International Journal of Microbiology and Biotechnology
2020; 5(1): 16-21
http://www.sciencepublishinggroup.com/j/ijmb
doi: 10.11648/j.ijmb.20200501.13
ISSN: 2578-9678 (Print); ISSN: 2578-9686 (Online)

Reliability of *in silico* Modeling Based on Restriction Patterns for the Study of the Carotenogenic Gene *pds* of *Haematococcus* sp

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To cite this article:
Bryan Pillacela, Janeth Galarza, Carolina Tufiño. Reliability of *In silico* Modeling Based on Restriction Patterns for the Study of the Carotenogenic Gene *pds* of *Haematococcus* sp. *International Journal of Microbiology and Biotechnology*. Vol. 5, No. 1, 2020, pp. 16-21.
doi: 10.11648/j.ijmb.20200501.13

Received: December 22, 2019; **Accepted**: January 17, 2020; **Published**: January 31, 2020

Abstract: Phytoeno desaturase (*pds* gene) is an enzyme involved in the biosynthesis of carotenoids such as β-carotene and astaxanthin in microalgae and some autotrophic organisms. Carotenoids have antioxidant, anti-inflammatory and anti-carcinogenic properties. Prior to biotechnological studies it is necessary to carry out the integral analysis of this carotenogenic gene and its molecules for future industrial applications. *In silico* modeling is an important bioinformatics tool that allows to adjust, summarize and organize experimental information, test theories and generally understand the interaction of molecules and biological systems. In this work, specific primers were used to amplify the phytoene desaturase (*pds*) gene of *Haematococcus* sp. (PM015). NEBcutter V2.0 and BioEdit provided the simulation design of the *pds* restriction patterns together with the restriction enzymes *FsPI*, *AlwI*, *HincII*, *BlpI* and *MluCI*. The amplification product resulted in two fragments one of 1200 bp and one of 1700 bp. The *in silico* design was reproduced and tested experimentally, in this way the identification of the *pds* gene of *Haematococcus* sp. (PM015) and the effectiveness of bioinformatics programs as an important alternative for *in silico* modeling of molecules and/or biological systems, which mapping predict reliable results, saving time and reducing costs in the experimental stage, in addition to allowing the selection of restriction enzymes with optimal enzymatic activity to digest DNA.

Keywords: *In silico* Model, Phytoene Desaturase, Restriction Enzymes, Bioinformatics, *Haematococcus* sp

1. Introduction

The phytoene desaturase PDS (*pds* gene) is a key regulatory enzyme in carotenoid biosynthesis. In plants and algae, PDS together with cis-carotene desaturase (ZDS) catalyze the desaturation of colorless carotenoids to colored carotenoids [1-3]. Carotenoids are a large group of natural pigments that range from yellow to red and have antioxidant, anti-inflammatory and anti-cancer properties, which in terms of applications are very attractive in the food, human and animal health industry [4, 5].

The expression of the *pds* gene in the biosynthesis of β-carotene and astaxanthin has been very well studied and characterized in different green microalgae such as *Dunaliella salina* [6], *Chlorella zofingiensis* [7], *Chlamydomonas reinhardtii* [8] and *Haematococcus pluvialis* (*H. pluvialis*) [9-11]. The activation of the *pds* gene depends on the conditions the microalgae were cultured. For example, it has been proved that *H. pluvialis* has a great capacity to produce the astaxanthin pigment at different stress conditions [12-14]. This reason, along with its ability of being grown on an industrial scale have made *H. pluvialis* one of the most important microalgae in the world [15-17].

The genes *pds, psy, bkt, CrtR, crtO* among others, are involved in the biosynthesis of astaxanthin in *Haematococcus pluvialis*, however, the phytoene desaturase gene (*pds*) is key for the passage of colorless carotenoids to colored carotenoids [7, 18, 3]. Based on this importance, our interest in the *pds* gene is to compare the effectiveness of *in silico* models versus experimental trials, in addition it suggests working with a

specific gene for genetic and / or molecular modeling.

As stated previously, one of the potential genes for the study of astaxanthin is the *pds* gene whose can be analyzed by using molecular markers or restriction enzyme. The traditional method to find these enzymes were trial and error laboratory tests, that involve more work time and additional costs [19]. At present, there are bioinformatic programs that allow to develop designs of restriction patterns *in silico*, in this way, the researcher is able to determine the number of cuts, the size of the fragments and the relative position of each enzyme before working on the laboratory [20-22]. Furthermore, the designs *in silico* can be friendly tools that allow to manipulate many variables in the DNA sequence and thus plan experiments, test hypotheses and evaluate results, optimizing time and costs [23, 10, 24].

In silico designs have been used in several publications with different application including the molecular identification of *Anopheles* spp. [25], evaluation of functions of the BAR gene in *Nicotiana benthamiana* [24] and the identification of different *Candida* species [26].

There are several programs for the analysis of restriction sites, some of the most recent include CisSERS [27], Restriction Digest [28, 29], among others. However, NEBcutter 2.0 is one of the most widespread tools and provides useful functionalities from cloning analysis to gel predictions based on different types of gels. NEBcutter is an online program (http://nc2.neb.com/NEBcutter2/) that uses the information of restriction enzymes available at NEB (New England Biolabs Inc.), in addition to REBASE recognition sites (www.neb.com / rebase) [30, 31]. Another widely used program is BioEdit Sequence Alignment Editor, which has several useful features, functions and tools that allow automatic integration with ClustalW programs and the BLAST platform, among others [32, 33].

Thus, the aim of this study was to evaluate experimentally the effectiveness of *in silico* designs, through the modeling of restriction patterns for the identification of the *pds* gene of *Haematococcus* sp., (PM015). This is done with consideration to the importance of bioinformatics application in molecular biological studies.

2. Materials and Methods

2.1. Cultivation Conditions of Haematococcus sp

The strain of *Haematococcus* sp., registered with code PM015, was isolated from the Santa Elena Peninsula (1°49'27.8" S 80°41'16.0" W) and grown in 200 mL of Bold 3N Basal Medium (BBM 3N). This medium was prepared following a previous work [11]. The cultivation conditions were maintained at 25 ± 0.5°C, with photoperiod 12/12 hours (light/dark), pH 7 and manual aeration twice a day. The growth was evaluated based on the cell concentration increasing of the culture (cell mL^{-1}). Daily cell counts were performed in an OMAX® brand optical microscope using a 0.1 mm deep Neubauer camera. The cultures were maintained in triplicate for eight days [34]. The biomass was obtained by centrifugation at 10000 rpm.

2.2. Obtaining the In silico Model

The sequence of the *pds* gene used for this study corresponds to the access number in GenBank X86783.1. This sequence was run in the BioEdit program to obtain the virtual restriction map and to select the enzymes. The enzymes were selected considering those that cut up to twice the *pds* gene [8]. Subsequently, the *in silico* model of the restriction patterns was obtained by running the NEBcutter 2.0 program.

2.3. RT-PCR Reverse Transcriptase

Experiments of reverse transcriptase PCR were performed with the total RNA extracted from the cultures of *Haematococcus* sp. using TRIzol reagent (Invitrogen™) according to the instructions provided by the manufacture. The first cDNA chain was synthesized from 2 µg of DNase treated RNA using the M-MLV reverse transcriptase enzyme and random primers according to the manufacturer's instructions (Promega, Madison, WI, USA). We used 1 µL of cDNA on a quencher in a final PCR of 10 µL. Primers PM005.F and PM006.R were design based on the sequence of GenBank No. X86783.1. Their sequences are 5´-CCGCCAAGCCTTTGCGCGTTGTCATT-3´ and 5´-CAGAATGCTCGCCCATATAGTGCCTGC-3´, respectively. We else contested our methodology with other primers, F. 5'-ATCAACTCTGACAATGCAGACAACA-3'and R. 5'-GGTGCCATTCTTTCATCACTTACA-3' [35]. The mixing of PCR and the amplification program for primers PM005.F/PM006.R and F/R was carried out according to previous protocol [11, 35]. The PCR fragments were separated on a 1% agarose gel stained with 0.5X GelRed (Biotium) and purified by Gel Purified Kit (Invitrogen™), following the instructions of manufacturer.

2.4. Experimental Restriction Pattern

The PCR product amplified with primers PM005. F/PM006.R was digested by mapping with restriction enzymes such as *FspI* and *AlwI*. On the other hand, the PCR product with primers designed by [35] (F/R) was digested with the enzymes *HincII, BlpI, MluCI*. All enzymes used in this study were produced to Thermo Fisher Scientific Inc, USA. Digestion was carried out following the instructions of manufacturer from 154 ng/µL of cDNA with 1U/enzyme in a final volume of 50 µL. The sample was incubated at 37°C for 4 hours, followed by an inactivation at 65°C for 15 minutes. The products were separated on a 1% agarose gel and were subsequently visualized by UV illumination after 0.5 X GelRed (Biotium) staining.

3. Results

3.1. Cellular Concentration of Haematococcus sp. (PM015)

The cultures exhibited a stationary growth phase during

the first three days with a cell concentration of 38 x 10^4 cell mL^{-1}. Then, the growth was followed by an exponential logarithmic phase that reached the highest cell concentration of 93 × 10^4 cell mL^{-1} on the seventh day, while the phase of plateau was observed during the eighth day when the cell concentration decreased to 92 × 10^4 cell mL^{-1} as shown (Figure 1). The relative number of mobile vegetative cells during growth was high (98%).

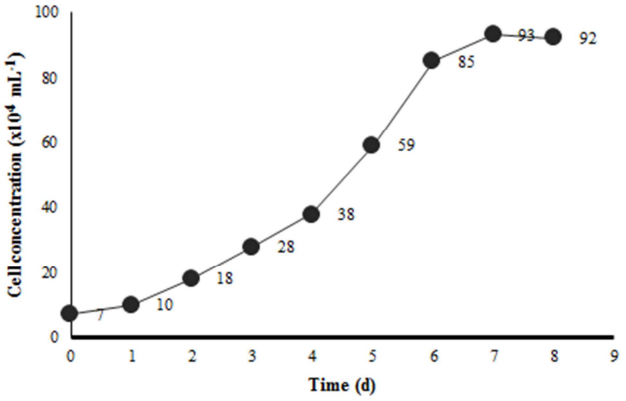

Figure 1. *Growth curve of Haematococcus sp. (PM015) during eight days of cultivation.*

3.2. RT-PCR Amplification of the pds Gene with Specific Primers

Polymerase chain reaction with reverse transcriptase (RT-PCR), using the specific primers (PM005.F/PM006.R) for the *pds* gene revealed a product of 1200 bp. In contrast, the primers (F/R) revealed a product of 1700 bp (Figure 2). These results indicate the presence of the *pds* gene in the sample under study.

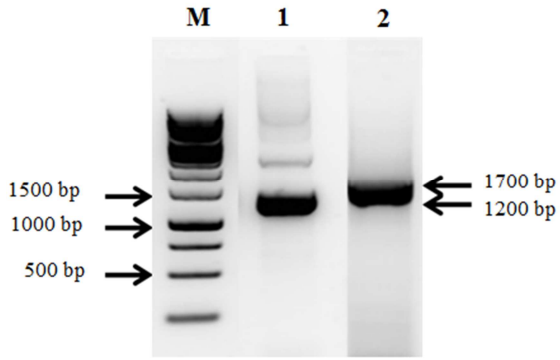

Figure 2. *Amplification of the pds gene. 1: PCR product amplified with specific primers; 2: amplified PCR product with other primers [35]. M: 1Kb molecular weight marker.*

3.3. Validation of Restriction Patterns Modeled in Silico

The *in silico* modeling of the restriction patterns for the *pds* gene with the 1200 bp amplified product was determined with the BioEdit and NEBcutter 2.0 programs, followed by the experimental assay with the *EspI* and *AlwI* enzymes. The *in silico* pattern of the *FspI* enzyme revealed three fragments: one of 500 bp and two of 350 bp that overlapped. On the other hands, the *AlwI* enzyme fragments of 700, 400 and 100 bp (Figure 3). The fragment sizes obtained from the NEBcutter software and those observed experimentally (Figure 4) showed congruence in the results. The only difference found was the lack of the smallest fragment that was difficult to discern in agarose gel. Although the 100 bp band was not observed in the gel, the results confirm that the gene being amplified is *pds*.

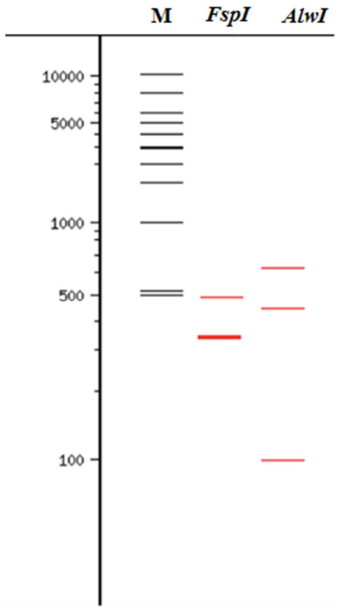

Figure 3. *In silico modeling of the restriction pattern using the 1200 bp RT-PCR product digested with the FspI and AlwI enzymes.*

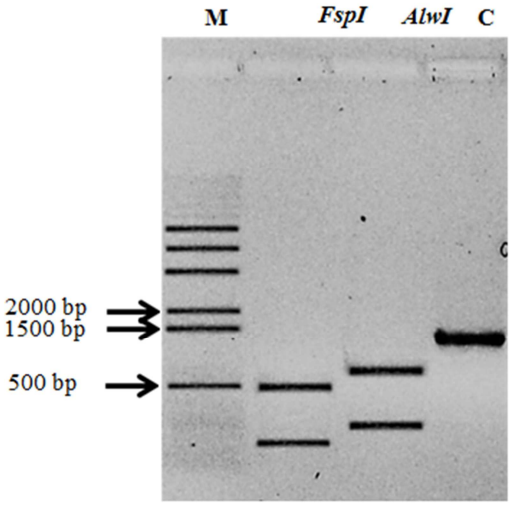

Figure 4. *Experimental digestion. FspI lane: fragments of 500 bp and two of 350 bp; AlwI lane: fragments of 700 bp and 400 bp; lane C: undigested amplicon.*

The *in silico* modeling of the 1700 bp RT-PCR product revealed fragments of 220 bp and 1480 bp digested with the enzyme *HincII*; 600 bp and 1100 bp with *BlpI* and the enzyme *MluCI* fragments of 950 bp and 750 bp (Figure 5). This restriction pattern *in silico* was confirmed experimentally, obtaining the expected fragments (Figure 6).

In the same way as in the previous design, there is only one restriction site for each of the enzymes and the smallest band (220 bp) was not observed in the gel. The results show again the *pds* gene and that the restriction patterns designed *in silico* are effective in identifying a gene of interest.

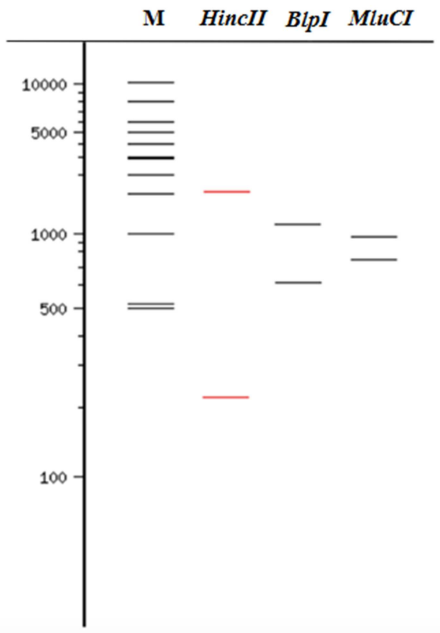

Figure 5. *A: In silico modeling of the restriction pattern using the 1700 bp RT-PCR product digested with the enzymes HincII, BlpI and MluCI.*

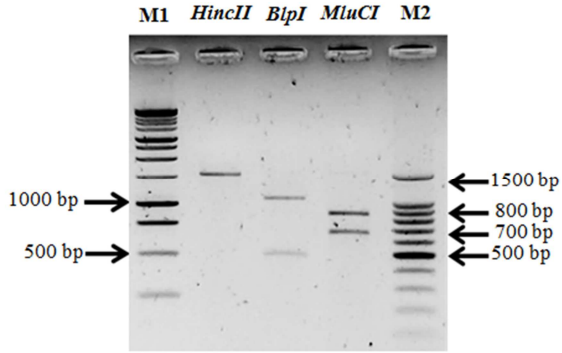

Figure 6. *Experimental digestion. HincII: 1480 bp fragment; BlpI: 600 bp and 1100 bp; MluCI: 950 bp and 750 bp. Lane M1: 1Kb molecular weight marker; M2: 100 bp molecular weight marker.*

4. Discussion

H. pluvialis cultivation for astaxanthin production include a two stages method. The first one establishes the conditions for vegetative growth and the second, several stress conditions to stimulate the production of carotenoids [11, 36, 37]. Following this methodology, it has been described that *H. pluvialis* achieves the highest cell concentration between five and six days of culture [38-40]. We found that *Haematococcus* sp. (PM015) reached a concentration of 93 × 10^4 cells mL^{-1} at seven days of culture (Figure 1). However, for molecular studies it was determined that the microalgae should be harvested at six days 85 × 10^4 cell mL^{-1}, near the end of the exponential phase. In this phase the cell wall is less rigid, and green and translucent cells [13, 41, 42]. In this work, the amplification of the *pds* gene using the primers (F/R), delivered a 1700pb product [35]. This data reveals the first approach to the identification of the gene. Also, using the specific primers designed in this study (PM005. F/PM006.R) revealed a 1200pb product (Figure 2). In both cases, the size of the *pds* gene is in relation to previous studies, which ensures the presence of the *pds* gene in our microalgae [11, 21].

Restriction patterns *in silico* were modeled with BioEdit and NEBcutter 2.0 programs to give the restriction enzymes *FsPI, AlwI, HincII, BlpI* and *MluCI.* The enzymes were selected irrespective for the type (I, II or III) and the type of cut, as suggested by [30, 31]. In addition, NEBcutter 2.0 was able to show the fragments and their size produced by enzymes in a 1% agarose gel. This allowed us to easily contrast the experimental test to the same conditions.

The pattern of bands predicted *in silico* coincided with most of the bands observed experimentally (Figures 4 and 6). The only difference was the lack of the smallest fragment in each RT-PCR product (100 bp for the 1200 bp product and 220 bp for the 1700 bp product), this fragment was difficult to distinguish in the agarose gel (Figures 4 and 6). This difference could be due to the fact that the small size fragments correspond to the ends of the sequence, left by the restriction enzyme cut-off and are generally not observable [24, 43]. On the other hand, the fragments that come from an *in silico* model are superimposed and provide an easily distinguishable and reliable pattern [22, 44], as shown in Figures 3 and 5. The large size fragments were clearly distinguishable in the two experiments, which allowed validating the efficacy of the *in silico* generated patterns for the *pds* gene.

Although DNA sequencing is efficient in obtaining information on functional genomics and identification of genes involved in the metabolic pathway of carotenoids in *Haematococcus pluvialis* [40, 45-47]. The identification of the *pds* gene through *in silico* genetic mapping predicted reliable results, saving time and reducing costs in the experimental stage, in addition to allowing the selection of restriction enzymes with optimal enzymatic activity to digest DNA.

5. Conclusion

The *pds* gene, of the strain *Haematococcus* sp. (PM015), was amplified with primers F/R and PM005.F/PM006.R obtaining a product of 1700 bp and 1200 bp respectively, revealing the presence of the *pds* gene. Bioinformatics programs, particularly NEBcutter 2.0, proved to be an effective, reliable and didactic tool for predicting an *in silico* modeling of restriction patterns, which were validated experimentally, identifying the *pds* gene of *Haematococcus* sp., (PM015) by digestion with the restriction enzymes *FsPI, AlwI, HincII, BlpI* and *MluCI.*

Acknowledgements

This research was supported by Center for Advanced Studies in Arid Zones (CEAZA-Chile), and was funded by the projects INCYT-PNF-2017M3112 (Ecuador); UPSE-CUP: 91870000.0000.384095 (Ecuador), with the Ecuadorian Ministry of Environment permit: MAE – DNB – CM – CM – 2018–0099.

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