stress that the microorganism is subjected either it is produced to give an advantage to the bacteria against the others (Choi et al., 2015a).

Usually the violacein production starts by a group of genes known as VioABCDE. Depending on the conditions that the microorganism is subjected, it triggers the production and imparts the substance's characteristics (August et al. 2000).

The violacein's biosynthetic pathway shows that the substance occurs after the metabolism of tryptophan. The violacein molecule itself it consisted by two molecules of tryptophan. Depending on the way the molecules are being arranged and the presence or the absence of the hydroxyl group, there are different derivatives generated, specifically deoxyviolacein (absence of hydroxyl group) and oxyviolacein (presence of two hydroxyl groups) (Hoshino 2011). The biosynthetic pathway is given in Figure 1.

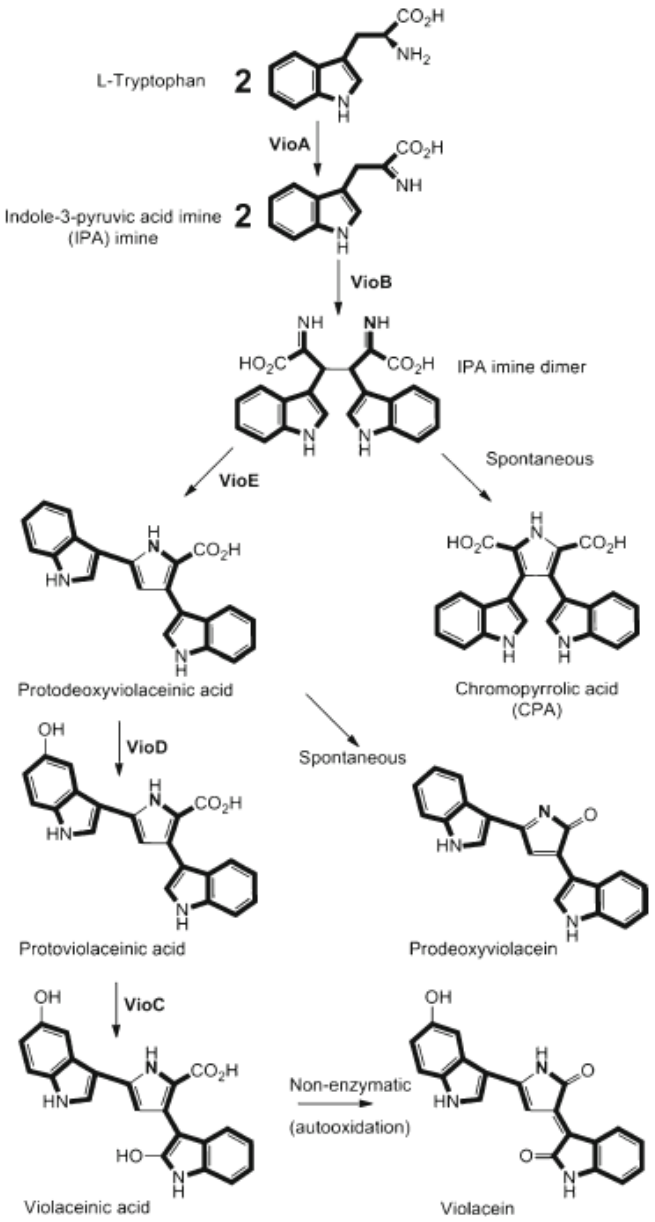

Figure 1. Violacein's biosynthetic pathway (Dantas et. al 2013)

1.2. VIOLACEIN APPLICATIONS

There are several reports about violacein's activity in the literature. It gained some attention mainly due to its antimicrobial, antifungal, antitumoral, antiviral, antiprotozoal effects (Table 2).

Type	Organism	Description	Reference
Fungi	Batrachochytrium dendrobatidis	Infects amphibians	Brucker et al. (2008)
Protozoa	Rhynchomonas nasuta	Flagellate	Matz et al. (2008)
	Tetrahymena sp.	Ciliate	
	Acanthamoeba castellanii	Amoeba	
	Leishmania amazonensis	Causative agent of leishmaniasis	Leon et al. (2001)
	Plasmodium falciparum	Causative agent of malaria in humans	Lopes et al. (2009)
	Plasmodium chabaudi chabaudi	Causative agent of malaria in mice	
Nematoda	Caenorhabditis elegans		Hornung et al. (2009)

Table 2. Violacein activity against certain Microorganisms.

It has been reported that in a combination with other antibiotics it was more efficient against pathogens (Subramanian et al. 2014). It was shown in vitro studies that the simple form of the substance was effective against the Gram positive bacteria but not against Gram negative (Duran et al. 2012). In the same study it was stated that violacein encapsulated in nanostructures was found to be three times more effective than in its simple form. Another research Martins et al. (2010), stated that violacein was effective against S. aureus, but there was no effectiveness mentioned against E. coli and Salmonella enterica. Also its antifungal activity was mentioned in the same study

Another study was reported on its anticancer ability by the National Cancer Institute (USA) and was confirmed that violacein had effect against cancer cells of leukemia, colon, and lung (Duran et al. 2012).

It was also stated the effect of violacein against malaria to in vitro and in vivo studies. Another important reference was that of its antiviral activity against the herpes virus (May et al. 1991).

As about its antiprotozoal activity it was mentioned by Kidder and Stuart (1939) and Matz et al. (2004).

Moreover, violacein was stated to have significant antioxidant activity as reported in various studies that were mentioned in Duran et al. (2012).

Finally, it was mentioned that violacein from C. violaceum had antipyretic analgesic effect (Antonisamy and Ignacimuthu 2010).

There are several reports in the literature about violacein's applications. Subsatnce produced by the Chromobacterium and Janthinobacterium was used as pigment for dyeing fibrous materials and nylon cloth, (Nomura 1994). The pigment showed antimicrobial activity against phytopathogenic fungus such as Rosellinia necatrix (Shirata et al. 1997, 1998).

Moreover, there are several reports about the use of the pigment in cosmetic products. An antibiotic activity against S. aureus and antioxidant effect on linoleic acid was shown, and so a cosmetic lotion with violacein was produced (Aoki and Nomura 1998). Also another cosmetic product was produced that contained violacein or its derivatives (Meiring et al. 2007).

Regarding the violacein applications in the food industry, a research was conducted to upscale the violacein's production (Venil et al. 2015). In this research the substance

was produced in a bioreactor. C. violaceum was cultivated in a 7 L bioreactor and finally was taken 500 mL of the pigment. Here the encapsulation was conducted with the spray drying method. Then the micro particles that came up as a result, were tested for their stability in pH, temperature and light. Also the micro particles showed solubility in water a characteristic that violacein in its simple form lacks off. After that the micro particles were utilized in coloring two model systems of food such as yogurt and jelly. After the two model systems were formulated then they were stored and it was observed that in one month of storage there was no change in conditions of temperature 25-30 ^{o}C, pH 7 and dark.

1.3. HPLC ANALYSIS OF VIOLACEIN

There is a vast majority of reports on characterizing the substance by the HPLC method (Rettori and Duran 1998, Yuan Lu et al. 2009, Rodrigues et al. 2012), providing fast and accurate results.

Until very recently the most widely used HPLC method was the one of Rettori and Duran (1998). As shown in Figure 2, the peaks of the substances could not be clearly distinct. For this method a mobile phase of 75% methanol and 25% water, a flow of 7 ml/min and a DELTA PAK C18 (30 mm×30 cm) column with pores of 100 A and particles with 15 μm diameter and in 230 nm wave length were used.

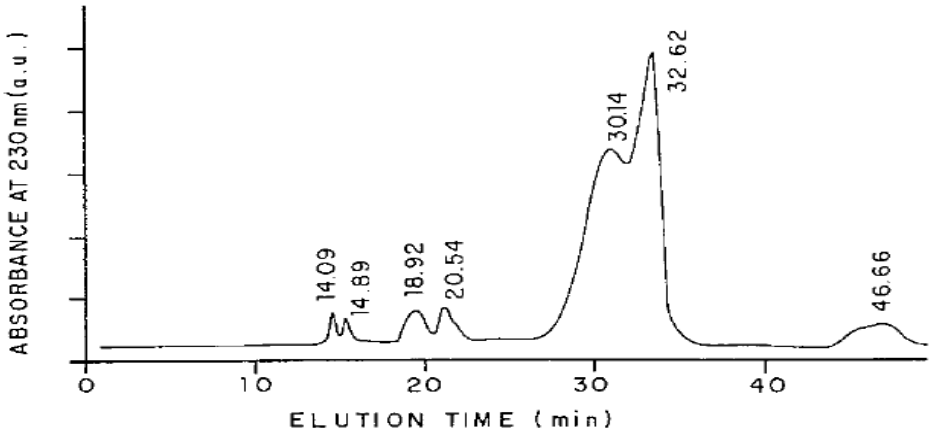

Figure 2. Chromatogram from the Rettori Duran (1998) method

An alternate column, either stationary phase or flow rate, was used by the researchers over the years so that the most accurate method could be found. This drove to a discussion about the best possible method. In the method of Yuan Lu et al. (2009) a 70% methanol and 30% water dissolve was used as mobile phase, with a flow of 1ml/min and an ODS (Inertsil ODS-35um, 250mm×4.6mm, Dikma) column, while the detector was set at the 576 nm.

A more accurate method was proposed by Rodrigues et al. (2012). According to that, the HPLC method for the violacein could be optimized by the use of a mobile phase of 50% ethanol and 50% water, a flow of 0.5 ml/min, a C18 column (Hypersil ODS, 5 μm, 250×4.6 mm), and a detection wave length of 575 nm. In this work only the picks of violacein, deoxyviolacein and that of the dissolver were reported. This

method was tested by Choi et al. (2015b) where violacein from several microorganisms was tested. Indicative results are given in Figure 3.

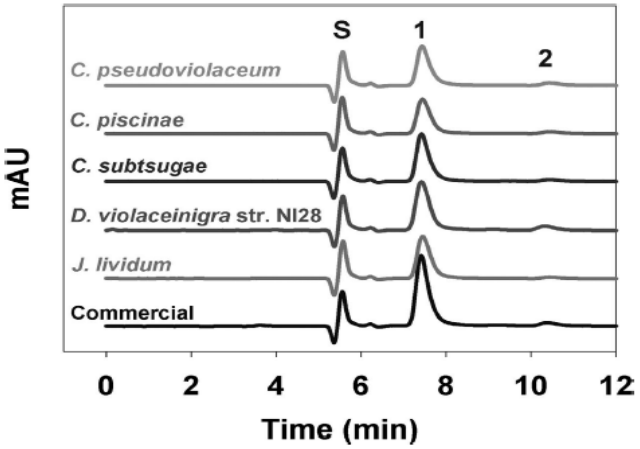

Figure 3. HPLC analysis of violacein taken from the Choi et al. (2015b). S peak is the ethanol, 1 the violacein and 2 the deoxyviolacein.

1.4. NANOLIPIDS

Solid lipid nanoparticles have been used as drug delivery systems since 1991 (MuÈller et al. 2000). Phospholipid vesicles, known as 'liposomes', were described in 1965 by Bagham and were used in the cosmetics since 1986 for an anti-aging product by Dior (Diederichs & Muller 1994).

The low solubility of violacein in water, makes its application more complicated in food systems or drugs, when there is a need for encapsulation and stabilization of the substance. On the other hand, it has a high solubility in DMSO (Dimethyl sulfoxide), a highly toxic agent. Due to their small size, violacein encapsulated in nanoparticles makes it more possible for use (Duran et al. 2012).

In a report by Martins et al., (2010), encapsulated violacein in nanoparticles of polymeric poly-(D,L-lactide-co-glycolide) was studied for its antibacterial activity against S. aureus strains, compared to violacein in a simple (free) form. Results, showed that the substance in its encapsulated form was up to three times more effective regarding its antibacterial activity than in its simple form. That could be a consequence of the higher uptake of the complex by bacterial cells.

1.4.1. ENCAPSULATION NANOLIPIDS

There are numerous studies about the encapsulation of various substances in nanoparticles, such as lycopene and selenium (Chaudhry et al. 2008). As it was stated in this research, the encapsulation of selenium in nanoparticles, enhances its uptake and its disposal in foods. Lycopene in its encapsulated form is used in soft drinks and refreshments as an additive that enhances the color and has a positive impact on health. It was found that the synthesis of lycopene with vitamin E treats prostate cancer in mice (Limpens et al. 2006).

Moreover, there have been studies for the same purpose on β- carotene from carrot, β- glucan from oats, omega-3 fatty acids from salmon oil, lactobacilli from yogurt (Neethirajan and Jayas 2011). In that research it was stated that omega-3 fatty acids nanoparticles in bread, when they reached the stomach, they released the fatty acids and in this way the unpleasant taste and odor of the omega-3 fatty acids could be avoided

However, the vast majority of studies found in the literature about violacein nanoparticles, has to do with pharmaceutical applications rather than food. Basically, it was tested in the cytotoxicity of the nanoparticles containing violacein against various cells. A research is Melo et al. (2009) reported the cytotoxicity of nanoparticles containing violacein against human leukemia cells.

Also in a review by Duran and others (2007) it is stated that the nanoparticles with PLGA–Pluronic® and of PLGA–Pluronic®–poly (vinyl alcohol) containing violacein were tested. Also it was stated that there were some applications of violacein with gold nanoparticles. The most recent approach is that of mono-(6-deoxy-6-[(mercaptohexamethylene)thiol])-β-cyclodextrin.

1.4.2. ENCAPSULATION METHOD

A variety of encapsulation methods is now available as applied in the pharmaceutical industry. The liposome formation occurs spontaneously when phospholipids are dispersed in water. Such multi-drug delivery nanocarriers are depicted in the Figure 4. Some of the methods that are used: thin film method, sonication, extrusion, injection methods, dehydrated-rehydrated vesicles, reverse phase evaporation and one step method (Nokhodchi et al. 2012).

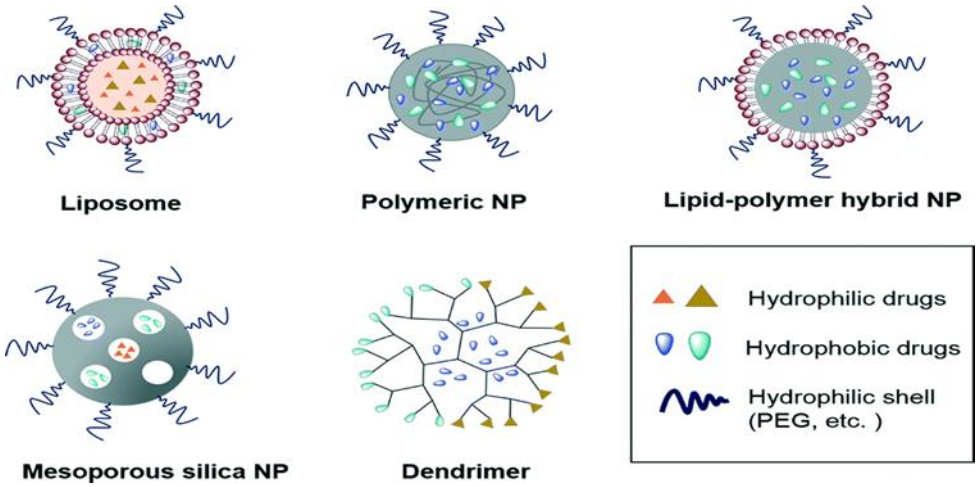

Figure 4. Multi-drug delivery nanocarriers (Gadde 2015)

In the thin liquid method, the liquids are dissolved in organic solvents and then the solvent is removed under vacuum or nitrogen stream to form a thin film on the wall of a flask or test tube. To complete the formation of liposomes aqueous phase is added to the lipid film at a temperature above the phase transition of the lipid (Bangham et al. 1965).

The sonication method is usually used to reduce the particle size and lamellarity of MLVs (multilamellar vesicles). By the use of the probe sonicator, the reduction in size of the liposomes can be guaranteed (Sharma et al. 1997).

The extrusion technique is used in order to achieve very homogeneous vesicles with a predetermined size. The MLVs are extruded under pressure through particular filter with well-defined pore sizes from 30 nm to several micrometers. But if the extrusion is repeated several times unilamellar liposomes can be formed (Olson et al. 1979).

The ethanol injection method could produce very small unilamellar vesicles with a particle size of 30 nm. In this method the lipids are dissolved in ethanol and then injected into the aqueous solution under stirring. Finally, the ethanol has to be removed from the system (Batzri and Korn 1973).

The dehydrated-rehydrated vesicles method is considered suitable for the drugs that are losing their activity under harsh conditions, since with this method the vesicles could hold high amounts of hydrophilic drugs under mild conditions (Sheltzer et al. 1988). In this method empty liposomes are disrupted during a freeze drying step in the presence of the drug meant to be encapsulated followed by a controlled rehydration in the presence of concentrated solution of the drug. With this technique large oligo lamellar liposomes of a size around 400 nm to several micrometers could be produced (Talsma et al. 1994).

Another method is this of reverse phase evaporation, which is similar to the thin liquid method. In this method the thin film is resuspended in diethyl ether followed by the addition of third of water and the suspension is sonicated in a bath sonicator. Then the emulsion is evaporated until a gel is formed and finally the gel is broken by the addition of water under agitation. The traces of organic solvent should be removed by evaporation (Ugwu et al. 2005).

All those methods have in common that are used for the formulation of liposomes, where the violacein would be encapsulated and are in use in the pharmaceutical science. So this is a main reason that those methods are been cited.

1.5. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

DSC (Differential Scanning Calorimetry) was used as the methodological technique to report the calorimetric parameters of the nanoparticles. DSC is the most frequent application in Pharmaceutical industry and scans the temperature and measure the difference between the heat flows to a sample and a reference pan, which is under the same temperature program, at atmospheric pressure, and the heat capacity of a material. Also it is a technic that provides quick and accurate information about the physical and the energetic parties of a material (Demetzos 2008). The thermodynamic parameters of a DSC curve are showed in the Figure 5.

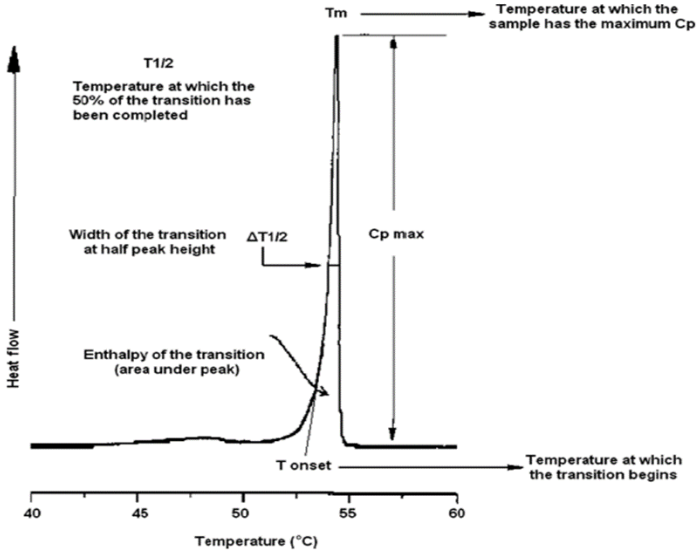

Figure 5. Characteristic thermodynamic parameters of a DSC curve. (Demetzos 2008)

Until very recently several researches aimed in explaining the behavior of these lipids by the DSC method. In specific, a study about the design and development of pH sensitive HSPC lipids was conducted using DSC, in order to form possible chimeric nanocarrier system. The researchers showed that the use of pH sensitive biomaterials could lead to a format of more accurate nanocarrier systems (Naziris et. Al, 2016). Moreover, a number of researches on the use of the DPPC lipids with other substances were presented in order to form drug nanocarrier systems. The behavior of the systems regarding their stability was adequately described via the thermographic DSC data (Pippa et. al., 2012; 2014; 2015). According to Demetzos (2008), the schematic presentation of the acyl chains and how they interacted in the DPPC lipids formation, which was one of the lipids used in this study, is depicted in the Figure 6.

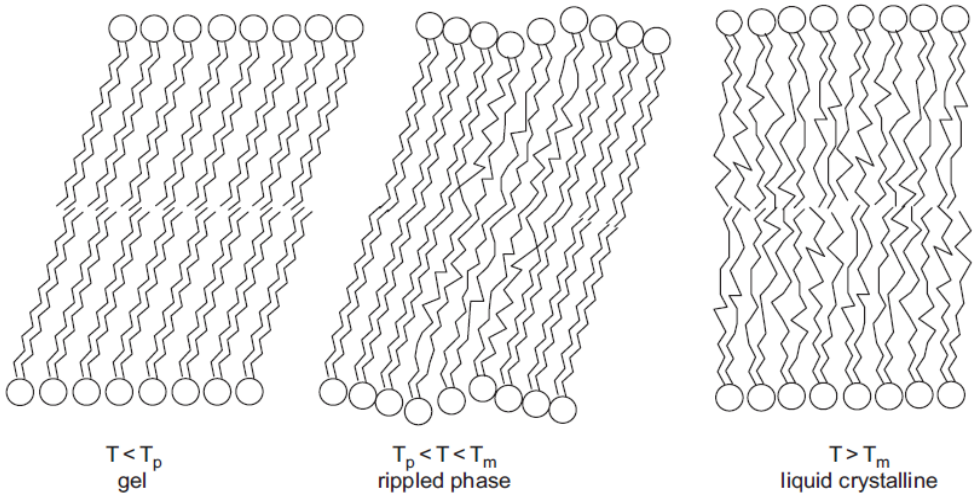

Figure 6. Schematic presentation of the alignment of acyl chains in a saturated phospholipid such as DPPC to form a double layer of molecules: gel in quasi-crystalline state (T< Tp), rippled phase (Tp<T<Tm) and in the liquid crystalline state (T>Tm). (Demetzos 2008)

2. PURPOSE OF THE STUDY

This study aims in the investigation of the potential violacein applications in food systems. For that, an initial target established regards the violacein's production to examine either what triggers the production, or the factors that had the most influence in the substance's production by the microorganisms. The development of a quantification method in order to determine the concentrations of the substance, that have been received after the cultivation, was the first step for the substance's examination. Therefore, the optimization of the substance production may be established and certain caltivation parameters may be proposed for future use. Furthermore, in order to exceed the problem of violacein's low solubility, nanocarriers were to be made using certain lipids, as suitable nanocarriers to encapsulate the violacein. Hence, the study of the thermodynamics of the nanolipids produced with different pH buffers, was considered an important objective for this study.

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

Violacein was received by microorganisms cultivated in 3 different pH rates of 6, 7 and 9, in different temperatures: 15, 20, 25 and 30º C and in different time intervals of cultivation. This lead in the output of 174 samples. Because there was a lack of a constant pattern in receiving the time intervals. Then the samples were unified according to the inoculum, the temperature and the pH. Therefore, 22 groups of samples codification post-unification are depicted in Table 3.

Sample	pH	Inoculum (log CFU)	Temperature (ºC)
6A-15	6	5	15
6A-20	6	4	20
6A-25	6	4	25
6A-30	6	4	30
7A-15	7	5	15
7A-20	7	5	20
7A-25	7	4	25
7A-30	7	4	30
9A-15	9	4	15
9A-20	9	4	20
9A-25	9	4	25
6B-15	6	6	15
6B-20	6	5	20
6B-25	6	5	25
6B-30	6	5	30
7B-15	7	6	15
7B-20	7	6	20
7B-25	7	5	25
7B-30	7	5	30
9B-15	9	5	15
9B-20	9	5	20
9B-25	9	5	25

Table 3: The groups of samples coding in relation to the microorganism conditions of growth and substances' production

3.2. VIOLACEIN SAMPLES

The samples from cultivated microorganisms at different time intervals, were collected according to Gkioka (2017). Samples were filtered 0.2 μm diameter filters before injecting them into the HPLC column, so that any cellular debris were removed. The same filtration step was also applied to the samples that were used for the nanostructures formulation.

3.3. PREPARATION OF NANOLIPIDS

Pure lipid and lipid/violacein bilayers were prepared by mixing the appropriate amounts of DPPC, HSPC and violacein in chloroform/methanol (1:1 v/v) solutions and the subsequent evaporation of the solvents under vacuum and heat. The chemical structure of the lipids in showed in the Figure 7.

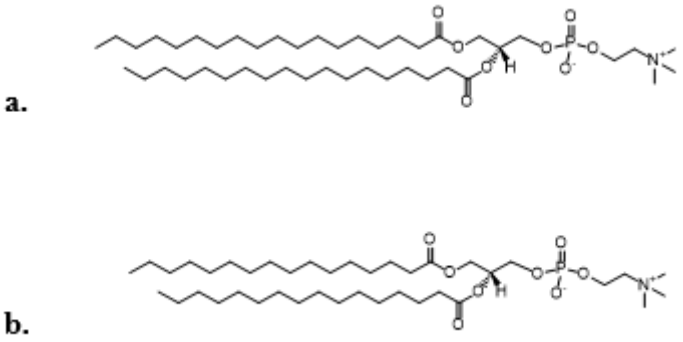

Figure 7. The chemical structure of a. HSPC and b. DPPC.

Appropriate amounts of these solutions were added to DPPC and to HSPC, weighted into HPLC vials, in order to obtain the desirable 9:0.01molar ratios, where necessary chloroform/methanol (1:1 v/v) solution was added. Then the vials were transferred to a vacuum machine (TechneDri-Block DB3Thermostat Teche Sample Concentrator). Lipid films were formed by removing the solvent at 50°C. The films were maintained under vacuum for 2h and then in a desiccator for at least 24h in order to remove traces of solvent. The obtained chimeric bilayers were hydrated into the appropriate medium and then were studied by differential scanning calorimetry.

3.3.1. AQUEOUS MEDIA PREPARATION

Acidified aqueous media at the predetermined values between pH 3 and 9, were prepared, by mixing 50 ml of 1M acetic acid, with 12.5 ml of 2M sodium hydroxide and, by adding 275 ml of deionized water. The pH was adjusted, with sodium hydroxide, to the selected values.

3.4. ANALYTICAL TECHNIQUES

3.4.1. QUANTITATIVE ANALYSIS OF VIOLACEIN AND DEOXY-VIOLACEIN BY HPLC

Samples used for the calibration curve were the standard commercial violacein (Sigma- Aldrich V9389) dissolved in different amounts of ethanol (Sigma- Aldrich HPLC-grade) in order to have the required rates for the calibration curve. These samples were injected in the HPLC device. Which was consisted by A JASCO PU 980 pump and injection system, with a JASCO FP920 fluorescence detector (Co. Ltd., Japan) supported by Clarity Lite software, ODS Hypersyl column (4.6 x 250 mm, 5 mm particle size, Thermo Scientific), 20 μl injection loop and a photochemical reactor (Multiwave length MD-915).

A mobile phase which by the Rodrigues' (2012) method consisted by 50% ethanol and 50% water, with a flow of 0.5 ml/min and at 575 nm wavelength was used as it is stated and in a previous chapter. The chromatogram collected by this method is depicted in the Figure 8.

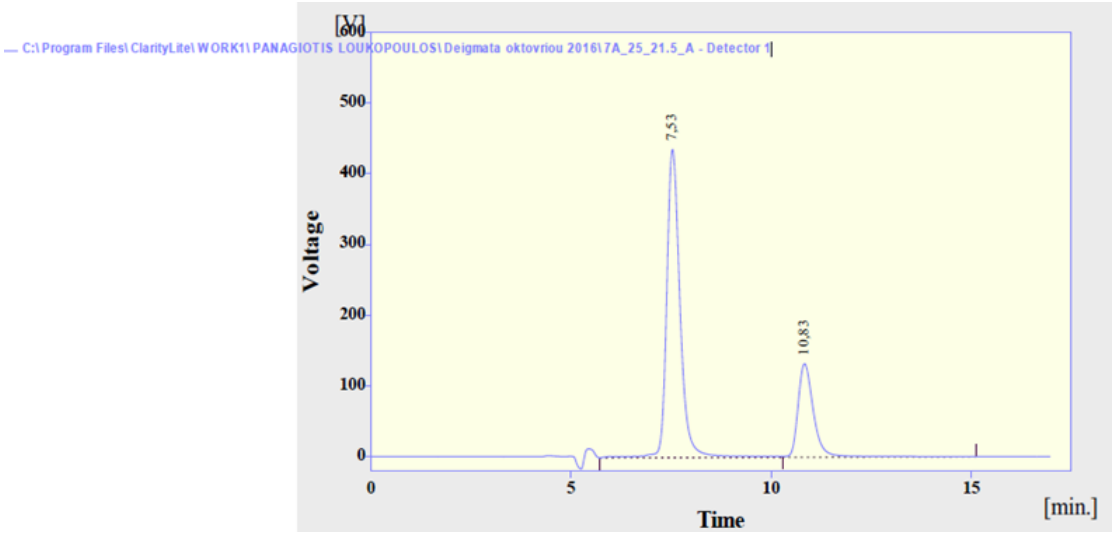

Figure 8. Sample with pH 7 of the A treatment, 25 ºC and on the 21st of May.

3.4.2. DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Differential Scanning Calorimetry (DSC) experiments, were carried out using an 822eMettler-Toledo (Schwerzenbach, Switzerland) calorimeter calibrated with pure indium (Tm=156.6 °C). Sealed aluminum 40μl crucibles were used as sample holders. Lipid fully hydrated bilayers were investigated. Thirty minutes prior to measurements each mixture, after it was placed in the crucible, was hydrated using the appropriate medium. Then the crucible was sealed. Two and a half heating-cooling cycles were performed in order to ensure good reproducibility of the data. The temperature range was from 20 ºC to 60 ºC and the scanning rate 5ºC/min. Before each cycle the samples were subjected to a constant temperature of 20ºC to ensure the equilibration, while an empty aluminum crucible was used as reference. The second heating and cooling runs were taken into account and the calorimetric data obtained (characteristic transition temperatures - Tm/s), enthalpy changes - ΔHm/s and the width at half-height of the Cp profiles - ΔT1/2 m/s), were analyzed using Mettler-Toledo STARe software.

3.4.3. STATISTICAL ANALYSIS

In order to determine if the mean for each factor (pH, temperature inoculum) of the tested samples was significantly different in the substance's production analysis of variance of the results for each individual factor among the samples (univariate tests) and the respective means were compared using LSD test at $p \leq 0.05$ at the 95% confidence limits was performed.

4. RESULTS AND DISCUSSION

In order for the samples to be compared according to the same factors (temperature, inoculum, pH), the following results will be given for samples of the same inoculum of 5 log CFU. Also the violacein's production will be described as the main product of the microorganism.

The samples that were produced from 5 log CFU inoculum and pH 6 are depicted in the Figures 9 and 10.

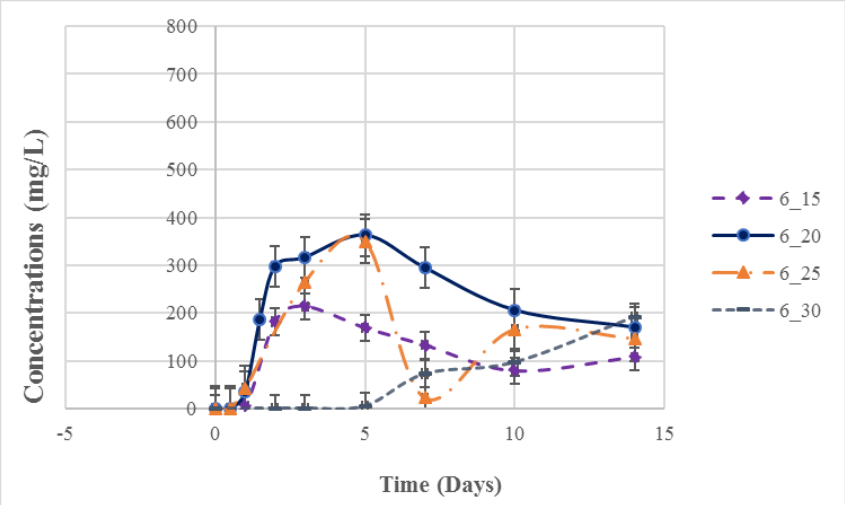

Figure 9. Violacein concentrations with pH 6 compared to the temperature of growth.

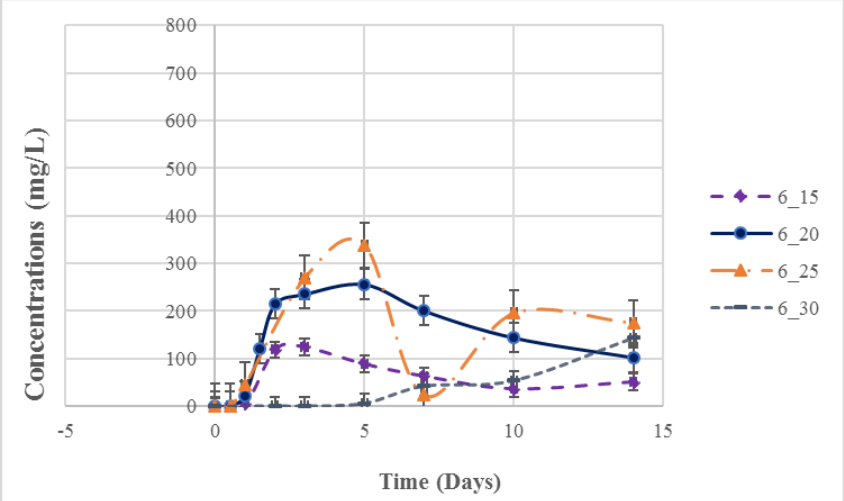

Figure 10. Deoxyviolacein concentrations for microorganisms cultivated at pH 6 and various temperatures of growth.

The samples that were produced at pH 6 at 15 °C, had the highest concentrations of violacein in the 3rd day. The production was decreased until the 10th day, when violacein's lowest concentration was appeared. On the 14th day the production started to increase. On the other hand, in the samples that were produced at pH 6 and at 20 ºC, the concentrations of violacein were increased until the 5th day where it had the highest concentration and then it decreased. As about the samples that were produced at pH 6

and at 25 ºC, on the 5th day an increase in the concentrations of violacein was noticed and then decreased until the 10th day, when started rising again. The samples that were produced at pH 6 and at 30 ºC, had a slow increase and the highest concentration of violacein was noticed on the 14th day.

The samples that were produced from 5 log CFU inoculum and pH 7 are depicted in the Figures 11 and 12.

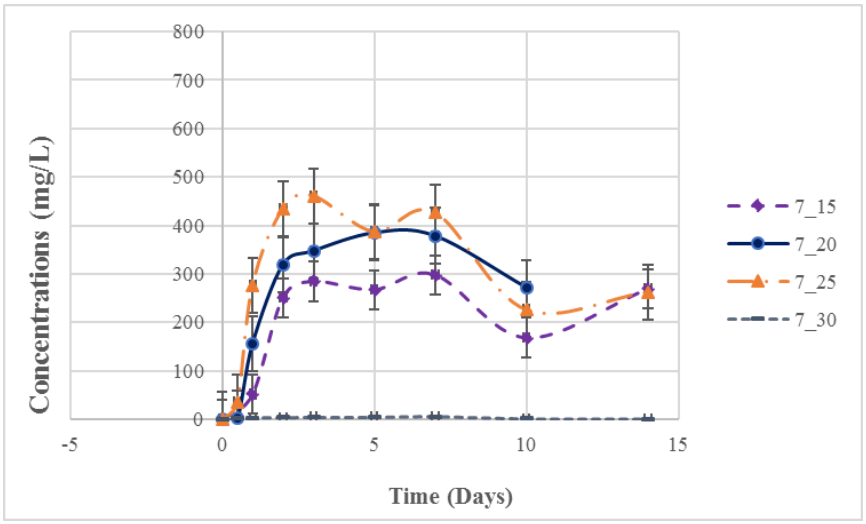

Figure 11. Violacein concentrations with pH 7 and various temperatures of growth.

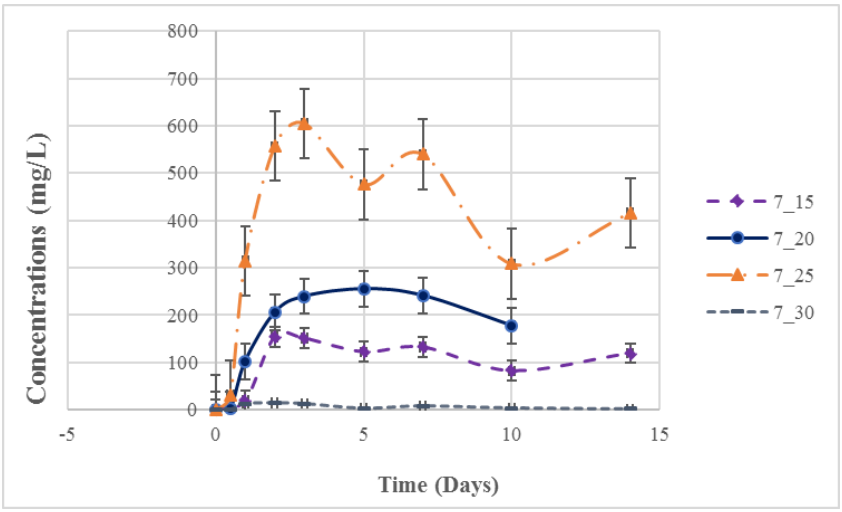

Figure 12. Deoxyviolacein concentrations with pH 7 and various temperatures of growth.

In the samples that were produced at pH 7 and at 15 ºC, the concentration of violacein was increasing from the 2nd day and remained in high levels until the 7th day where the samples had the highest concentration. Then decreased again until the 14th day where the samples' concentration started to increase once more. In the samples that were produced at pH 7 and at 20 ºC, the concentration of violacein was increasing from the 2nd day, then stayed in high levels until the 7th day where started to decrease again. The highest concentration of violacein was noticed on the 5th day. As about the samples that were produced at pH 7 and at 25 ºC, the concentration of violacein was increasing until the 3rd day where its highest concentration was appeared. Then showed a decrease

on the 5th day and then started to increase again until the 7th day. In the 10th day a decrease was noticed and finally on the 14th day it started to increase again. Also in these samples the deoxyviolacein's curve had higher values than the violacein's curve. The samples that were produced at pH 7 and at 30 ºC, the concentration of violacein was increasing until the 2nd day and then decreased on the 5th day. Then started to increase until the 7th day when the concentration of violacein decreased again. And in these samples the deoxyviolacein's curve appeared to have higher values than the violacein's curve.

The samples that were produced from 5 log CFU inoculum and pH 9 are depicted in the Figures 13 and 14.

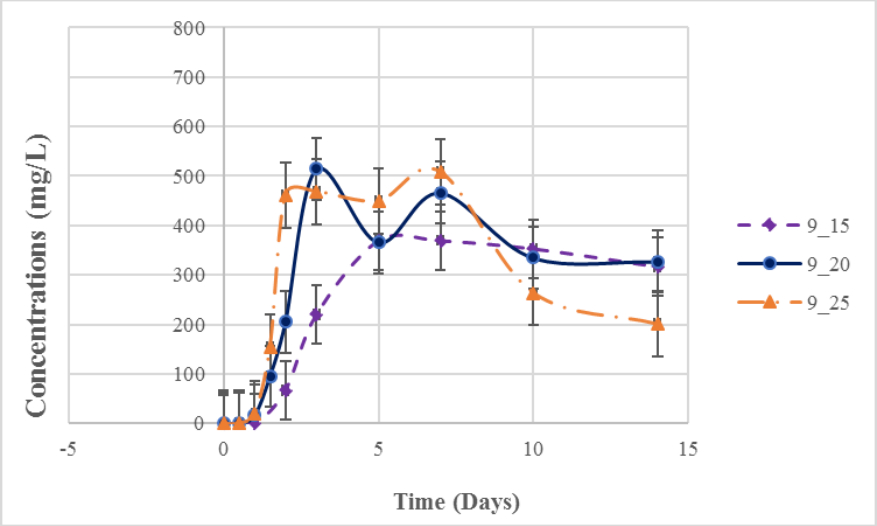

Figure 13. Violacein concentrations with pH 9 and various temperatures of growth.

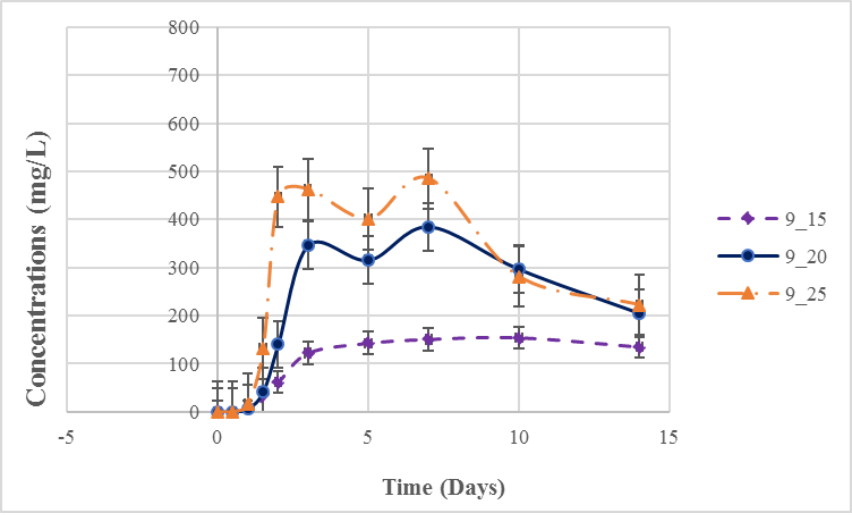

Figure 14. Deoxyviolacein concentrations with pH 9 and various temperatures of growth.

As about the samples that were produced at pH 9 and at 15 ºC, the concentration of violacein was increased until the 5th day and it remained in high values. In the samples that were produced at pH 9 and at 20 ºC, the concentration of violacein showed an increase until the 3rd day where had its highest value. Additionally, another increase in the concentration of violacein was noticed on the 7th day. It showed a decrease on the

5th day. In the samples that were produced at pH 9 and at 25 ºC, the concentration of violacein was increasing until the 2nd day and then it remained in high values. The highest concentration of violacein was on the 7th day and then it decreased again.

In the Figures 15 to 16 it is easily observed that if we compare all the samples together, we notice that the samples that were produced at pH 7 and at 25 ºC had the highest deoxyviolacein's concentrations. The samples that were produced at pH 9 and at 20 ºC also appeared to have the highest violacein's concentrations.

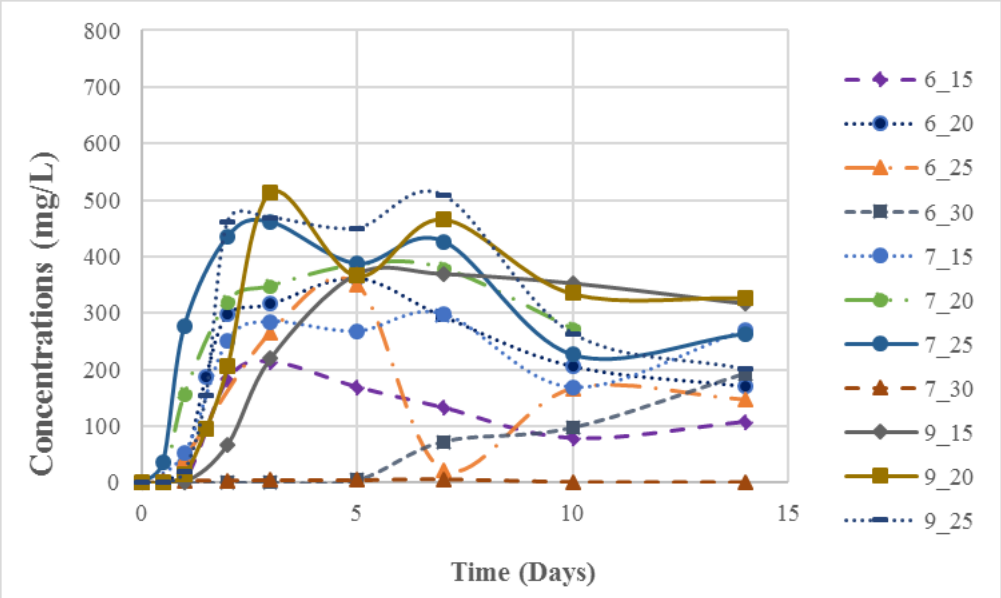

Figure 15. Violacein concentrations compared to the temperature.

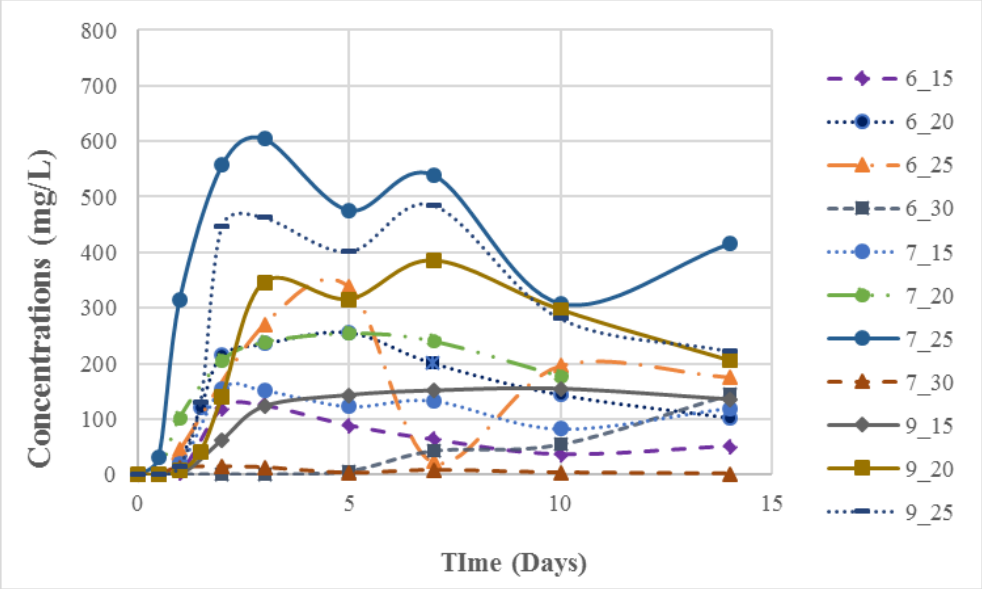

Figure 16. Deoxyviolacein concentrations compared to the temperature.

4.1. STANDARD QUANTIFICATION CURVES

Three concentration levels of the compounds' mixture (violacein/deoxyviolacein:85/15) were used. The three groups used were: 0,1 to 10mg/L, 10-50 mg/L and 50-

400 mg/L, as in Table 4. All the samples were analyzed by the HPLC method stated before. The response areas from the chromatograms were reported for both violacein and deoxyviolacein. The results were plotted as area of detection versus the amount of the compound injected in the HPLC column. Thus, 3 best-fit-line equations in each substance were applied: for the areas between 0.1 to 10 mg/L, for the areas between 10-50 mg/L and for the areas between 50-400 mg/L. In this way the possibility for a quantification error was minimized.

The results per group of each of the two compounds and group of concentrations are given in Table 4. The quantification curves were checked for linearity (R2 > 0.94) for each of the 6 equations. Only one of the equations, though, had not adequate linearity, this of deoxyviolacein (0,1-10 mg/L).

Violacein			
Area (mVxsec)	**Equations**	**Concentations (mg/L)**	**Linearity**
3.11-200	$y = 0.041x + 0.2783$	0.1- 10 mg/L	R^2= 0.9888
200-828	$y = 0.0539x - 2.6833$	10- 50 mg/L	R^2= 0.9924
828<	$y = 0.0511x + 17.309$	50 mg/L <	R^2=0.9752
Deoxyviolacein			
Area (mVxsec)	**Equations**	**Concentations (mg/L)**	**Linearity**
1.93-5.35	$y = 0.2957x - 0.4827$	0.1- 10 mg/L	R^2=0.7454
5.35-28.33	$y = 0.247x + 0.4757$	10- 50 mg/L	R^2=0.9837
28.33<	$y = 0.21x + 3.1055$	50 mg/L <	R^2=0.9953

Table 4. The standard quantification curves.

A similar evolution pattern in violacein's and deoxyviolacein's production is observed. This pattern shows that the substances' production in every sample tested was decreased and then increased again. We assumed that because the substance is consisted by two molecules of tryptophan the microorganism started to consume the substance, because it already had consumed the nutrient medium, as it is depicted in the Figure 1. This is a phenomenon that is frequently observed in the microbiology science related to the microorganism's survival (Knowles 1988).

Interestingly enough, violacein's and deoxyviolacein's production had showed higher concentrations at pH 7 and 9. Specifically the highest values were grown at pH 7 and at 25 ^{o}C. The lowest substance's production was proved to be at pH 7 and at 30 ^{o}C.

It is also interesting to report that for samples produced by microorganisms' cultivation at pH 7 and in every temperature, the patterns of the curves of the violacein and deoxyviolacein production were similar, regardless the difference between the inoculums.

The charts of violacein and deoxyviolacein samples are appeared in the appendices in the Figures 22 to 32.

4.2. STATISTICAL ANALYSIS

Statistical analysis on which of the factors was affecting the violacein's production was performed. Samples 6A-25, 7A-25, and 9A-25 were compared. The results indicated that there were no statistically significant differences (Table 5).

pH	Count	Mean	Homogenous Groups
6A-25	24	198,013	A
9A-25	24	261,078	AB
7A-25	24	329,717	B

Table 5. Statistical analysis results when applying the method 95,0 percent LSD for the 6A-25, 7A-25, 9A-25 samples.

Then the samples 6B-25, 7B-25 and 9B-25, were compared. There were not statistically significant differences between the samples. However, there was a difference in the homogenous groups between the pH 6 and the other two pH values, as is depicted in Table 6.

pH	Count	Mean	Homogenous Groups
6B-25	21	142,018	A
7B-25	21	292,29	B
9B-25	21	296,709	B

Table 6. Statistical analysis results when applying the method 95,0 percent LSD for the 6B-25, 7B-25, 9B-25 samples.

As the statistical analysis indicated, the acidity was apparently not significantly affecting the substances' production. Samples 7A-25 and 7B-25 were compared in order to examine the inoculum's role in the violacein's production. The analysis showed that there were no statistically significant differences between the inocula of 4 and this of 5 log CFU. The last analysis that had to be conducted was in the samples 7A-15, 7A-20, 7B-25, 7B-30 that had the same pH, the same inoculum but with different temperature. In order to examine if the temperature is the factor that mostly affected the violacein's production.

At the examination of the temperature as a factor in the statistical analysis it was observed that the temperature was statistically significant different. Specially, the temperature of 30 ºC. The other values had not many statistically significant differences. Something that was expected and according to the Figures 15 and 16.

4.3. THERMAL BEHAVIOUR OF VIOLACEIN LIPID BIOLAYERS

4.3.1. INFLUENCE OF THE pH ON THE THERMOTROPIC BEHAVIOR OF DPPC AND HSPC BILAYERS

Previous work (Koynova and Caffrey 1998) indicated the thermal transitions DPPC may undergo with temperature increase. In particular, the DSC heating profiles obtained in this work for the pure DPPC and HSPC fully hydrated liposomes bilayers are given in Figure 17 and for DPPC:violaceine and HPSC:violaceine in Figure 18. Data captured regarding the $T_{onset,m}$/ ºC, T_m/ºC, $\Delta T_{1/2,m}$/ºC, ΔH_m/Jmol^{-1}, $T_{onset,s}$/ºC, T_s/ºC, $\Delta T_{1/2,s}$/ºC, ΔH_s/Jmol^{-1} calorimetric parameters are summarized in Tables 7 and 8 (heating and cooling calorimetric parameters).

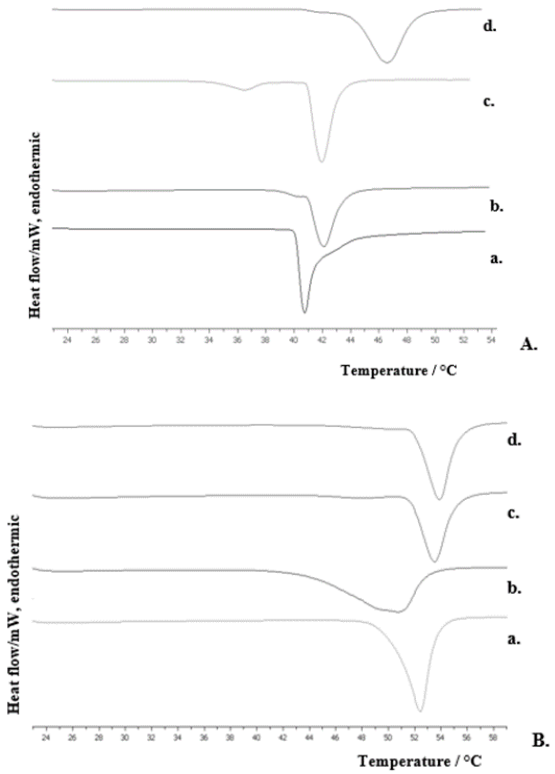

Figure 17. DSC heating scans of **A.** DPPC and **B.** HSPC fully hydrated bilayers at pH **a.** 3, **b.** 5, **c.** 7.4 and **d.** 9. The limits for the calculation of thermotropic parameters are from 25°C to 60°C.

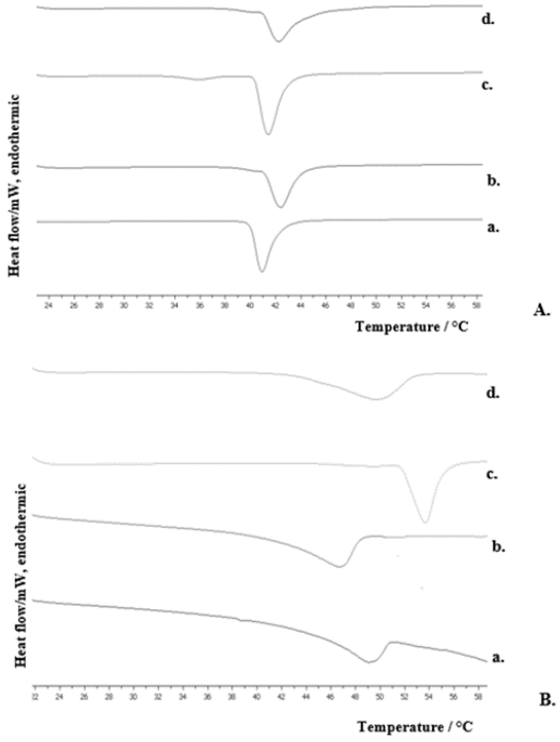

Figure 18. DSC heating scans of **A.** DPPC:violacein and **B.** HSPC:violacein fully hydrated bilayers at pH **a.** 3, **b.** 5, **c.** 7.4 and **d.** 9. The limits for the calculation of thermotropic parameters are from 25°C to 60°C.

Table 7. Calorimetric profiles of DPPC and HSPC fully hydrated bilayers in different pH (heating).

Composition	pH	$T_{onset,m}$ /°C	T_m /°C	$\Delta T_{1/2,m}$ /°C	ΔH_m / J mol^{-1}	$T_{onset,s}$ /°C	T_s /°C	$\Delta T_{1/2,s}$ /°C	ΔH_s /J mol^{-1}
DPPC	3.0	40.1	40.8	0.99	58.0	-	-	-	-
DPPC	5.0	40.8	42.1	1.53	47.5	-	-	-	-
DPPC	7.4	40.9	41.9	1.33	179.8	34.2	36.5	2.07	27.5
DPPC	9.0	44.2	46.6	2.56	244.2	-	-	-	-
HSPC	3.0	50.2	52.5	1.99	213.2	-	-	-	-
HSPC	5.0	44.2	50.5	5.56	236.7	-	-	-	-
HSPC	7.4	51.8	53.6	1.88	127.7	45.2	48.0	2.97	43.0
HSPC	9.0	52.0	54.0	1.91	176.5	-	-	-	-

T_{onset}: temperature at which the thermal event starts; T..: temperature at which heat capacity (ΔC_p) at constant pressure is maximum; $\Delta T_{1/2}$: half width at half peak height of the transition; ΔH: transition enthalpy normalized per mol of liposomal system. m: main transition; s: secondary transition

Table 8. Calorimetric profiles of DPPC and HSPC fully hydrated bilayers in different pH (cooling).

Composition	pH	$T_{onset,m}$ /°C	T_m /°C	$\Delta T_{1/2,m}$ /°C	ΔH_m / J mol^{-1}	$T_{onset,s}$ /°C	T_s /°C	$\Delta T_{1/2,s}$ /°C	ΔH_s /J mol^{-1}
DPPC	3.0	45.5	38.7	2.35	140.5	-	-	-	-
DPPC	5.0	39.9	38.6	1.50	123.9	-	-	-	-
DPPC	7.4	39.7	38.6	1.42	184.7	-	-	-	-
DPPC	9.0	43.7	41.7	2.09	164.0	-	-	-	-
HSPC	3.0	45.1	44.2	1.42	280.0	50.1	49.2	1.94	15.6
HSPC	5.0	49.1	46.2	5.98	230.8	-	-	-	-
HSPC	7.4	51.5	49.8	2.12	148.5	-	-	-	-
HSPC	9.0	52.0	50.4	2.23	163.8	-	-	-	-

T_{onset}: temperature at which the thermal event starts; T..: temperature at which heat capacity (ΔC_p) at constant pressure is maximum; $\Delta T_{1/2}$: half width at half peak height of the transition; ΔH: transition enthalpy normalized per mol of liposomal system. m: main transition; s: secondary transition

In previous works, it has been demonstrated that DPPC bilayers undergo phase transitions when exposed of increased temperatures (Koynova and Caffrey 1998). I was also demonstrated that pure DPPC lipid exhibited a pre-transition event at 36.1ºC followed by a main transition at 41.7ºC (Matsingou and Demetzos 2007, Pippa et al. 2014). The presence of the two transitions corresponded to different thermotropic behavior attributed to the different thermo-dynamic content of the lipid bilayers. The assumption made on this phenomenon refers to the liposomal structure curvature that plays an important role for this thermodynamic content difference. Therefore, according to Ivanova and Heimburg (2001), the geometric characteristics of the liposomes

impact the cooperativity of the phase transition, which increases with increased vehicle's curvature.

Furthermore, as seen in Table 7, the rest of the calorimetric parameters corresponding to the non-polar, lipid phase of the membranes recorded during heating showed that, $T_{onset,m}$/ºC and T_m/ºC, significantly increased with increasing pH, compare to the initial values recorded for samples obtained with pH 3.0 hydration water. The most significant and profound increase of almost 4-times fold was recorded for the ΔH_m/Jmol^{-1} values. In a similar trend the $\Delta T_{1/2,m}$/ºC also increased from 0.99 to 2.56, while the sharpness of the main peaks also differ from rather sharp at pH 3.0 and 7.4 to more broad and shorter for pH 5.0 and 9.0.

Transitions of DPPC attributed to the phospholipid hydrocarbons acyl chains, contain a number of gauche conformations during their thermodynamic transitions to the liquid crystalline state. Van der Waals interactions of these chains are reduced due to their intra- and intermolecular endothermic increased motion. The apparent sharp peaks in the thermographs are the corresponding result of such events, indicating high cooperativeness of the molecules (Demetzos 2008).

As shown in Figure 17A, and reported in Table 7**,** metastable phase could only be recognized for pure DPPC lipid bilayers when exposed to buffer of pH 7.4, while no such apparent transition may be reposted for pH 3.0, 5.0 or 9.0. Such metastable phase, may be due to the pre-transitions of polar head groups of the phospholipids, but with different thermodynamic contribution. In the end, it is the structure/morphology changes that create such metastable phases, which in our case may most likely be related to the pH effect. The relative calorimetric parameters' values of $T_{onset,s}$/ºC, T_s/ºC, $\Delta T_{1/2,s}$/ºC and ΔH_s/Jmol^{-1} can also be found in Table 7.

In comparison, when HSPC was used (Figure 17B), no such behavior may be reported for the pure fully hydrated bilayers during heating, where no pre-transitions were recorded, while only a rather comparatively smaller, increase in the main transition temperature was recorded with increasing pH for pH 3.0, 7.4 and 9.0. These samples had a distinctly different behavior when hydrated in citrate buffer pH 5.0 compared to the rest of the three samples.

The endothermic transitions have been reported by Naziris et al. (2016) and confirmed to references therein, for pure HSPC, and both the T_m and T_s values of theirs are in full agreement to the one reported in the present work for hydration occurring in pH 7.4. In specific, the pure HSPC fully hydrated lipid bilayers at pH 5.0, had the lowest values for $T_{onset,m}$/ ºC and T_m/ºC and the highest values for the $\Delta T_{1/2,m}$/ºC, ΔH_m/Jmol^{-1} calorimetric parameters, both being close or even more than double compared to those recorded for the samples with pH 3.0, 7.4 and 9.0. Furthermore, the only samples showing a pre-transition curve where those hydrated in citrate buffer of pH 7.4. These curves, as seen in Table 7, had calorimetric parameters' values higher than those for the DPPC samples obtained hydrated with citrate buffer (pH=7.4)

In general, a key characteristic of pH-sensitive groups may be described as a preferential protonation/deprotonation mechanism at a pH value depending on the pKa value, in very close dependency to the resulting structural alterations in the nanosystems. Therefore, mimicking the pH of surrounding to bilayers environments, one may firmly assume the resemblance of such environments to particular areas of the human body or other natural structures.

In general, for either type of bilayers, changes in T_m could be attributed to the potential decrease in the non-polar head groups, a strengthening of the packing density within the bilayer and reduced membrane fluidity caused by using hydration medium with pH away from the pK value of the DPPC phosphate groups and the decrease of

the content of the positively charged head groups. The alterations for the ΔH_m were following a more arbitrary order of no specific pattern in relation to the pH of the hydration medium.

Moreover, whenever an increase in the $\Delta T_{1/2,m}$/ºC values was recorded, it could be indication of a lower chain cooperativity in the bilayer and a very small amount of molecules are correspondingly melting with increased pH and more non-polar head group charge screening by the ions of the buffer. The pH increase in the hydration medium caused a narrowing and higher temperature shift of the pre-transition temperatures and lowering of the main transition.

4.3.2. INFLUENCE OF THE VIOLACEIN ON THE THERMOTROPIC BEHAVIOR OF DPPC AND HSPC BILAYERS

Through this work we tried to show the impact of violacein when incorporated into two distinct lipid bilayers structures, through their thermodynamic behavior as the main index of stability and potential release.

The DSC heating profiles obtained for the DPPC and HSPC violacein fully hydrated liposomes bilayers at various pH, are given in Figure 18A and Figure 18B, respectively. The values for the main thermographic parameters $T_{onset,m}$/ ºC, T_m/ºC, $\Delta T_{1/2,m}$/ºC, ΔH_m/Jmol^{-1}, $T_{onset,s}$/ºC, T_s/ºC, $\Delta T_{1/2,s}$/ºC, ΔH_s/Jmol^{-1} calorimetric parameters are summarized in Tables 9 and 10 (heating and cooling calorimetric parameters).

Table 9. Calorimetric profiles of DPPC:violacein and HSPC:violacein fully hydrated bilayers in different pH (heating).

Composition	pH	$T_{onset,m}$ /°C	T_m /°C	$\Delta T_{1/2\,m}$ /°C	ΔH_m / J mol^{-1}	$T_{onset,s}$ /°C	T_s /°C	$\Delta T_{1/2\,,s}$ /°C	ΔH_s /J mol^{-1}
DPPC:violacein	3.0	40.0	40.9	1.31	109.8	-	-	-	-
DPPC:violacein	5.0	41.0	42.4	1.69	109.9	-	-	-	-
DPPC:violacein	7.4	40.3	41.4	1.47	133.2	34.6	35.8	2.37	10.7
DPPC:violacein	9.0	41.0	42.3	1.74	74.1	-	-	-	-
HSPC:violacein	3.0	45.4	49.0	3.29	101.4	-	-	-	-
HSPC:violacein	5.0	47.3	50.3	3.77	211.4	-	-	-	-
HSPC:violacein	7.4	51.7	53.7	1.90	185.2	-	-	-	-
HSPC:violacein	9.0	43.7	49.7	5.33	200.6	-	-	-	-

T_{onset}: temperature at which the thermal event starts; T..: temperature at which heat capacity (ΔC_p) at constant pressure is maximum; $\Delta T_{1/2}$: half width at half peak height of the transition; ΔH: transition enthalpy normalized per mol of liposomal system. m: main transition; s: secondary transition

Table 10. Calorimetric profiles of DPPC:violacein and HSPC:violacein fully hydrated bilayers in different pH (cooling).

Composition	pH	$T_{onset,m}$ /°C	T_m /°C	$\Delta T_{1/2\,m}$ /°C	ΔH_m / J mol^{-1}	$T_{onset,s}$ /°C	T_s /°C	$\Delta T_{1/2,s}$ /°C	ΔH_s /J mol^{-1}
DPPC:violacein	3.0	38.8	37.8	1.58	112.7	-	-	-	-
DPPC:violacein	5.0	39.7	38.2	1.85	106.2	-	-	-	-
DPPC:violacein	7.4	39.1	38.0	1.61	138.5	-	-	-	-
DPPC:violacein	9.0	43.0	41.3	2.13	149.2	-	-	-	-
HSPC:violacein	3.0	49.1	47.5	3.12	256.9	-	-	-	-
HSPC:violacein	5.0	45.0	43.8	2.16	411.7	-	-	-	-
HSPC:violacein	7.4	51.4	49.7	2.15	183.4	-	-	-	-
HSPC:violacein	9.0	49.6	46.5	5.30	253.6	-	-	-	-

T_{onset}: temperature at which the thermal event starts; T.: temperature at which heat capacity (ΔC_p) at constant pressure is maximum; $\Delta T_{1/2}$: half width at half peak height of the transition; ΔH: transition enthalpy normalized per mol of liposomal system. m: main transition; s: secondary transition

Concerning the characteristics of the lipid bilayers, the direction of changes may vary in accordance to the nature of the dispersion medium and therefore the specific calorimetric parameters values could be accordingly explained. Furthermore, the compounds incorporated into the lipid bilayer could as well impact the properties at various dispersion media through the compounds' amphiphilicity or ionic strength of the acid used for pH adjustments that may well impact on the solubility of compounds into bilayers. Depending on the pH values in relation to the pKa a number of events may be present such as hydrogen bonding between the unionized –COOH and phosphadiester groups, hydrophobic interactions, electrostatic repulsions or adhering to cationic polar groups such as phospholipids, previously described in relevant works (Fujiwara et al. 1997, Tribet and Vial 2008).

The effect of the type of acid used for pH adjustments on the physico-chemical interactions, has also been demonstrated for lipid bilayer destabilization phenomena in particular, by Felber et al. (2012).

In particular, when comparing Figure 7 to the transition peaks for heating DPPC:violacein lipid bilayers of Figure 18A, an apparent shift of the main transition to higher temperatures may be claimed for pH 9.0, compared to all of the rest pH, while liposomes at pH 7.4 and 5.0, although did not differ between each other, they were both higher compared to the results of DCS for pH 3.0.

The transition peaks for heating DPPC-violacein lipid bilayers, indicated quite similar T_m for samples at pH 3.0, 7.4 and 9.0, while samples at pH 5.0 had a slightly but clearly higher T_m. Changes in the enthalpy during phase transitions in the presence of incorporated compounds into lipid bilayers most likely indicate modifications of phospholipid bilayers' structures as a result of the structural conformation of the additive. Then, as a result, grater thermotropic changes in T_m may be recorded (Gardikis et al. 2006).

The ΔH_m for DPPC:violacein was increased when hydrated in citrate buffer of pH 5.0 and 9.0 was used, though it was lower when hydration was done with citrate buffer of pH 3.0 and similar that with pH 7.4, that according to Maswadeh et al. (2002) could be due to an increased interdigitated gel phase, giving rise to more rigid bilayers as he recorded for DPPC:vinblastine model membranes studies. The calorimetric data showed that violacein induced thermal modification of DPPC lipid bilayers which was hydrated in aqueous media in a pH dependent manner (Table 9). A decreased cooperativity of the transitions while practically also destabilize the gel phase. According to Kyrikou et al. (2004), such phenomena cannot be recorded for lipids with shorter acyl chains such as DMPC or longer chains in the case of DSPC, but it is due to the active compounds of vinblastine that the inter-digitation phenomena can occur. On the other hand, when HSPC was used (Figure 17B), no such behavior may be reported for the pure fully hydrated bilayers during heating, where no pre-transitions were recorded, while only a rather comparatively smaller, increase in the main transition temperature was recorded with increasing pH for pH 3.0, 7.4 ad 9.0.

As shown in Figure 18B, there are still no pre-transitions, but the main transition temperatures were lower for samples at pH 5.0 compared to samples at pH 3.0 and lower for samples at pH 9.0 compared to samples at pH 7.4. Nevertheless, T_m for samples at either pH 9.0 or 7.4 were higher compared to either of the T_m for samples at pH 3.0 or 5.0.

In general, interaction of active compounds with liposomes affect the mobility of the lipid groups, where the main transition temperature corresponds to the phospholipids acyl chains' mobility. The decrease in the ΔH values may be related to the reduction in the "effective volume fraction" of the lipid nanodomains, when an active compound (i.e. violacein) is incorporated in the lipid bilayers (Pippa et al. 2014).

Notably, a pre-transition was not present of HSPC: violacein fully hydrated bilayers (Table 9). Apparently, violacein also altered the thermodynamic behavior of HSPC lipids bilayers as can be recorded through the rather significant reduction of ΔH_m values during heating, for all pH (Table 9**)**. Such thermal events, most likely indicate the impact of the ionic strength at low and high pH environments on the mobility of the polar groups of lipids (Pippa et al. 2014, 2015).

Similar trends were also recorded for the $\Delta T_{1/2}$ values for the HSPC: violacein fully hydrated bilayers compared to pure HSPC. These values were significantly increased for sample hydrated in citrate buffer of pH 3.0 and 9.0, most likely suggesting that the cooperativity between HSPC lipids and violacein decreased (Naziris et al. 2016).

Moreover, the onset temperature for the thermal event ($T_{onset,\ m}$) started at lower values for HSPC: violacein fully hydrated bilayers hydrated at pH 3.0 and 9.0. There were no significant differences for 7.4, compared to pure HSPC fully hydrated bilayers, while it was slightly higher when citrate pH 5.0 was used.

On the other hand, DSC cooling profiles obtained for the pure DPPC and HSPC fully hydrated liposomes bilayers at various pH, without are given in Figure 19 and for the DPPC:violacein and HSPC: violacein fully hydrated lipid bilayers are given in Figure 20. The values for the main thermographic parameters $T_{onset,m}$/ ^oC, $T_m/^oC$, $\Delta T_{1/2,m}/^oC$, $\Delta H_m/Jmol^{-1}$, $T_{onset,s}/^oC$, $T_s/^oC$, $\Delta T_{1/2,s}/^oC$, $\Delta H_s/Jmol^{-1}$ are given in Tables 8 and 10, respectively.

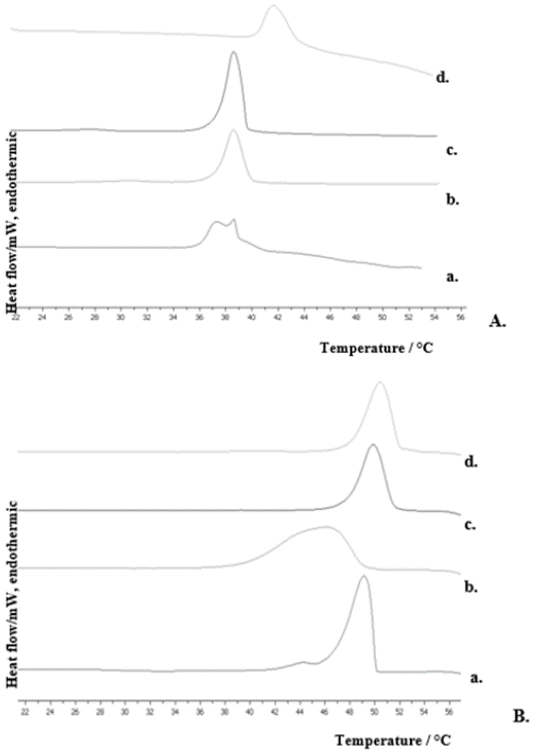

Figure 19. DSC cooling scans of **A.** DPPC and **B.** HSPC fully hydrated bilayers at pH **a.** 3, **b.** 5, **c.** 7.4 and **d.** 9. The limits for the calculation of thermotropic parameters are from 25°C to 60°C.

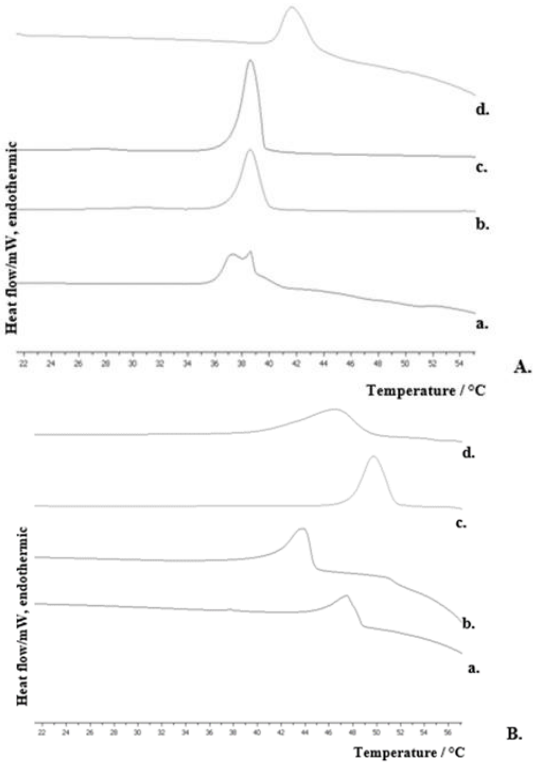

Figure 20. DSC cooling scans of **A.** DPPC:violacein and **B.** HSPC:violacein fully hydrated bilayers at pH **a.** 3, **b.** 5, **c.** 7.4 and **d.** 9. The limits for the calculation of thermotropic parameters are from 25°C to 60°C.

We have to point out that in DSC cooling curves (see experimental data in Tables 8 and 10) small hysteresis was observed, for DPPC:violacein and HSPC:violacein at all pH ratios most likely due to the amphiphilic character of the compound, a suggestion on the formation of interdigitated phase could be made (Pippa et al. 2012). Violacein penetrating into the interior of the bilayers, forming hydrophobic nanoclusters and finally causing membrane disruption and phase separation, especially at the high pH values (Table 8 and 10). These nanoclusters if heterogeneously distributed to the bilayer lipid interior, they may be forming highly concentrated violacein regions, known as nanodomains as previously reported for polymers (Pippa et al. 2014, 2015).

As data in Tables 7 and 9 indicate that the main transition temperature for both the DPPC and the HSPC were slightly decreased, the specific enthalpy was significantly in certain pH increased and the transition peak reappeared at 49.2^{o}C for HSPC hydrated at pH of 3.0, while no other transition peak was recorded for either of the two bilayers used at any pH.

Further addition of violacein renders the pretransition undetectable for both bilayers at pH of 7.4 (Table 7 and Table 8). The previously reported metastable phases at these pH levels were not again reappeared (Figure 18). The main transition temperature T_m and $T_{onset,m}$ were both shifted to rather lower temperatures in a way much depended on the hydration water pH (Table 8), while the main peak was actually also following a similar pattern. The main transitions were appearing sharper for the pH 7.4 for both types of lipid:violacein bilayers.

The enthalpy change (ΔH_m) of the main transition seemed to be significantly increased for DPPC:violacein only for pH 9.0, while very significant increase was recorded for HSPC:violacein at pH 5.0, followed by pH 3.0 and 9.0 but no change could be claimed for pH 7.4. Furthermore, the peaks became broader or narrower in a very much pH depended way, as the $\Delta T_{1/2}$ values in Table 10 indicated.

5. CONCLUSION- FUTURE RESEARCH

This study is divided in two main parts: the HPLC analysis and the DSC analysis part. The HPLC analysis for the quantification of the compounds demonstrated the influence of the environmental conditions in the substance's production while on the other hand, DSC analyzed the nanostructures thermal-stability in the different pH values.

Regarding the production of the substances by the microorganisms for this work, the results of Pantanella et. al (2007) were confirmed. The suggested temperature for the violacein production was the 25 ^{o}C. In the present study it was observed that temperature was the factor which mainly affects the violacein's production, compared to the different inoculum amount and the pH values. That was also suported by the difference in the color of the samples, as showen in Figures 33-35 of the appendices and in particular, Figure 35, where samples of the substance with the same inoculum 5 log CFU, same pH but different temperatures (15, 20, 25, 30 ^{o}C) are depicted.

Another interesting observation was that the substances' production curve was not constant. But on the contrary, fluctuations were observed in every sample's production curve. The absence of tryptophan could be responsible. Because as was stated the molecule of the violacein is consisted by two molecules of tryptophan as in Figure 21 in the appendices. Possibly, the microorganism started the violacein's degradation, as it

was depicted in Figure 1. This is a common characteristic behavior of the microorganisms (Knowles 1988). Specifically, the Chromobacterium violaceum was known for degrading the cyanide, a secondary metabolite just as violacein, either to detoxify cyanide or to use it as a source of nitrogen for growth (Macadam and Knowles 1984). In our case we hypothesise that Janthinobacterium livindum due to its lack of nutrient medium, that was consisted by tryptophan and in order to ensure its survival, it started to degrade the violacein for receiving the tryptophan needed.

In conclusion, the HPLC analysis results indicated that the microorganism was strongly affected by the temperature of the environment in order to produce the substances, which played a key role in the microorganism survival.

Furthermore, the thermal behavior of violacein incorporated into DPPC and HSPC lipids'fully hydrated bilayers, was investigated. When hydration water was acidified with acetic acid at pH 3.0, 5.0, 7.4 and 9.0, we managed to assess a set of main calorimetric parameters using DSC, in order to picture the areas of potential design and development interest for usage in various biological structures as delivery systems.

Due to its high hydrophobicity, violacein use raises some obstacles to reside the compound inside the bilayers of the phospholipids, causing significant alterations of their physicochemical properties as recorded via the thermal alterations during the DSC analysis. Therefore, the incorporation of violacein into such systems presents certain advantages. Differentiating the acidic environment at a range of pH 3.0 to 9.0, we have managed to achieve a quite clear view regarding the pH-sensitivity of the chimeric DPPC and HSPC liposomes in relation to their behavior at temperature scans from 20 to 60 ^{o}C.

The per case presence of pre-transitions for the two lipid bilayer at different pH and relevant thermotropic behavior, suggested the existence of cooperativity at very few conditions, namely for pH 7.4 for pure lipids and only at the same pH for DPPC:violacein fully hydrated bilayers, while lack of such pre-transitions indicate the loss of cooperativity for most cases studied.

Therefore, proper justification of the results could also reveal the potential interactions of the active compounds of the liposomes when present in various acidic environments. Such as in the case of food matrices, affecting the availability of the active compound is questionable. The concluded stability of the system, could significantly affect the delivery process of the violacein at different environments.

Future studies that could be planned following this research, may consider the violacein's effectiveness in the encapsu-lated form should be tested; in certain packaging material or directly in foods. If the directly-in-the-food approach will be chosen, the challenge of how to spread the substance and not contain it exclusively on the surface of the food, will be faced. In addition, thermal treatments and/or chemical environment influence could be considered. On the other hand, if the packaging-material approach will be chosen, an investigation on how the substance would interact with the food system, should be conducted.

Additional studies may concern the release mechanism for the substance from the nanostructures. While a need to examine whether the release of the substances would be conducted in the stomach or in the food before consumption, could also be of interest.

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APPENDICES

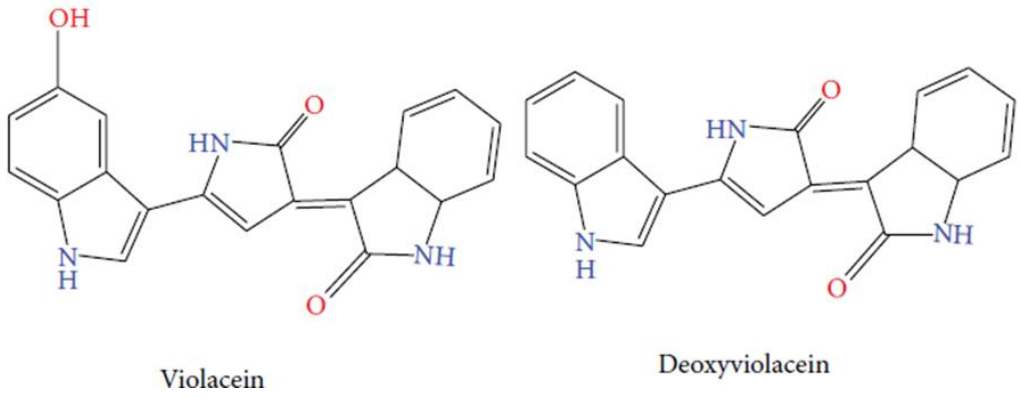

Figure 21. The chemical structure of violacein and deoxyviolacein. The structures were prepared using ChemDoodle 2D sketcher (http://web.chemdoodle.com/demos/sketcher). (Choi *et al.* 2015a).

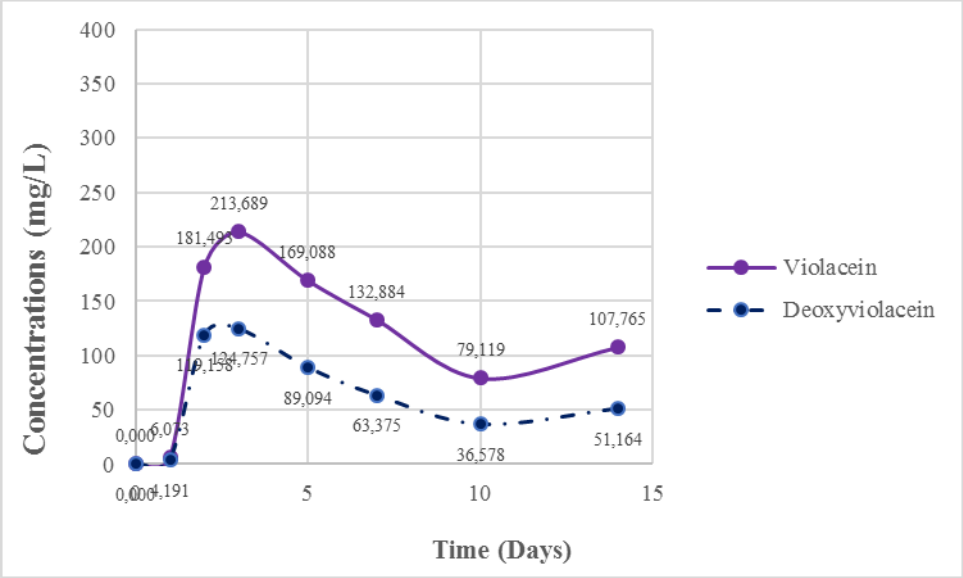

Figure 22. Violacein and Deoxyviolacein of sample 6- 15 ºC

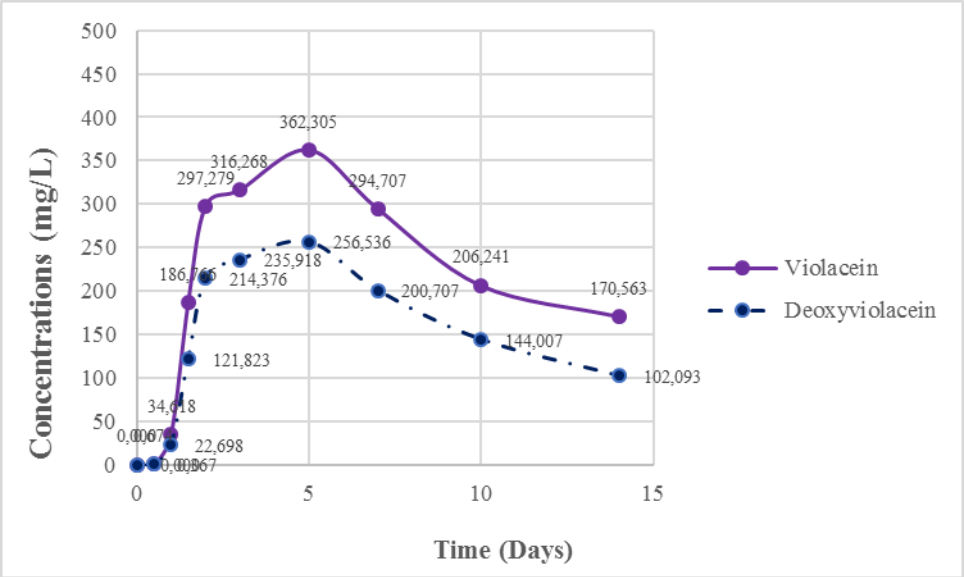

Figure 23. Violacein and Deoxyviolacein of sample 6- 20 ºC

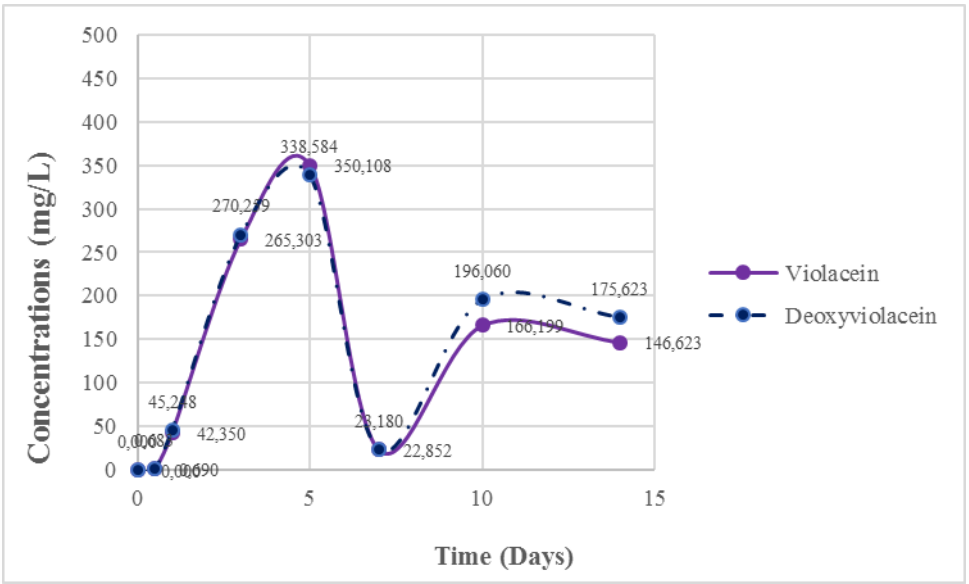

Figure 24. Violacein and Deoxyviolacein of sample 6- 25 ºC

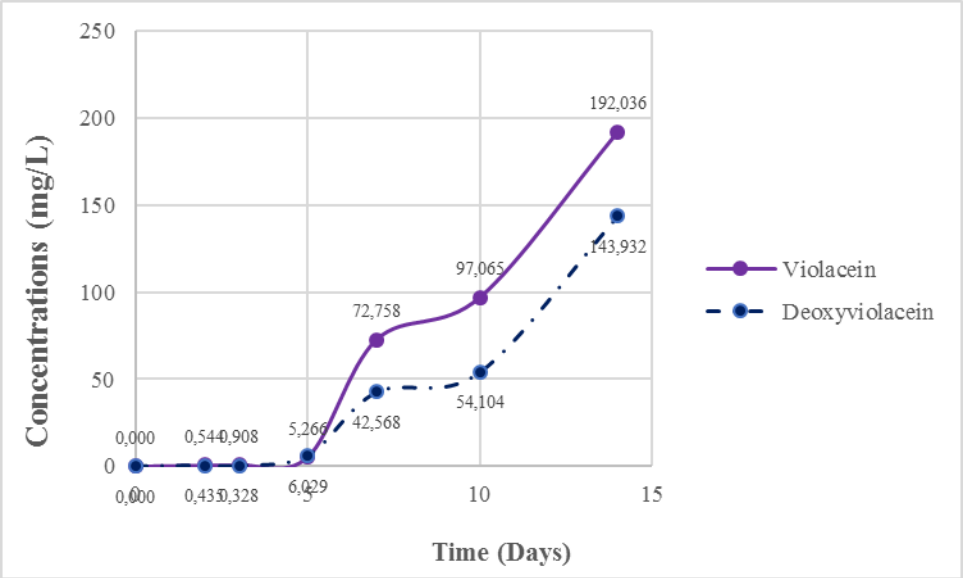

Figure 25. Violacein and Deoxyviolacein of sample 6- 30 ºC

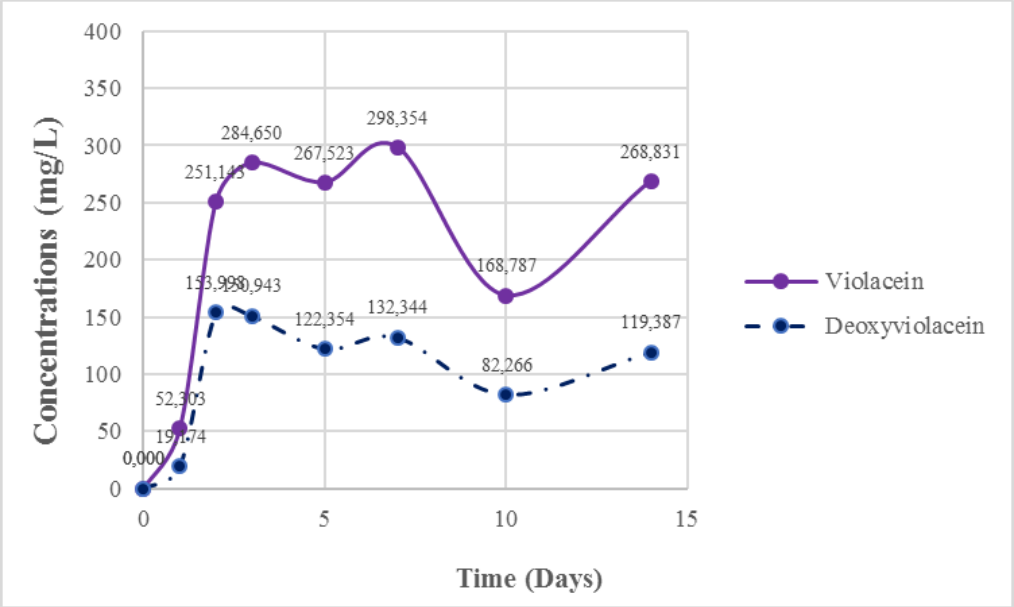

Figure 26. Violacein and Deoxyviolacein of sample 7- 15 ºC

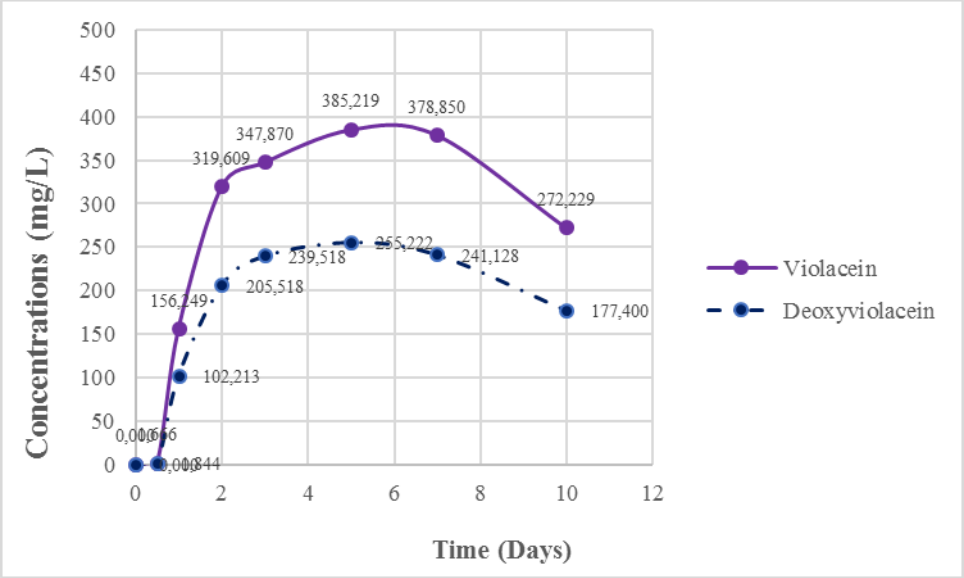

Figure 27. Violacein and Deoxyviolacein of sample 7- 20 ^{o}C

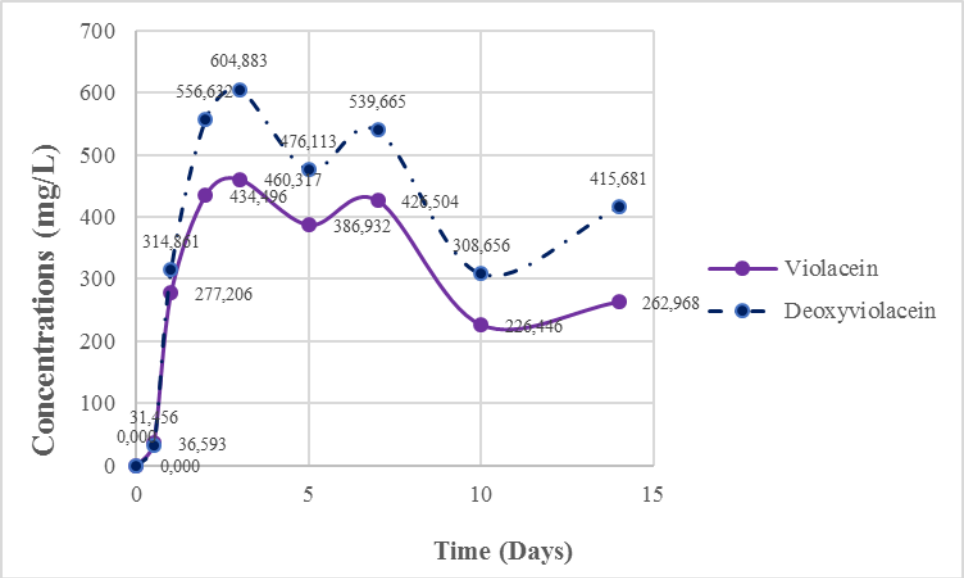

Figure 28. Violacein and Deoxyviolacein of sample 7- 25 ^{o}C

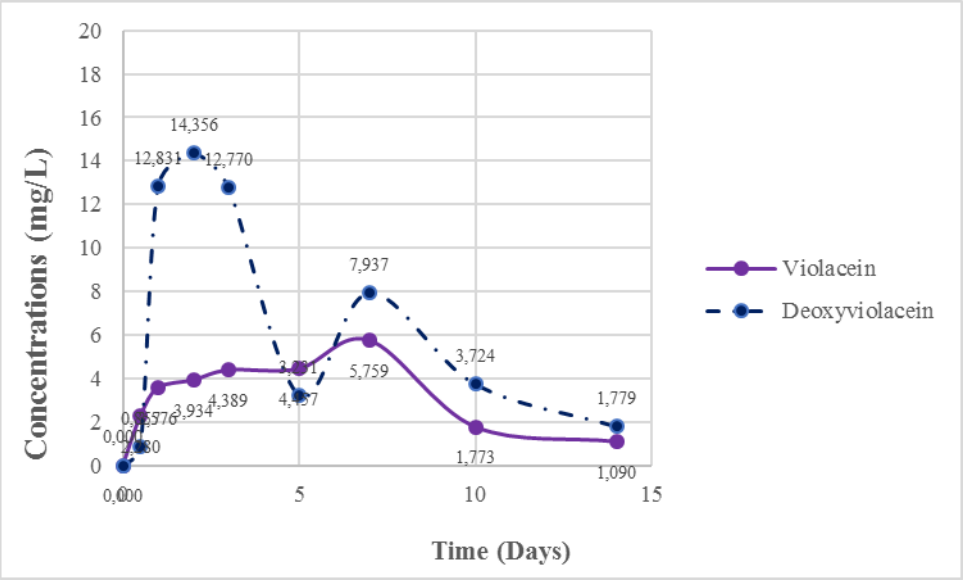

Figure 29. Violacein and Deoxyviolacein of sample 7- 30 ^{o}C

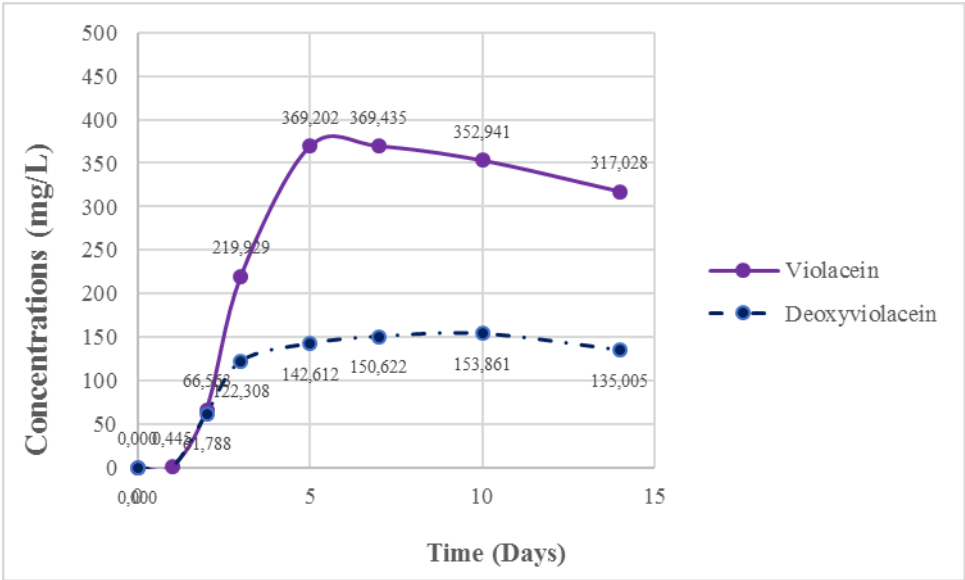

Figure 30. Violacein and Deoxyviolacein of sample 9- 15 ^{o}C

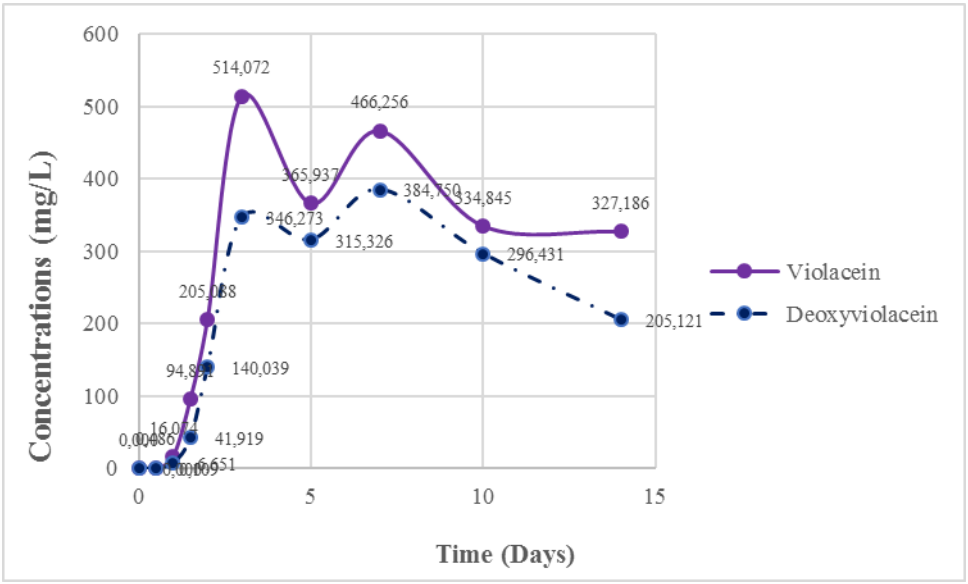

Figure 31. Violacein and Deoxyviolacein of sample 9- 20 ºC

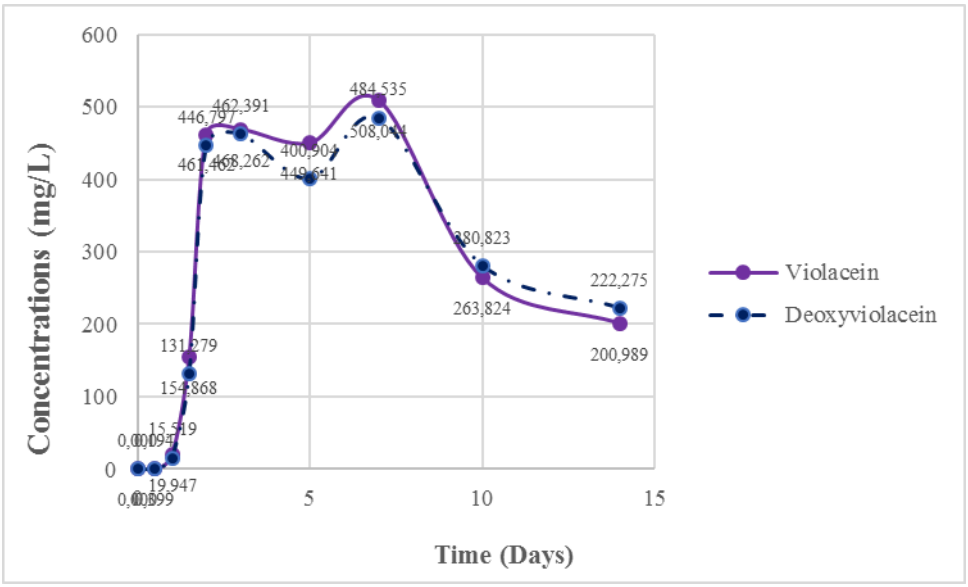

Figure 32. Violacein and Deoxyviolacein of sample 9- 25 ºC

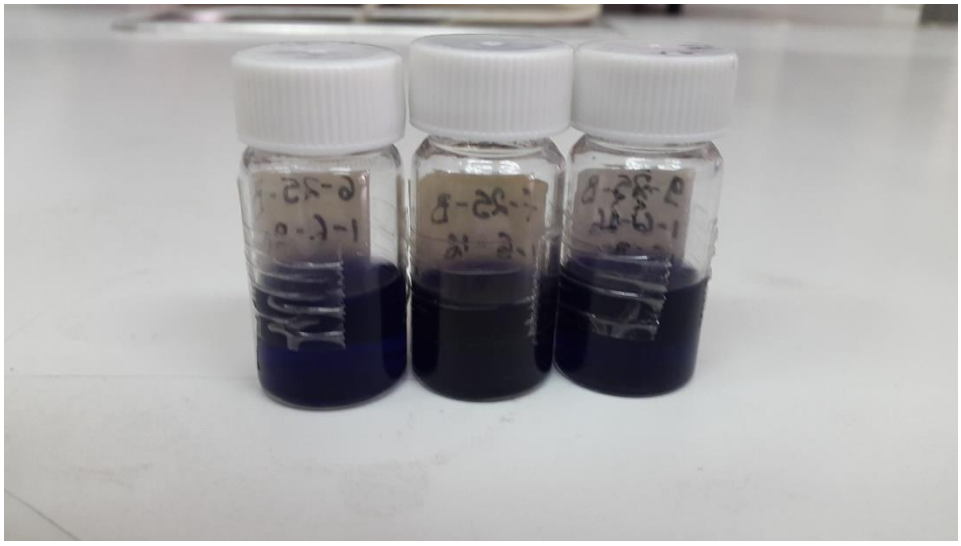

Figure 33. Samples of Violacein of the same temperature (25 ^{o}C) in the same day (14th) but in different pH and with the same inoculum 5 log CFU.

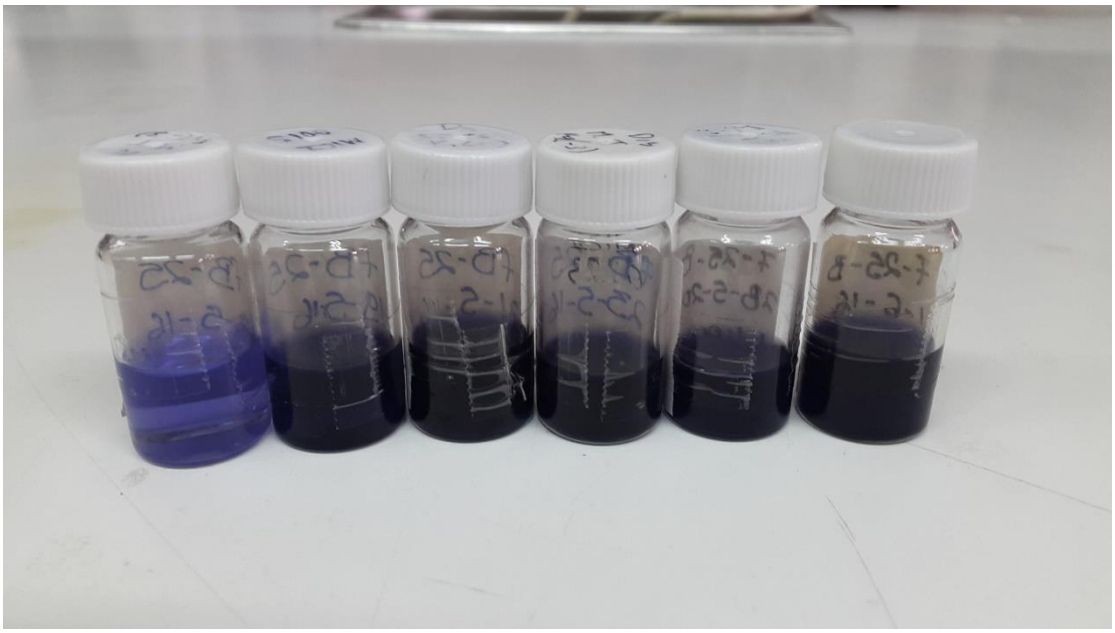

Figure 34. Samples of Violacein in the same inoculum 5 log CFU, same pH but in different days (0,1,3,5,10,14) and in the same temperature (25 ^{o}C).

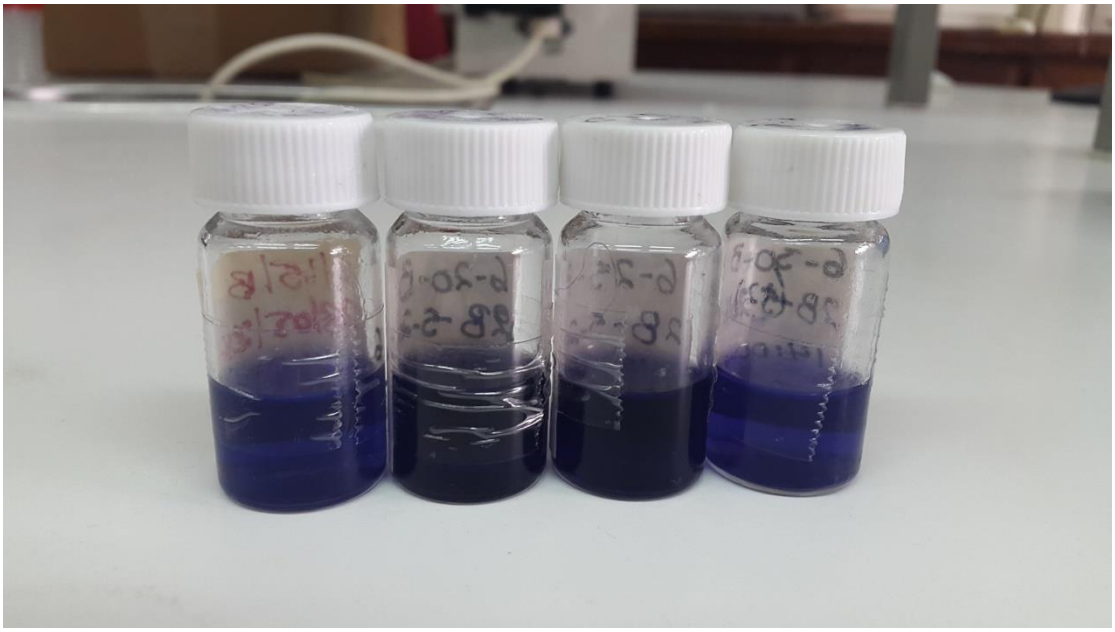

Figure 35. Samples of Violacein with the same inoculum 5 log CFU, same pH but different temperatures (15, 20, 25, 30 ºC).