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An extraction of the violacein sequence from pJP101 for expression and analysis in pHSG398

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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

analysis, expression, pjp101, extraction, sequence, phsg398, violacein

Disciplines

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An extraction of the violacein sequence from pJP101 for expression and analysis in pHSG398

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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 pJP101 for expression and analysis in pHSG398 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; *vioA* (1,257 bp), *vioB* (2,997 bp), *vioC* (1,290 bp), *vioD* (1,122 bp) [15] and *vioE*, 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 *vioA* removes an amine group from the terminal end of one L-tryptophan and replacing it with a ketone,
- On another L-tryptophan, *vioD* adds an alcohol group to the hexane ring, and then *vioE* causes a 1-2 shift with the carboxylic acid and amine group,
- vioB* combines the two L-tryptophan to create a violacein intermediate, an in the process removes carboxylic groups from each terminal end, and
- vioC* 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.

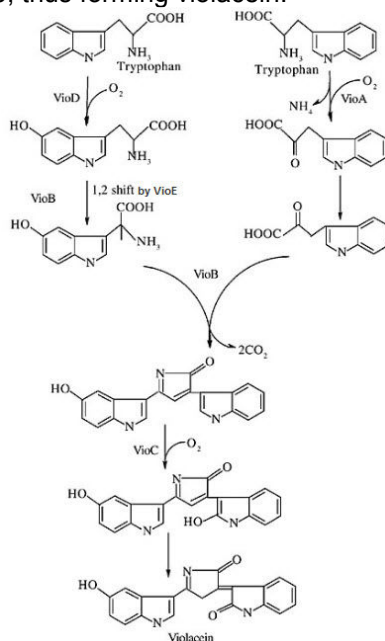


Figure 2. Biosynthetic pathway for the production of violacein in *C. violaceum*, showing products, reagents and gene products used, also shows changes in structure of violacein as the reaction [18].

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.

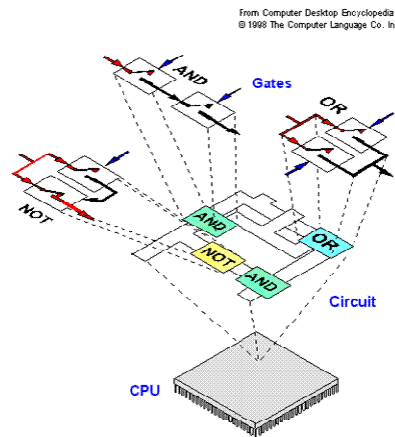


Figure 3. logic gates within a 'Central processing Unit' (CPU) hierarchy (logic device) [21].

3. Experimentation

The aim of this experiment was to try and extract the smallest possible fragment from pJP101 which contained the violacein genes.

3.1. Fragmented pJP101 context

Plasmid JP101 was the created by Pemeberton and colleauges (1991) [22], it was the smallest plasmid created expressing violacein in the study. Plasmid JP101 (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 pJP100, which contained a 30 kb insert.

A smaller insert was obtained by partially digesting pJP100 with *Sau*3AI and ligating it into pBR332 which had been digested with *Bam*HI. This clone was named pJP101, the DNA fragment containing the violacein genes in pJP101 was 14.5 kb.

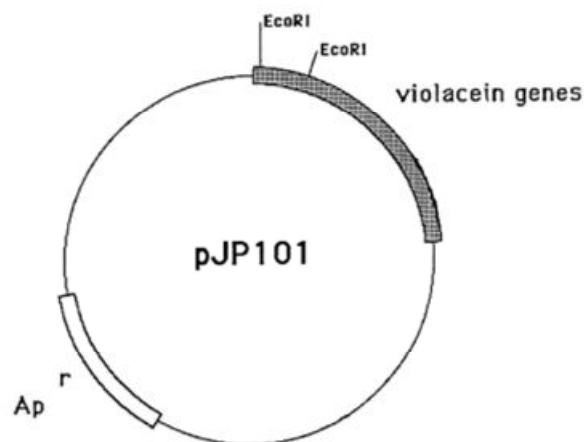


Figure 4. Diagram of pJP101 showing ampicillin resistance (Ap^r) and violacein genes. The diagram also shows *EcoRI* sites. Plasmid JP101 also has multiple *Sau3A*I sites scattered throughout its genome (not shown) [22].

3.2. Vector pHSG398 context

Plasmid *HSG398* was used as the vector for the violacein genes (Figure 5). Plasmid *HSG398* was selected as it has a different antibiotic resistance than that of pJP101, to allow for antibiotic screening. Plasmid *HSG398* was also selected for this experiment as it only has one *Bam*HI restriction site in its multi cloning region [23].

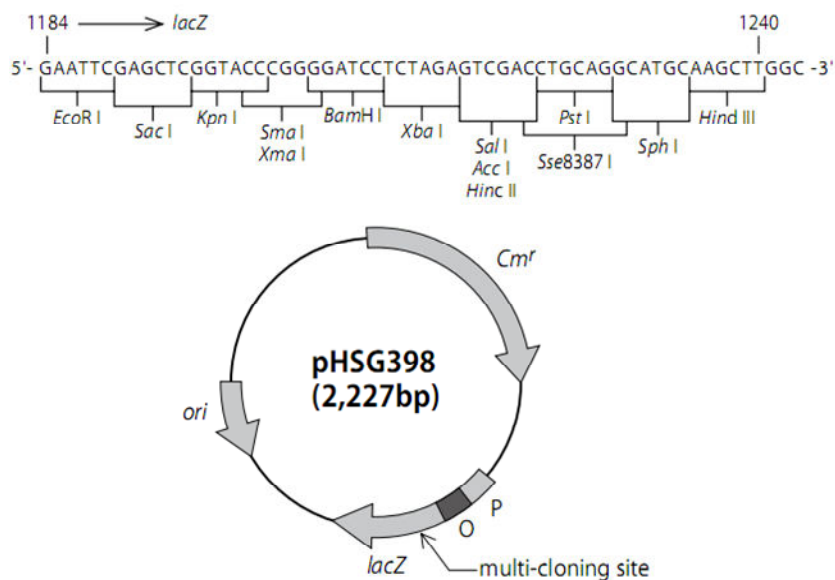


Figure 5. Plasmid map of pHSG398, showing; plasmid size, origin of replication, antibiotic resistance (Cm^r), and *LacZ* region. Shown inside the *LacZ* region is pHSG398 multiple cloning sites, shown in greater detail above plasmid map [23].

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 pHSG398) for size and sequence analysis [1].

These results can then be compiled 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 pJP101, and inserted into pHSG398. In this experiment it was expected that the clones that expressed violacein, when digestion would give two fragments. One fragment from the digestion would pHSG398, 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 pJP101 digested using serially diluted *Sau*3AI (1:1) for digestion, starting with a 60 µL master mixture containing; 40 µL of pJP101, 10 µL buffer, 10 units of *Sau*3AI. 30 µL of the master solution was added to a 30 µL solution containing; 20 µL of pJP101, 1x buffer, giving dilution factor 0.5.

This dilution was performed 5 times. Samples were 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 were selected for ligation.

4.2. Ligation of Vector into target plasmid

The vector fragment ligation (pHSG398) was digested for 1 hour at 37 °C in; 10 units of *Bam*HI, 1x restriction buffer and 10 µL pHSG398. Samples were heated at 70 °C for 10 minutes. Digested pHSG398 and the serial digests of pJP101 were 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, pJP101, pHSG398 and TE buffer were added to separate 200 µL of JM109 cells, sample were 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 were added to agar plates with either Ampicillin_{100µg/mL} to check for contamination by pJP101 or Kanamycin_{100µg/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 were re-suspended in a 200 µL solution; 50 mM glucose, 25 mM TrisHCl and 10 mM EDTA. 200 µL of a lysis 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 H₂O. Isolated plasmids had undigested and samples digested with *Bam*HI electrophoresed for analysis.

5. Results

After the dilution digests with *Sau*3AI on pJP101 was performed, the digests were run on an electrophoresis gel to see if there were any digests that produced fragments large enough to contain the whole violacein sequence (fragments ≥ 8kb).

When the serial enzyme dilution digests were 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 samples from this digest mainly show bands 9 kb or larger (Figure 6). These samples could then be ligated with pHSG398 digested using *Bam*HI.

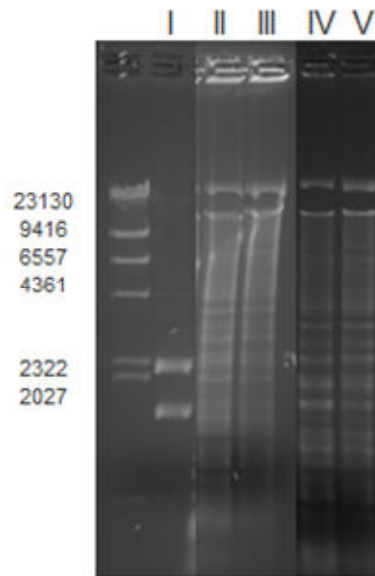


Figure 6. The partial digests of pHSG398 with *Sau3AI*. The contents of each gel lane are; pHSG398 digested with *Bam*HI (I), pJP101 digested with 0.500 units of *Sau3AI* (II), pJP101 digested with 0.250 units of *Sau3AI* (III), pJP101 digested with 0.125 units of *Sau3AI* (IV), pJP101 digested with 0.063 units of *Sau3AI* (V).

Following the ligation of genetic material into pHSG398 and the uptake recombinants by JM109 cells, the clones were plated along with control cultures (Table 1). There was no unexpected growth on the Ampicillin_{100µg/mL} control plates. Clones expressing violacein were found in all Kanamycin_{100µg/mL} plates with 400 µL of culture. Sample pJP101^{0.5*Sau3AI*} 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 _{100µg/mL}	Plate (cfu/mL) Kamermycin _{100µg/mL}
pJP101	100 µL	Purple	80	0
		White	330	0
pHSG398	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 pHSG398 and another between 9416 and 23130 bp. Cut ligation samples I and III had the smallest inserts from pJP101. 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).

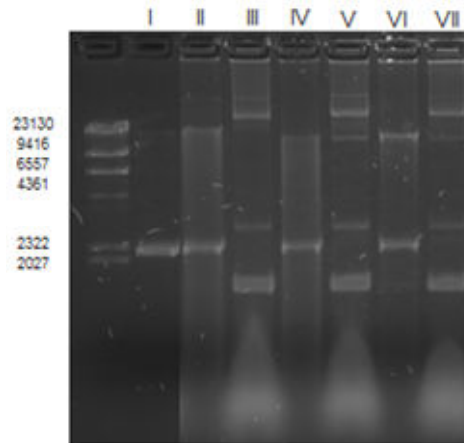


Figure 7. The re-digests of the successful ligations where digested with *HindIII*, gel contents are; Digested pHSG398 (I), cut pJP101^{0.5Sau3AI} (II), uncut pJP101^{0.5Sau3AI} (III), cut pJP101^{0.25Sau3AI} (IV), uncut pJP101^{0.25Sau3AI} (V), cut pJP101^{0.125Sau3AI} (VI), uncut pJP101^{0.125Sau3AI}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

6. Discussion

The results from the partial digestion showed that pJP101 had been fragmented. In each gel the largest band (~20 kb) this band was uncut pJP101, 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 pJP101^{0.5Sau3AI} also gave bands between 8 kb and 9 kb.

6.1. Digestion

The results of the serial digestion of pJP101 (Figure 6) had given fragments that could possibly contain violacein [11]. These fragments produced where smaller than the one originally inserted into pJP101, 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 pJP101 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 pHSG398 band digested with *BamHI*. 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 *Sau3AI*, 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 JP101 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 pJP101 for expression and analysis in pHSG398 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 pJP101, 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 quorum 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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