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An extraction of the violacein sequence from p*JP*101 for expression and analysis in p*HSG*398

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Abstract. *Violacein is a pigment found in Chromobacterium violaceum, it has a number of properties, such as; anti-bacterial, anti-viral, anti-protozoa, anti-tumoral. Violacein is produced by a series of genes named vioA, vioB, vioC, vioD and vioE. A violacein sequence is 8 kilo-bases (kb) in size. The goal of this study was to isolate the smallest possible fragment of 'Deoxyribonucleic acid' (DNA) that would produce violacein from pJP101 and making a connection to DNA biological logic gates.*

Keywords. Quorum sensing, Polymerase chain reaction, *Escherichia coli* (E.*coli*), Plasmid, Violacein, logic gates. biorheology

1. Introduction

The hypothesis posed is as follows, 'the extraction of the violacein sequence from p*JP*101 for expression and analysis in p*HSG*398 determine the smallest 'Deoxyribonucleic acid' (DNA) fragment responsible for purple pigmentation'. This paper aims to develop an experimentation process and applies the process to validate the hypothesis within a hybrid biology and rheology context.

In molecular biology it is important to be able to identify and isolate DNA sequences of interest for expression and analysis. This can be achieved through the amplification and isolation of genetic material, with techniques such as 'Polymerase chain reaction' (PCR) and restriction digests [1].

The techniques PCR and restriction digest can be used to identify gene products, such as resistance genes in bacteria [2], Furthermore, these techniques are important in the development of antibiotics, as pathogens continue to develop resistance to many common antibiotics [3], due to this it is important to look for different types of antibiotics. One such example of this is violacein.

Violacein is a purple pigment produced by *Chromobacterium violaceum* which is a Gram-negative bacterium found in soil and water in tropical and subtropical regions [4]. *C. violaceum* is rarely infectious in mammals and comes in 2 strains; pigmented and un-pigmented. Violacein has been extensively researched, which has shown it has many properties and potential uses, such as;

- Protecting *C. violaceum* by triggering cell death and lysis in bacterivorous flagellate after ingestion [5]
- Inhibit the growth of the plasmodium parasites, such as the ones which cause malaria [6]

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- Exhibits some antiviral properties as demonstrated against HSV-1, may exhibit greater anti-viral activity by incasing violacein in a liposome [7]
- Demonstrated the ability to affect HL60 leukemia cells, it achieves this by activating tumor necrosis factor receptor signaling, causing apoptosis [8]

2. Background and Contextualization

One way of finding new antibiotics is by studying bacterium to see if they have any antibiotic properties [9]. By isolating a bacterium's genetic material it is possible to generate a genomic library, where the genetic material responsible for the antibiotics production can be identified and cloned for expression [10]. The size of a piece of genetic material is generally shown as the number of DNA base pairs it consists of, this measurement is usually given either as a single unit ('base pairs' [bp]) or in units of a thousand (kilo-bases [kb]).

2.1. Molecular biology context

To obtain genetic material for identification, a restriction digest maybe performed. Restriction digests utilizes a restriction enzymes ability to cleave DNA at defined sites usually 4 to 8 bp in length (restriction sites) [11], multiple 'restriction sites' may be scattered throughout an organisms genome. Multiple types of restriction enzymes can be used to fragment an organisms genome. This fragmented genetic material can then be inserted into a vector plasmid for sequencing and/or to identify an organism/gene of interest [12]. Once a gene has been identified, it may be amplified via PCR.

There are three basic steps involved in PCR [13]:

- Initially DNA is denatured from its usual double stranded form into a single stranded form,
- Then specific primers (short sequences of single stranded DNA) anneal to the single stranded DNA, and
- Once annealed a heat dependent polymerase is used to replicate DNA from where the primers have annealed.

Figure 1. PCR tubes with reaction mixture placed in thermal cycler that is used in targeted DNA sequence amplification [9].

The PCR cycles are repeated multiple times to generate a large number of copies of the sequence of interest, which then can be either inserted into a vector

for uptake and expression or sequenced to identify its genetic sequence. Primers can be designed to include specific restriction enzyme sites, which can be used to ensure the genetic material is inserted in the right direction [14].

2.2. Violacein context

The genes responsible for the production of violacein form a large metabolic pathway, which has 5 specific regions, these regions are; *vio*A (1,257 bp), *vio*B (2,997 bp), *vio*C (1,290 bp), *vio*D (1,122 bp) [15] and *vio*E, giving a total sequence length of ~8 kb [16].

Violacein is produced by combining two L-tryptophan (Figure 2), the process by which it is synthesized is as follows [17]:

- Initially *vio*A removes an amine group from the terminal end of one L-tryptophan and replacing it with a ketone,
- On another L-tryptophan, *vio*D adds an alcohol group to the hexane ring, and then *vio*E causes a 1-2 shift with the carboxylic acid and amine group,
- *vio*B combines the two L-tryptophan to create a violacein intermediate, an in the process removes carboxylic groups from each terminal end, and
- *vio*C then adds an alcohol group to the N-ring attached to the hexane ring without any functional groups, this alcohol is then transformed into a ketone group, thus forming violacein.

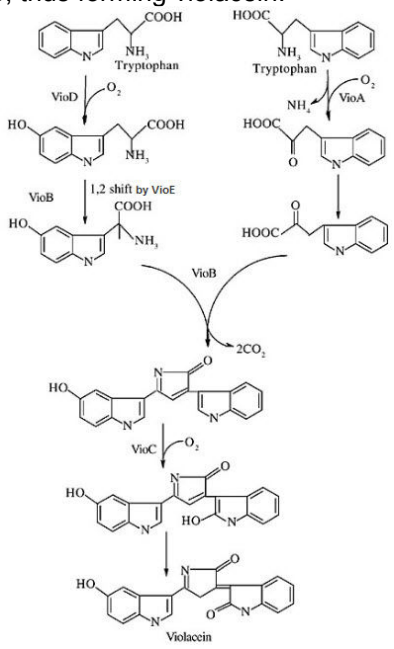

Figure 2. Biosynthetic pathway for the production of violacein in *C.violaceum*, showing products, reagents and gene products used, also shows changes in structure of violacein as the reaction [18].

2.3. Logic gate context

A nexus appears to exist between the ‘waking up’ of a sleeping virus (genes are turned on and off) [19] and DNA ‘switches’ that act as biological logic gates.

Violacein may be the switching mechanism as it appears to activate tumor necrosis factor receptor signaling [16] and an alignment to a traditional approach of dealing with logic gates within a 'Central processing Unit' (CPU) hierarchy (logic device). Once mapped (Figure 3), the violacein gene domains could have causality with NOT [inputs opposite to output] logic gate blocks [20] and be extracted as the smallest possible bp fragment in a bio machine.

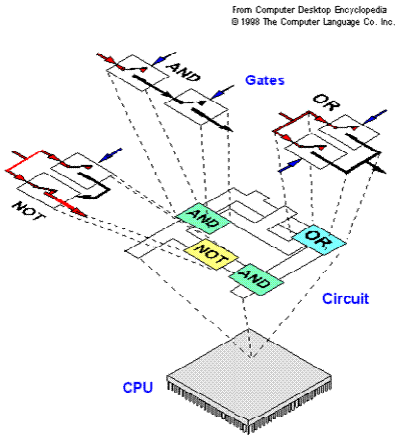

Figure 3. logic gates within a 'Central processing Unit' (CPU) hierarchy (logic device) [21].

3. Experimentation

The aim of this experiment was to try and extract the smallest possible fragment from p*JP*101 which contained the violacein genes.

3.1. Fragmented pJP101 context

Plasmid *JP*101 was the created by Pemeberton and colleauges (1991) [22], it was the smallest plasmid created expressing violacein in the study. Plasmid *JP*101 (Figure 4) was assembled by ligating chromosomal DNA into the *Bam*HI site of the Trasnposon n5, this was followed up by infection of E.coli of the clones of the experiment (JMP2540) carried the cosmid p*JP*100, which contained a 30 kb insert.

A smaller insert was obtained by partially digesting p*JP*100 with *Sau3*AI and ligating it into p*BR*332 which had been digested with *Bam*HI. This clone was named p*JP*101, the DNA fragment containing the violacein genes in p*JP*101 was 14.5 kb.

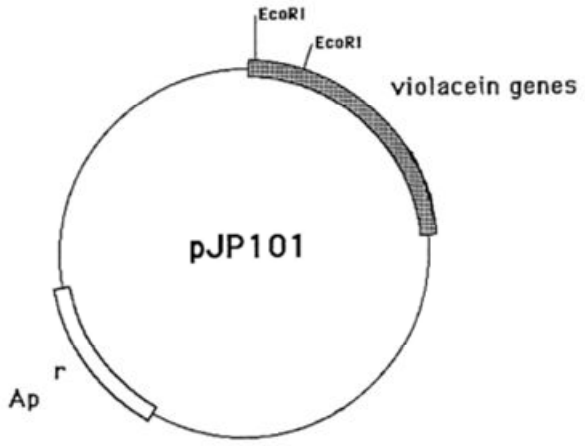

Figure 4. Diagram of p*JP*101 showing amphilcilin resistance (Ap^r) and violacein genes. The diagram also shows *Eco*RI sites. Plasmid *JP*101 also has multiple *Sau*3AI sites scattered throughout its genome (not shown) [22].

3.2. Vector pHSG398 context

Plasmid *HSG*398 was used as the vector for the violacein genes (Figure 5). Plasmid *HSG*398 was selected as it has a different antibiotic resistance than that of p*JP*101, to allow for antibiotic screening. Plasmid *HSG*398 was also selected for this experiment as it only has one *Bam*HI restriction site in its multi cloning region [23].

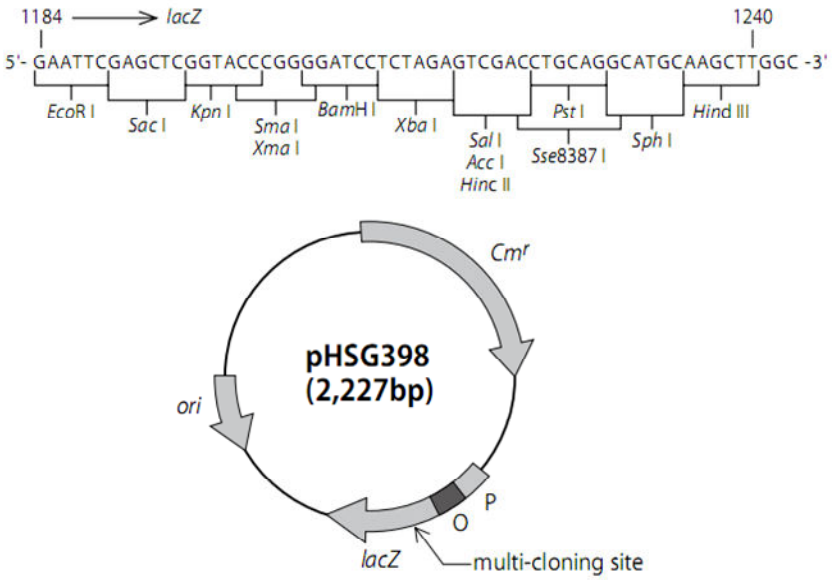

Figure 5. Plasmid map of p*HSG*398, showing; plasmid size, origin of replication, antibiotic resistance (Cm^r), and *Lac*Z region. Shown inside the *Lac*Z region is p*HSG*398 multiple cloning sites, shown in greater detail above plasmid map [23].

3.3. Techniques context

The techniques used in this experiment are usually used to make a genetic library. This can be done by performing enzyme dilution digests on DNA of interest. Serial digests work by using varying amounts of enzyme to fragment a plasmid, these fragments can then be ligated into a vector plasmid (for this experiment p*HSG*398) for size and sequence analysis [1].

These results can then be complied to construct genetic library, which can be used as a reference point for further research into specific genes as well as comparisons between different genomes [24]. Similar techniques can be used if a recombinant is easily identifiable (colour expression), in this case only clones expressing the recombinant would be analysed [22].

This experiment has similar methodology to that of Pemberton and colleagues (1991), except for this experiment violacein is being extracted from p*JP*101, and inserted into p*HSG*398. In this experiment it was expected that the clones that expressed violacein, when digestion would give two fragments. One fragment from the digestion would p*HSG*398, as it has only one *Bam*HI restriction site, and another between 8,000 to 14,500 bp.

4. Methodology

The methodology focuses on using:

- Digestion of vector,
- Ligation of Vector into target plasmid,
- Transformation and plating, and
- Cell lysis and electrophoresis.

4.1. Digestion of vector

The cosmid p*JP*101 digested using serially diluted *Sau*3AI (1:1) for digestion, starting with a 60 µL master mixture containing; 40 µL of p*JP*101, 10 µL buffer, 10 units of *Sau3*AI. 30 µL of the master solution was added to a 30 µL solution containing; 20 µL of p*JP*101, 1x buffer, giving dilution factor 0.5.

This dilution was performed 5 times. Samples where digested at 37°C for 15 minutes, after this time digestion was stopped by incubating at 85°C for 15 minutes. Each digestion was run on an electrophoresis gel. Digestion samples which had gel bands ≥8 kbp where selected for ligation.

4.2. Ligation of Vector into target plasmid

The vector fragment ligation (p*HSG*398) was digested for 1 hour at 37°C in; 10 units of *Bam*HI, 1x restriction buffer and 10 µL p*HSG*398. Samples were heated at 70°C for 10 minutes. Digested p*HSG*398 and the serial digests of p*JP*101 where ligated in a solution of; 10 µL of each DNA sample, 1x ligation buffer, 1 unit of T_4 ligase. This solution was left for 16 hours at 16°C.

4.3. Transformation and plating

For transformation 25 µL of the following; ligation samples, p*JP*101, p*HSG*398 and TE buffer where added to separate 200 µL of *JM*109 cells, sample where left

on ice for 10 minutes, then incubated at 37°C for 5 minutes. Each sample had 500 µL of LB broth added, after which they were incubated at 37°C for 45 minutes.

The ligation cell cultures where added to agar plates with either $Ampercillin_{100\mu g/mL}$ to check for contamination by p*JP101* or $Kamermycin_{100\mu g/mL}$ to screen for recombinants (see table 1 for set up). Cultures were re-plated every 24 hours for 3 days. Positive ligation results were picked for further analysis.

4.4. Cell lysis and electrophoresis

Clones that produced a purple pigment where re-suspended in a 200 µL solution; 50 mM glucose, 25 mM TrisHCl and 10 mM EDTA. 200 µL of a lyse solution containing; 200 mM NaOH and 1% SDS, was then added to the cell mixture. This new solution was left on ice for 5 minutes. The solution was centrifuged at 20,000 rpm, the supernatant was transferred to a fresh 1.5 mL tube, and pellet was discarded.

The supernatant was left to incubate at room temperature for 10 minutes. 450 µL of isopropanol was added to the supernatant, and left at room temperature for 10 minutes. This solution was then centrifuged at 20,000 rpm for 10 minutes. The supernatant was discarded and the pellet was rinsed with 70% ethanol centrifuged at 20,000 rpm for 5 minutes, supernatant was discarded and pellet was air dried and re-suspended in sterile H_2O. Isolated plasmids had undigested and samples digested with *Bam*HI electrophoresed for analysis.

5. Results

After the dilution digests with *Sau3*AI on p*JP*101 was performed, the digests where ran on a electrophoresis gel to see if there were any digests that produced fragments large enough to contain the whole violcein sequence (fragments ≥ 8kb).

When the serial enzyme dilution digests where examined with gel electrophoresis gave four samples containing fragments large enough to contain the violacein sequence (≥8 kbp). Sample $pJP101^{0.5Sau3AI}$ gave a range of bands from 8 to 10 kb and another 20 kb. All other sample from this digest mainly shows bands 9 kb or larger (Figure 6). These samples could then be ligated with p*HSG*398 digested using *Bam*HI.

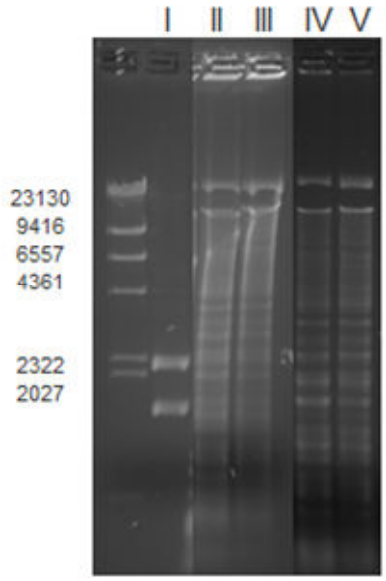

Figure 6. The partial digests of p*HSG*398 with *Sau3*AI. The contests of each gel lane are; p*HSG*398 digested with *Bam*HI (I), p*JP*101 digested with 0.500 units of *Sau3*AI (II), p*JP*101 digested with 0.250 units of Sau3AI (III), pJP101 digested with 0.125 units of *Sau3*AI (IV), p*JP*101 digested with 0.063 units of *Sau3*AI (V).

Following the ligation of genetic material into p*HSG*398 and the uptake recombinants by *JM*109 cells, the clones where plated along with control cultures (Table 1). There was no unexpected growth on the $Ampercillin_{100\mu g/mL}$ control plates. Clones expressing violacein were found in all $Kamermyicin_{100\mu g/mL}$ plates with 400 µL of culture. Sample $pJP101^{0.5Sau3AI}$ also showed purple colonies when plated using 200 µL of culture.

Table 1. Results of plating the ligations, the table show contents of plates, antibiotic resistance and cfu/mL. When plates where not use, they were marked with 'not applicable' (N/A).

Sample	Volume	Colour	Plate (cfu/mL) $Ampicillin_{100\mu g/mL}$	Plate (cfu/mL) $Kamermycin_{100\mu g/mL}$
p*JP*101	100 µL	Purple	80	0
		White	330	0
p*HSG*398	100 µL	Purple	0	0
		White	0	760
TE buffer	100 µL	Purple	0	0
		White	0	0
I	100 µL	Purple	N/A	0
		White	N/A	170
I	200 µL	Purple	N/A	0
		White	N/A	200
I	400 µL	Purple	N/A	3
		White	N/A	293
II	100 µL	Purple	N/A	0
		White	N/A	270
II	200 µL	Purple	N/A	5
		White	N/A	251
II	400 µL	Purple	N/A	3
		White	N/A	238
III	100 µL	Purple	N/A	0
		White	N/A	340
III	200 µL	Purple	N/A	5
		White	N/A	270
III	400 µL	Purple	N/A	5
		White	N/A	245
IV	100 µL	Purple	N/A	0
		White	N/A	315
IV	200 µL	Purple	N/A	0
		White	N/A	335
IV	400 µL	Purple	N/A	5
		White	N/A	270

Clones expressing violacein were re-plated, re-suspended in culture and lysed to extract there genetic material for analysis by electrophoresis. The lysed clones had two samples of each on a electrophoresis gel; undigested and digested.

All cut ligation samples showed two bands, one the size of p*HSG*398 and another between 9416 and 23130 bp. Cut ligation samples I and III had the smallest inserts from p*JP*101. The uncut ligation sample I showed two bands, one near 9416 bp and another near 23130 bp, all other uncut samples showed smears, blodges and multiple bands (Figure 7).

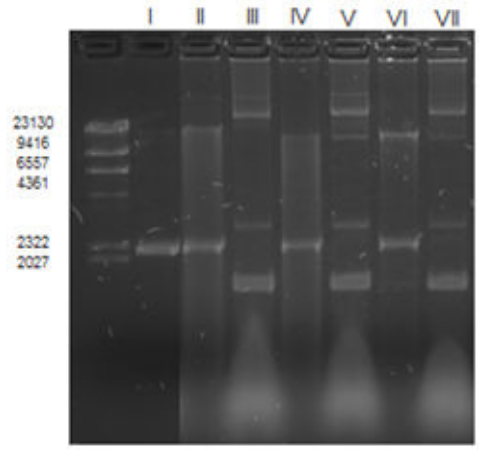

Figure 7. The re-digests of the successful ligations where digested with *Hind*III, gel contents are; Digested pHSG398 (I), cut pJP101$^{0.5Sau3AI}$ (II), uncut pJP101$^{0.5Sau3AI}$ (III), cut pJP101$^{0.25Sau3AI}$ (IV), uncut pJP101$^{0.25Sau3AI}$ (V), cut pJP101$^{0.125Sau3AI}$ (VI), uncut pJP101$^{0.125Sau3AI}$ (VII), measurements to calculate molecular weights where taken from the checkered line. Molecular weights where compared to those produced by λ/ *Hind*III marker. Regions of interests (bands other than p*HSG*398) where outlined.

6. Discussion

The results from the partial digestion showed that p*JP*101 had been fragmented. In each gel the largest band (~20 kb) this band was uncut p*JP*101, which has a molecular weight of 19 kb [22]. Every digest sample gave bands that where large enough to contain the violacein gene, these bands where ≥9 kb. Sample p*JP*101$^{0.5Sau3A}$I also gave bands between 8 kb and 9 kb.

6.1. Digestion

The results of the serial digestion of p*JP*101 (Figure 6) had given fragments that could possibly contain violacein [11]. These fragments produced where smaller than the one originally inserted into p*JP*101, which was 14 kb, meaning we had not simply re-extracted that sequence [22].

The data from the plating analysis (Table 1) shows that even though all the p*JP*101 had contains the violacein sequence, not all colonies would be purple. This result had also been show in other research [25]. The low rates of expression of the violacein where mostly due to insertions containing only some, or even none of the biosynthetic pathway. What was highlighted was that the rates of production may be reduced as violacein production is low and unstable in E.*coli* [26]. This indicates that identifiable clones would occur in small numbers, as not all clones containing the violacein genes would give a positive result. Each of the ligation samples showed clones expressing the violacein gene in small concentrations (between 3-5 cfu/mL).

The re-digested samples (Figure 7) gave two bands, the smaller of the two bands was 2.2 kbp, this band was the cut vector, as it was the same size and the p*HSG*398 band digested with *Bam*HI. As an outcome, the smallest functional violacein fragment obtained from re-digestion was 9.5 kbp. This fragment is larger than the complete violacein sequence [11].

6.2. Stability

This study has shown that it is possible to further isolate the violacein sequence using *Sau3*AI, and express it in a clone organism using similar techniques used to create a genome library. A cautionary note is made, that is there where technical problems with this experiment, mainly centered with the production rates and stability of violacein.

Both wild types and clones of violacein have low, unstable production rates. These problems occur because non-violacein producing variants occur spontaneously. This usually happens at a higher frequency when hyper-producing violacein colonies are replanted [26].

Recent studies have showed it is possible to produce high levels of stable violacein in E.*coli*. This may be done by combining the genetic material from stable lines of E.*coli* that produce low levels of violacein with strains that over produce violacein, but are unstable. The resulting strains of E.*coli* could produce high levels of violacein stably for multiple generations [25].

6.3. DNA Logic gates

The connection to DNA biological logic gate is made via an experiment that had the aim of trying and extracts the smallest possible fragment from Plasmid *JP*101 created by Pemeberton and colleagues (1991) which contained the violacein genes [22].

Both the instability and stability states could be used as 'switches' as part of a DNA (biorheological) logic gate block(s) as suggested in Figure 3. With an increase production rates this may be an enabler to assist organ additive fabrication or biofabrication of DNA (biorheological) logic gates. Organ additive fabrication or biofabrication has provided the opportunity for rheology to play a key role in developing polymer scaffolds to light medical biorheological devices.

This may additionally suggest biorheology logic gates reprogramming (encoding-decoding) vaccine pathways. Such an approach may enable the development of biorheological creams, gels, glues, slims or foams that could be biofabricated as programmed logic controllers through to artificial wisdom intelligent based systems to overcome various related patient medical conditions [27].

7. Conclusions

The hypothesis has been validated as plausible (*extraction of the violacein sequence from pJP*101 *for expression and analysis in pHSG*398 *determine the smallest DNA fragment responsible for purple pigmentation*). It may also be possible to regulate and increase the production of violacein, via the use of quorum sensing.

Quorum sensing is where small diffusible signaling molecules are used as activators and it is used in both intra- and extra cellular communication [28]. It has been suggested that introduction of certain quorum sensing particles can increase the production rates of violacein [29].

This experiment showed it is possible to:

- Further isolate the genes responsible for producing violacein from p*JP*101, and has hinted that it may be possible to increase production rates via genetic manipulation and introduction of quorum sensing particles, and
- Use the instability and stability states as 'switches' as part of a DNA (biorheological) logic gate block(s) for gene reprogramming. Logic gates (input to output logic operations) are a growing research field to enable biological devices using scalable DNA combinational logic gates. These devices tend to have biochemistry drivers to distinguish devices within a circuit [30].

8. Future research

Future research is proposed in using this papers conclusion. The focus is on Organ additive fabrication or biofabrication. This provides the opportunity for a hybrid biology and rheology to play a key role in developing polymer scaffolds to light medical biorheological devices.

The developed biorheological material and devices might not only be used in biofabrication but might take the next step as a countermeasure or intervention. As an example, by developing biorheological creams, gels, glues, slims or foams that could be biofabricated as programmed logic controllers through to artificial wisdom intelligent based systems to overcome various related patient medical conditions.

It may also be possible to further control the expression of engineer microbes, using DNA logic gates which are activated by qurom sensing particles and/or proteins, to express antimicrobials as a means of treating resistant microbes [4], and as a means to have greater control over expression in a constantly fluctuating cellular environment [31].

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References

[1] Morales, J.C. Patton, J.C. and Bickham, J.W. 'Partial endonuclease digestion mapping of restrction sites using PCR-amplified DNA', *Genome Research*, vol. 2, (1993), pp. 228-233.

[2] Stubbs, S. L.J. Brazier, J. S. Talbot P. R. and Duerden, B. I. 'PCR-Restriction Fragment Length Polymorphism Analysis for identification of bacteroides spp. and Characterization of Nitroimidazole Resitance Genes', *Journal of Clinical Microbiology*, vol. 38, no. 9, September, (2000), pp. 3209-3213.

[3] Neu, H.C. 'The crisis in antibiotic resistance', *Science*, vol. 257, no. 5073, August, (1992), pp. 1064-1073.

[4] Lee, J. Kim, J. Nahm, C. Choi, J. J, K. Pai, S. Moon,. K. K, L and Chong, Y. 'Two case studies of Chromobacterium violaceum infection after injury in a subtropical region', *Journal of Clinical Microbiology*, vol. 37, no. 6, June, (1999), pp. 2068-2070.

[5] Matz, C. Deines, P. Boenigk, J. Arndt, H. Eberl, L. Kjelleberg, S. and Jurgens, K. 'Impact of violacein-producing bacteria on survival and feeding of bacterivorous nanoflagellates', *Applied and Environmental Microbiology*, vol. 70, no. 3, March, (2004), pp. 1593-1599.

[6] Lopes, S.C.P. Blanco, Y.C. Justo, G.Z. Nogueira, P.A. Rodrigues, F.L.S. Goelenitz, U. Wunderlich, G. Facchini, G. Brocchi, M. Duran, N. and Costa, F.T.M. 'Violacein extracted from Chromobacterium violaceum inhibits Plasmodium growth In Vitro and In Vivo', *Antinicrobial Agents and Chemotherapy*, vol. 53, no. 5, March, (2009), pp. 2419-2512.

[7] Andrighetti-Frohner C., Antonio, R. Creczynski-Pasa, T. Barardi, C. and Simoes, C. 'Cytotoxicity and potential antiviral evaluation of violacein produced by chromobacterium violaceum', *Mem Inst Oswaldo Cruz*, vol. 98, no. 6, September, (2003), pp. 843-848.

[8] Ferreira, C.V. Bos, C.L. Versteeg, H.H. Justo, G.Z. Duran, N. and Peppelenbosch, M.P. 'Molecular mechanism of violacein-mediated human leukemia cell death', *Blood*, vol. 104, no. 5, May, (2004); pp. 1459-1464.

[9] Clardy, J. Fischbach M. A. and Walsh, C. T. 'New antibiotics from bacterial natural products', Nature Biotechnology, vol. 24, no. 12, December, (2006), pp. 1541-1550.

[10] Saeidi, N. Wong, C.K. Lo, T. M. Nguyen, H.X. Ling, H. Leong, S.S.J. Poh, C.L. and Chang, M.W. 'Engineering microbes to sense and eradicate Pseudomonas aeruginosa, a human pathogen', *Molecular Systems Biology*, vol. 7, June, (2011), pp. 1-11.

[11] Pingoud, A. Fuxreiter, M. Pingoud, V. and Wende, W. 'Type II restriction endonucleases: structure and mechanism', *CMLS Cell and molecular life sciences*, vol. 62, (2005), pp. 685-707.

[12] Lu, J. Perng, C. Lee, S. and Wan, C. 'Use of PCR with Universal primers and restriction Endonuclease Digestions for Detection and Idebtification of Common Bacterial Pathogens in Cerebrospinal Fluid', Journal of Clinical Microbiology, vol. 38, no. 6, June, (2000), pp. 2076-2080.

[13] Russell, P.J, '*i*Genetics, third edition', A molecular approach (2010).

[14] Wikipedia, 'Polymerase chain reaction', Wikipedia, the free encyclopedia, en.wikipedia.org/wiki/Polymerase_chain_reaction, (2013). p. 1.

[15] August, P.R. Grossman, T.H. Minor, C. Draper, M.P. MacNeil, I.A. Pemberton, J.M. Call, K.M. Holt, D. and M.S., O. 'Sequence analysis and functional characterization of the violacein biosynthetic pathway from chromobacterium violaceum', *Journal of molecular microbiology and biotechnology*, vol. 2, no. 4, October, (2004). pp. 513-519.

[16] Asamizu, S. Kato, Y. Tgarashi, Y. and Onaka, H. 'VioE, a prodeoxyviolacein synthase invilved in violacein biosynthesis, is responsible for intremolecular indole rearrangement', *Tetrahedron letters*, vol. 48, no. 16, April, (2007); pp. 2923-2926.

[17] Sanchez, C. Brana, A.F. Mendez, C. and Salas, J.A. 'Reevalutation of the violacein biosynthetic pathway and its relationship yo indolocarbazole biosynthesis', *CHEMBIOCHEM*, vol. 7, (2006), pp. 1231-1240.

[18] Duran, N. and Menck, C.F.M. 'Chromobacterium violaceum: A review of pharmacological and Industrial Perspectives', *Critical Reviews in Microbiology*, vol. 27, no. 3, (2001) ; pp. 201-222.

[19] OOM&C; *Melbourne researchers make HIV wake up call*; Office of Marketing and Communications OOM&C; .Monash University; http://www.monash.edu.au/news/show/melbourne-researchers-make-hiv-wake-up-call; 4 March 2013; p. 1.

[20] Als; A. 'Logic Gates', *Uni. of West Indies*, www.scitec.uwichill.edu.bb, (1999), p.1.
[21] Answers; 'Boolean circuit'; *Computer Desktop Encyclopedia;* http://www.answers.com/topic/boolean-circuit; (2012), p. 1.
[22] Pemeberton, J.M. Penfold, K.M. Penfold, R.J. and Penfold, V.J. 'Cloning and heterologous expression of the violacein biosynthesis gene cluster from chromobacterium violaceum', *Current Microbiology*, vol. 22, (1991), pp. 355-358.
[23] Takeshita, S. Sato, M. Toba, M. Masahashi, W. and Hashimoto-Gotoh T., 'High copy number and low copy number plasmid vectors for lacZa complementation and chloamphenicol or kanamycin resitance selection', *Gene*, vol. 61, no. 1, (1987), pp. 63-74.
[24] Shepherd, N.S. Pfrogner, B.D. Coulby, J.N. Ackerman, G.V. Sauer, R.H. Balkenhol, T.C. and Sternberg, N. 'Preparation and screening of an arrayed human genomic library generated with P1 cloning system', *Proceedings of the National Academy of Sciences of the USA*, vol. 91, no. 7, March, (1994), pp. 2629-2633.
[25] Ahmetgic, A. and Pemeberton, J.M. 'Stable high level expression of the vioacein indolocarbazole anti tumor cluster and the Streptomyces lividans amyA gene in E.coli K12', *Plasmid*, vol. 63, December, (2012); pp. 79-85.
[26] Jiang, P.-x. Wnag, H.-s. Zhang, C. Lou, K. and Xing, X.-H. 'Reconstruction of the violacein biosynthetic pathway from Dunganella sp.B2 in different heterologous hosts', *Applied Microbiology and Biotechnology*, vol. 86, no. 4, (2012), pp. 1077-1088.
[27] Kittleson, J. T. Cheung S. and Anderson, J C. 'Rapid optimization of gene dosage in E. coli using DIAL strains; *Journal of Biological Engineering 2011*, 5:10 doi:10.1186/1754-1611-5-10, http://www.jbioleng.org/content/5/1/10, (2011); p. 1.
[28] Mcclean, K.H. Winson, M.K. Fish, L. Taylor, A. Chhabra, S.R. Camara, M. Daykin, M. Lamb, J.H. Swift, S. Bycotf, B.W. Stwart, G.S.A.B. and Williams, P. 'Quorum sensing and Chromobacterium vilaceum: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones', *Microbiology*, vol. 143, (1997), pp. 3703-3711.
[29] Blosser, R.S. and Gray K.M., 'Extraction of the violacein from Chromobacterium violaceum provides a new quantitative bioassay for N-acyl homoserine lactobe autoinducers', *Journal of Microbiological Methods*, vol. 40, no. 1, March, (2000), pp. 47-55.
[30] Anderson, J. C. 'Combinational Logic Design in Synthetic Biology', *Department of Bioengineering University of California*, Berkeley www.ddensmore.net/dza/wp-content/uploads/2009/05/iscas_ieee.pdf, (2009), p. 1.
[31] Dari, A. Kia, B. Bulsara, A.R. and Ditto, W. 'Creating morphable logic gates using logical stochastic resonance in an engineered gene network', EPL (Europhysics Letters), vol. 93, no. 1, January, (2011), 18001