BIOTECH STUDIES

VOL 31 ISSUE 1 JUNE 2022 ISSN 2687-3761 E-ISSN 2757-5233

Published by Field Crops Central Research Institute, Ankara, TURKEY

TAGEM JOURNALS

Aims and Scope

"Biotech Studies" is the successor to the "Journal of Field Crops Central Research Institute" which has been published since 1992. The journal publishes articles on agro-biotechnology, plant biotechnology, biotechnology for biodiversity, food biotechnology, animal biotechnology, microbial biotechnology, environmental biotechnology, industrial biotechnology and bioprocess engineering, applied biotechnology, omics technologies, system biology, synthetic biology, nanobiotechnology and bioinformatics.

The journal of Biotech Studies has been published twice a year (June & December). Original research papers, critical review articles, short communications and scientific research of the journal are published in English. It is an international refereed journal.

The journal is aimed for researchers and academicians who work in or are interested in the topics of research in biotechnology.

Biotech Studies is indexed in ULAKBIM TR Index, CrossRef, and Google Scholar.

Further information for "***Biotech Studies***" is accessible on the address below indicated:

http://www.biotechstudies.org/

You can reach table of contents, abstracts, full text and instructions for authors from this home page.

Corresponding Address

Address : Field Crops Central Research Institute, (Tarla Bitkileri Merkez Araştırma Enstitüsü)
Şehit Cem Ersever Cad. No:9/11
Yenimahalle, Ankara, Turkey
Web : www.biotechstudies.org
E-mail : info@biotechstudies.org
Phone : + 90 312 327 09 01-2222
Fax : + 90 312 327 28 93

BIOTECH STUDIES

UN SDG Publishers Compact

As a Publisher, Biotech Studies is pleased to declare its commitment to the United Nations Sustainable Development Goals (UN SDGs) Publishers Compact. The purpose of this Compact is to accelerate progress to achieve SDGs by 2030. Signatories aspire to develop sustainable practices and act as champions of the SDGs during the Decade of Action (2020-2030), publishing books and journals that will help inform, develop, and inspire action in that direction. Furthermore, as a signatory of SDG Publishers Compact, Biotech Studies commit to:

1. **Committing to the SDGs**: Stating sustainability policies and targets on our website, including adherence to this Compact; incorporating SDGs and their targets as appropriate.
2. **Actively promoting and acquiring content** that advocates for themes represented by the SDGs, such as equality, sustainability, justice and safeguarding and strengthening the environment.
3. **Annually reporting on progress towards achieving SDGs**, sharing data and contribute to benchmarking activities, helping to share best practices and identify gaps that still need to be addressed.
4. **Nominating a person who will promote SDG progress,** acting as a point of contact and coordinating the SDG themes throughout the organization.
5. **Raising awareness and promoting the SDGs among staff** to increase awareness of SDG-related policies and goals and encouraging projects that will help achieve the SDGs by 2030.
6. **Raising awareness and promoting the SDGs among suppliers,** to advocate for SDGs and to collaborate on areas that need innovative actions and solutions.
7. **Becoming an advocate to customers and stakeholders** by promoting and actively communicating about the SDG agenda through marketing, websites, promotions, and projects.
8. **Collaborating across cities, countries, and continents** with other signatories and organizations to develop, localize and scale projects that will advance progress on the SDGs individually or through their Publishing Association.
9. **Dedicating budget and other resources towards accelerating progress** for SDG-dedicated projects and promoting SDG principles.
10. **Taking action on at least one SDG goal**, either as an individual publisher or through your national publishing association and sharing progress annually.

Besides these 10 action points, Biotech Studies endorses the SDGs, and explicitly concentrate on eight of 17 goals:

BIOTECH STUDIES

Editorial Information

BIOTECH STUDIES

Editorial Information

BIOTECH STUDIES

Volume 31 Issue 1 June 2022

CONTENTS

Biotech Studies 31(1), 1-9
https://doi.org/10.38042/biotechstudies.1062792

BIOTECH STUDIES

RESEARCH PAPER

Analysis of the carbon metabolism of *Rhodopseudomonas palustris* for biohydrogen production

Ezgi Melis Dogan-Guner, Harun Koku*

Department of Chemical Engineering, Faculty of Engineering, Middle East Technical University, 06800, Ankara, Turkey.

How to cite

Dogan-Guner, E. M., & Koku, H. (2022). Analysis of the carbon metabolism of *Rhodopseudomonas palustris* for biohydrogen production. *Biotech studies*, *31*(1), 1-9. https://doi.org/10.38042/biotechstudies.1062792

Article History
Received 24 August 2021
Accepted 15 December 2021
First Online 10 January 2022

Corresponding Author
Tel.: +90 312 210 2610
E-mail: harunk@metu.edu.tr

Keywords
R. palustris
Flux balance
Hydrogen production
Sucrose

Abstract

Hydrogen can be produced renewably and sustainably by the purple non-sulfur bacterium *Rhodopseudomonas palustris* from sucrose. To improve hydrogen production, detailed insight is needed, which can be obtained by studying the coupling of carbon fluxes with the light utilization apparatus and the hydrogen producing enzymes. In this study, the flux balance analysis approach was used to construct a model of the central carbon metabolism of this organism and solve the resulting network for a chosen objective function. The model was able to closely reproduce key qualitative and quantitative aspects of an independent experimental study. Further insight was obtained by additional case studies. Specifically, it was found that extreme light intensities resulted in the decrease of hydrogen production, that hydrogen production could be possible even when no light is provided, and a mix of sucrose and an organic acid could improve hydrogen production, which can be explained and supported by prior work on this organism. Further investigation is necessary to investigate the connections between metabolic network components, such the antagonistic relationship between hydrogen and polyhydroxybutyrate, which is a reserve product of this microorganism.

Introduction

Energy consumption worldwide has been rising and depends mostly on non-renewable fossil fuels. Hydrogen (H_2) is one of the prospective replacement alternatives for fossil fuels (Das & Veziroglu, 2001). It has a high per-mass energy content (122 kJ/g) and evolves only water in combustion, suggesting a clean, sustainable and environmentally friendly fuel for energy demand in the future (Argun et al., 2008; Kotay et al., 2008). However, in order for hydrogen to become a viable option, it has to be produced renewably.

Biological hydrogen production from microorganisms has the advantages of renewable substrates, low energy consumption and mild operating conditions (Azwar et al., 2013). Several families of microorganisms, such as the photosynthetic purple non-sulfur bacteria (PNSB), algae and cyanobacteria, produce hydrogen (Sparling et al., 2012). PNSB are promising candidates for biological hydrogen production (Basak et al., 2007) as they accomplish light-driven conversion of organic compounds to hydrogen with high efficiency and are metabolically versatile, which enables them to use a large variety of simple and complex feedstock including food and agricultural wastes. PNSB include species such as *Rhodobacter capsulatus, Rhodobacter sulfidophilus, Rhodobacter sphaeroides, Rhodopseudomonas palustris* and *Rhodospirillum rubrum*, all capable of producing hydrogen using energy from light and a source of organic carbon, such as sugars and short chain organic acids, which can be found in agricultural and industrial wastes (Eroglu et al., 2014).

Despite their versatility, enhancing the hydrogen productivities (rate per volume of culture) from PNSB remains a challenge due to their complex metabolism. Hence, a thorough understanding of the metabolism of these species plays a vital role in assessing this challenge and identifying the prospects for metabolic engineering to improve productivity. The present study is an analysis of the hydrogen-production metabolism of PNSB, using metabolic analysis.

In PNSB, hydrogen is produced via a light-driven metabolism utilizing the hydrogen-producing enzymes hydrogenase and nitrogenase in the absence of oxygen (Vignais et al., 1985). The overall hydrogen metabolism results from the interaction of several components along with the enzymes, as summarized in Figure 1, adapted from the study of Koku et al. (2002). Sucrose is assimilated by the sucrose pathway, glycolysis and the TCA cycle. The electrons that are extracted from the substrate are transferred to the electron carriers NAD (Nicotinamide adenine dinucleotide) and Fd (Ferredoxin) and in turn utilized by nitrogenase and hydrogenase in addition to other pathway blocks such as the Calvin cycle. Light is converted in the Photosynthetic unit (PSU) to ATP, which in turn is directed to nitrogenase along with the protons and electrons. Protons are supplied by the TCA cycle and ATP-synthase, the latter a part of the photosynthetic apparatus. Over-reduction due to excess electrons in the system is the main cause of hydrogen production (McEwan, 1994). Various strategies have been previously implemented, based on targeting of the primary systems of Figure 1, such as the overexpression of genes, elimination of competitive pathways and implementation of new hydrogen production pathways and engineering the genes of nitrogenase and hydrogenase (Ozturk et al., 2006). Oh et al. (2011) have summarized various metabolic engineering studies on PNSB for improved bio-hydrogen production.

While an overall schematic is conceptually useful, for a more thorough understanding of hydrogen metabolism, genome-based metabolic models are necessary (Hallenbeck & Liu, 2016). Metabolic flux analysis is such a computation-assisted method for this purpose. In this method, a single cell is treated as a system that exchanges mass with its surroundings, as well as carrying out internal (intracellular) reactions. The magnitudes of the calculated rates of the reactions, called *fluxes* are calculated by linear programming techniques and reveal detailed information on resource utilization and product turnover. The model, and the procedure employed in this study can thus provide insight into biological hydrogen production.

Several prior studies have investigated the metabolism of PNSB, with various goals and model sophistication. Klamt et al. (2002) identified metabolic constraints via a metabolic flux analysis applied to the central photogermentative metabolism of PNSB. Golomysova et al. (2010) applied FBA to the first comprehensive model of PNSB metabolism using *R. sphaeroides* where 314 metabolic reactions were accounted for, with 287 compounds involved. The computed metabolic fluxes were compared with those in previous experimental studies. Similarly, Imam et al. (2011) used a model with 796 metabolites and 1158 reactions of *R. sphaeroides*. Their results were close to experimental observations and the authors pointed out competing pathways as a cause of low yields of H_2 production. Navid et al. (2019) studied aspects of anaerobic photoheterotrophic growth of *R. palustris* on acetate with a genome-wide metabolic model and compared the allocation of resources for several cases.

The model species of the present study is *R. palustris* due to its good hydrogen yield on sucrose (Sagir et al., 2017, Zhang et al., 2015). Furthermore, the genome sequence of *R. palustris* has been published and available (Larimer et al., 2004). Based on this sequence it has been possible to distinguish several metabolic capabilities of *R. palustris*, not in common with other PNSB. For instance, *R. palustris* can harvest light of differing qualities and intensities. It carries out asymmetric cell division and secretes a surface adhesive allowing it to stick to solid substrates, an advantage for hydrogen production (Larimer et al., 2004). Sagir et al. (2017) experimentally observed *R. palustris* having the maximum hydrogen productivity (0.78 mmol/h) from

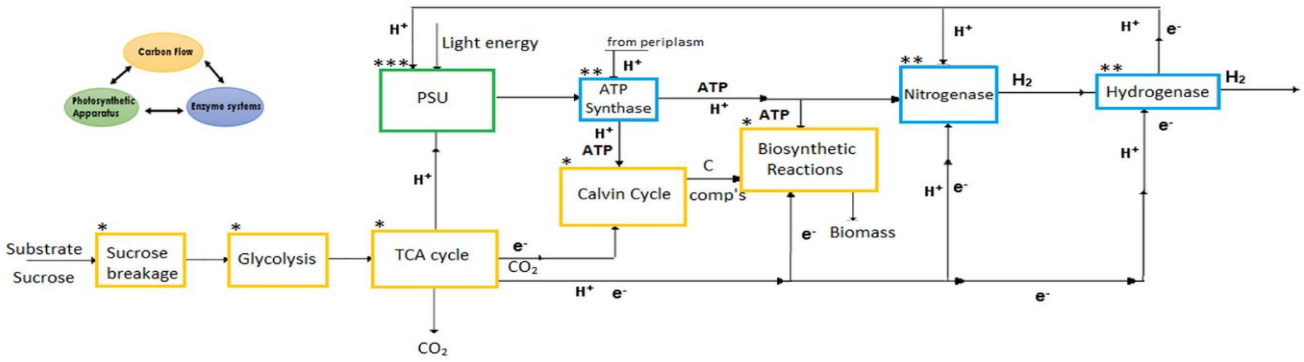

Figure 1. The overall scheme for hydrogen production.
Boxes: (*) Carbon flow, (**) Enzyme systems, (***) Photosynthetic unit (PSU)

sucrose among the PNS species studied, namely *R. capsulatus*, *R. capsulatus* YO3 (hup-), and *R. sphaeroides*.

In the present study, the starting concentrations of the carbon source sucrose and the nitrogen source glutamate were chosen such that the carbon-to-nitrogen ratio values were close to those typically used in experimental hydrogen production studies. Sucrose, the principal component of molasses, is a relatively cheap feedstock, thus resulting in a lower cost process for hydrogen production (Keskin et al., 2012).

Materials and Methods

Flux balance analysis and underlying assumptions

We used flux balance analysis (FBA), where bacterial metabolism is analogous to a set of biochemical reactions occurring in a reactor. Details of the algorithm are well-established (Stephanopoulos et al., 1998). A key assumption is the pseudo steady-state condition for cellular metabolism, leading to constant fluxes and a set of linear mass balance equations, and obviating the need for kinetic parameters. This set of equations can be presented in matrix form, with the m × n stoichiometric matrix ($\underline{\underline{S}}$) and the unknown n × 1 flux vector ($\underline{v}$), where m is the number of metabolites and n is the number of the reactions. Equation (1) displays the system of equations in compact form, with each row corresponding to the sum of the fluxes for a particular metabolite (equations) and each column to a particular flux.

$$\underline{\underline{S}} \cdot \underline{v} = 0 \quad (1)$$

To consolidate the equation system, intermediate metabolites that are produced and consumed in irreversible reactions without branching are neglected (Varma & Palsson, 1993), and compounds that are not key metabolites are excluded. Some biosynthetic reactions can be lumped into a single reaction when intermediates are not of interest. Furthermore, cofactor carrier species such as coenzyme A and NAD^+ have their fluxes intrinsically balanced and can therefore be ignored in the network. Inclusion of such metabolites only leads to the generation of dependent rows (Varma & Palsson, 1994).

Objective function and constraints

Typically, as in this study, the number of equations is less than the number unknowns (m<n). Thus an objective function is defined and mathematically optimized, with the known flux equations (i.e. the set formulated as equation 1) constraining the solution space. Subsequently a unique optimum solution and a corresponding optimal flux distribution for the metabolic network are obtained.

The objective function is usually set to maximize the growth rate of the biomass since this is arguably the natural goal of the cell (Varma & Palsson, 1994). The case for other objective functions such as maximization of certain products, maximization of ATP production or minimization of substrate consumption, has been argued for and against, as discussed in (Feist et al., 2010). In the present study, most solutions were obtained for the default objective function of maximized growth, except for a few explicitly stated cases with maximum hydrogen production as the objective.

There are no well-defined rate limitations for a specific enzyme or a set of enzymes in the metabolic network of *R. palustris*, thus the flux vector values were allowed a large range of -1000 to +1000, respectively to ensure convergence and obtain finite values. No solutions were found when unconstrained sets were used. Reversible reactions in the metabolic network can have positive or negative flux values, with the sign of the flux indicating the direction (Edwards *et al.*, 2000). In the model of the present study, 50 out of 148 reactions are reversible and thus their resulting values can be positive or negative.

Optimization

The simplex method is most commonly used, but the interior-point method was preferred in this study. Trial solutions are corner-point feasible solutions in the simplex method, whereas in the interior-point algorithm they originate inside the feasible domain, which promotes a better chance of reaching a solution when corners or edges are not well-defined, or when the unknown vector contains negative values (Venkataraman, 2009).

Modeling methodology

The initial stage was the reconstruction of the cellular metabolism in terms of metabolic pathways and their reactions. Biochemical reactions were identified from databases to construct a general, overall metabolic network for PNS bacteria (*R. palustris* CG009) and listed in standard stoichiometric notation. Most reactions were obtained from the KEGG database (Kanehisa et al., 2015) whereas MetaCyc (Caspi et al., 2014) and BRENDA (Schomburg et al., 2013) databases were used to check enzymes/reactions not available in KEGG database.

In the preparation stage, the network was simplified for *R. palustris* and the conditions of interest. For each a reaction/enzyme among metabolic reactions, the databases as well as the literature data for this species were scanned. If the presence of this enzyme was confirmed either via the genomic map or the literature data, the selected reaction was included in the network. Pathways inactive under hydrogen-producing conditions were eliminated, for example, since anaerobic conditions are required for photofermentative hydrogen production, aerobic respiration were excluded.

In the development stage, the metabolic network was formulated with the selected reactions and the stoichiometric matrix was constructed. Stoichiometric reductions were carried out to form a fully independent

linear system. An objective function and constraints were set up as described above.

In the calculation/result stage, first an optimization script was formulated using MATLAB R2016. The script was verified using by a prior test-case with known results (Varma et al., 1993b) and the resulting flux values were found to be almost identical.

After verification, the script was applied to the stoichiometric matrix for *R. palustris*. The final metabolic model consists of 148 reconstructed biochemical reactions with 128 compounds within the reaction network. Eliminating dependent reactions reduced the number of rows in the stoichiometric matrix from 128 to 121. Pseudo steady-state was assumed for the metabolic network and the objective function was assumed as either maximal growth of biomass or hydrogen production, as explained above.

Results and Discussion

Metabolic network

The reconstructed metabolic network consists of the core metabolic blocks necessary for growth and hydrogen production. These blocks include the sucrose assimilation pathway, the pentose phosphate pathway, glycolysis, Calvin cycle, TCA cycle, PHB synthesis, lumped biosynthetic (growth) reactions, photosynthetic reactions and hydrogen production reactions. Pathways in which maltose, pectin and cellulose are consumed, and the pathways that require aerobic respiration were not included. In the central metabolic network, sucrose was considered as the primary carbon source and glutamate the nitrogen source in a growth medium with low nitrogen to carbon ratio, to emulate typical conditions used for hydrogen production.

Figure 2 displays the reconstructed carbon flow network. Gray boxes contain biosynthetic precursors for biomass growth. Some metabolites reacting at separate regions of the network are color-coded to represent the network in two dimensions. Each major pathway block is letter-coded (A-F in Figure 2): Carbon utilization starts with external sucrose which is broken down in the sucrose pathway (A). Phosphorylated glucose enters glycolysis (B) and the pentose phosphate pathway (C), the latter being specific to *R. palustris* among the PNS bacteria. Following 3-Phospho-D-glycerate, carbons are utilized in the Calvin cycle (D). Organic acids are produced in TCA cycle (E). Fluxes 39 and 40 in the TCA Cycle are the glyoxylate shunt, which is specific to *R. palustris* among PNSB. ATP, CO_2 and electrons are produced and consumed throughout these pathways while NAD and FAD shuttle electrons to the reactions. Residual electrons and ATP are consumed for hydrogen production by nitrogenase and hydrogenase. The production of PHB (F) is also accounted for in the model. The hydrogen production reactions are outside the scope of the central carbon network and summarized in Table 1.

Table 1. Reactions for hydrogen production with their resulting fluxes

Enzyme	Reaction	Flux (mmol/h)
Mo-Nitrogenase	$-16ATP + 8H^+ + 8e^- \rightarrow 16ADP + 4H_2$	0.048
V- Nitrogenase	$-16ATP + 8H^+ + 8e^- \rightarrow 16ADP + 4H_2$	0.048
Fe- Nitrogenase	$-16ATP + 8H^+ + 8e^- \rightarrow 16ADP + 4H_2$	0.048
Hydrogenase	$2H^+ + 2e^- \rightarrow H_2$	0.11

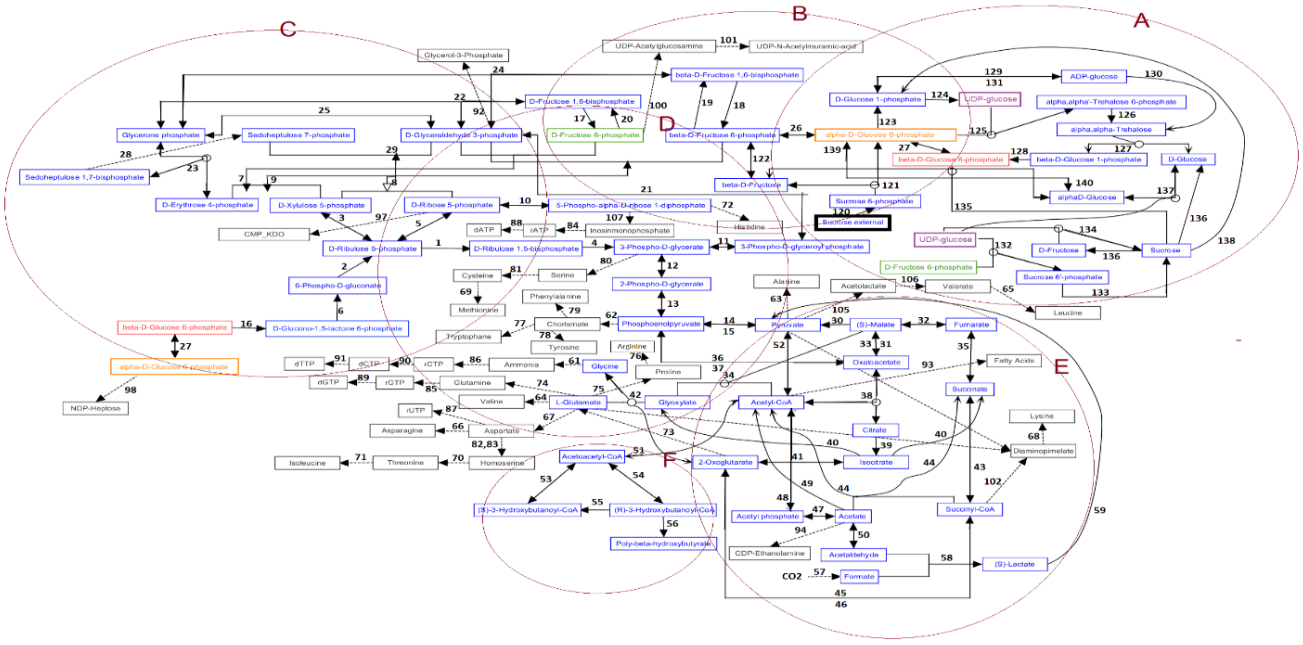

Figure 2. Central carbon flow in *R. palustris* for growth on sucrose (A:Sucrose pathway, B:Glycolysis, C:Pentose phosphate pathway, D: Calvin cycle, E: TCA cycle, F: PHB synthesis).

Model parameters and cases

An experimental work was carried out by Sagir et al. (2017), to produce hydrogen from sucrose and molasses by *R. palustris* in 50 ml small-scale batch photobioreactors. The researchers reported the time-dependent profiles of the medium pH, bacterial growth, hydrogen production and observed the soluble end-products acetic acid, formic acid and lactic acid. This experimental study was used for the input parameters (known fluxes) and formed the basis of comparing the outputs of the model calculation results with the experiment.

The three main input fluxes calculated from the actual values of the bacterial growth medium used in the experimental hydrogen production setup of Sagir et al. (2017) are the glutamate uptake rate, the initial sucrose consumption rate and the photon flux all in units of mmol/h per culture liter. The default values of these inputs derived from the experimental data are listed as Case 0, in Table 2 below, with the remaining cases corresponding to the studies where the effect of changes in input fluxes were analyzed.

Note that in all cases, the medium is nitrogen-limited in order to mimic natural hydrogen production conditions, which is the primary focus of this study. As shall be seen below, this limitation is a central constraint that governs the features of the model solution.

Table 2. List of model cases

Case	Glutamate uptake rate (mmol/h)	Initial sucrose flux (mmol/h)	Photon flux (mmol/h)	Organic acid uptake rate (mmol/h)
0 (Base)	0.1	0.25	5	0
1	0.1	0.25	varied	0
2	0.1	0.25	5	varied

Comparison of the model with experimental data

The performance of the model was tested using the aforementioned experimental data (Sagir et al., 2017). For comparison of the model results, the times of maximum bacterial growth rate (24th hour) and maximum hydrogen production rate (48th hour) for 5 mM sucrose in the experimental study were selected, since FBA is essentially based on the steady-state approximation, as mentioned previously. For the production of organic acids such as acetic acid, lactic acid and formic acid, the lower and upper values of the ranges given in the tables correspond to the organic acid fluxes at 0 and 72nd hours of the experimental data, which is the targeted time period for this model.

Table 3 compares the experimental and computed hydrogen production rates with sucrose concentration and glutamate concentrations of 5 mM and 2 mM, respectively. The model results agree well with experimental data. The percent errors between the experimental and model rate results are 11.8% for production of biomass, 2.9% for hydrogen production and 4.2%, for the maximum hydrogen production, in which the maximum hydrogen production objective was set. Furthermore, the model correctly predicts organic acid production, and their fluxes are within the given range of experimental results. The sucrose conversion efficiency, obtained from the sucrose uptake flux of the base case of the model, and defined as the rate of hydrogen production (0.68 mmol/h) divided by theoretical (that is, maximum possible) rate of hydrogen production (1.2 mmol/h), was compared to the efficiency observed by Sagir et al. (2017). The efficiency calculated from the model was 56%, very close to the experimental efficiency of 53 %.

Growth and hydrogen production

The growth rate (flux 147) was the same (0.0118 g dry cell weight/h) for two different objective functions of maximum growth and maximum hydrogen production, presumably due to the nitrogen limitation. It is customary to classify bacterial growth into the lag, exponential and stationary phases. The exponential phase is where maximum biomass production rate is observed, whereas maximum hydrogen is produced in the late-exponential and stationary phases (Waligorska et al., 2009). It may be speculated that in the present model, the nitrogen-limited environment causes a low biomass growth rate, which brings the point of maximum biomass growth rate closer to the point of maximum hydrogen production rate. Therefore, having the same growth rate for two different objective functions is expected in the case of high nitrogen to carbon ratio.

Hydrogen is produced by the enzymes nitrogenase and hydrogenase when excess electrons and ATP are available. The *R. palustris* genome contains genes encoding three separate nitrogenases based on the different metals in their active sites (Mo, V and Fe). The presence of alternative nitrogenase enzymes was

Table 3. Experimental and computed output parameters for the base case

Control parameters	Units	Experimental result	Model result
Biomass production rate	(gdcw/h)	0.0104	0.0118
Hydrogen production rate[a]	(mmol/h)	0.70	0.68
Max H_2 production[b]	(mmol/h)	0.86	0.83
Acetic acid production	(mmol)	0.004-0.108	0.0128
Lactic acid production	(mmol)	0.008-0.09	0.0127
Formic acid production	(mmol)	0.02-0.12	0.087
Sucrose conversion efficiency	-	53%	56%

[a] Using biomass growth as the objective function
[b] Using hydrogen production as the objective function

supported by the microarray analysis of *R. palustris* during nitrogen starvation (McKinlay, 2014), as similar to the present modeling case. All nitrogenase isozymes use the same number of electrons for H_2 production. The relevant reactions and the corresponding fluxes are displayed in Table 4.

Table 4. Significant changes between the flux distributions of the maximum growth objective and the maximum H_2 production objective

Reaction	Objective: Maximum Growth (mmol/h)	Objective: Maximum H_2 (mmol/h)
H_2 Production	0.678	0.784
PHB Production	0.0011	0
Acetate Production	0.00053	0
Lactate Production	0.00053	0
Formate Production	0.0036	0

The hydrogen production rate of one of the nitrogenase isoenzymes (0.047 mmol/h) is lower than the hydrogen production rate of hydrogenase (0.11 mmol/h). This agrees with the literature stating that the catalytic rate of nitrogenase is lower than the rate of hydrogenase (Basak et al., 2007).

When the objective function was switched to maximization of hydrogen production, the total hydrogen production increased as expected, compared to the results of the growth objective function. The most noteworthy changes (Table 4) included the complete elimination of PHB and organic acids production. For a real physical case, this would represent the maximum possible diversion of reducing equivalents generated from the substrate to hydrogen production, including the consumption of organic acids produced during growth. The PHB production rate is zero in the case of maximum hydrogen production since PHB production competes with H_2 for reducing equivalents and ATP.

Effect of illumination (photon flux)

A range of photon fluxes (0 – 200 mmol/h) was utilized to observe the effect of photon flux on the specific growth rate, H_2 production rate and PHB production rate. Results are presented in Figure 3. Rates for specific growth and sucrose consumption were constant at each photon flux for both objective functions, due to the nitrogen-limited conditions.

For maximum biomass growth as the objective, the highest H_2 production flux was obtained with a photon flux of 10 mmol/h, and 5 mM sucrose and 2 mM glutamate initially. Sasikala et al. (1995) stated that hydrogen production saturated around 5000 lux, corresponding to a photon flux of 13 mmol/h, close to the value found in this work. At higher photon fluxes, H_2 production decreases because of the inhibitory effect on H_2 production. The highest irradiation of the day (0.9 kW/m^2), corresponding to a photon flux of 30 mmol/h, was experimentally observed to deteriorate H_2 production (Miyake et al., 1999). Model results are consistent with this observation, showing a significant decrease in hydrogen production at photon fluxes of 10-30 mmol/h. It is interesting that this inhibition effect is reproduced in the model without an additional, explicit constraint. Presumably, when the photon flux is too high, the model cannot balance the equations within the set of constraints.

PHB production was found to be antagonistic to H_2 production as it competes with the latter for reducing equivalents and ATP. According to the flux distribution, when the photon flux increases, more ATP is produced by ATP synthase. However, H_2 production decreases after a certain photon flux (10 mmol/h) because of the increase in PHB production. Nitrogenase cannot utilize the excess ATP converted from high intensity light (McKinlay & Hardwood, 2010) and therefore ATP is used for other pathways such as organic acid and PHB synthesis, which in turn decreases hydrogen production.

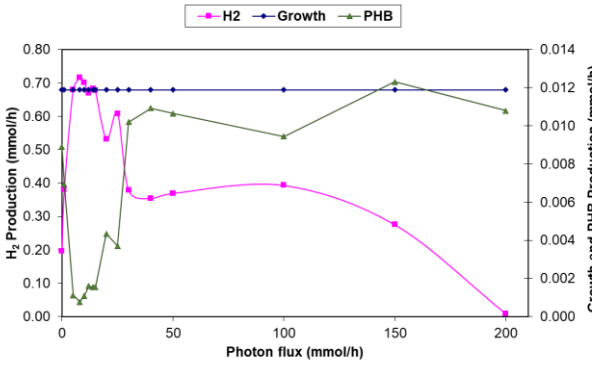

Figure 3. The effect of the value of photon flux on growth, H_2 production and PHB production.

Even with zero photon flux, the simulation predicts growth and H_2 production. The reason is that bacteria use energy from organic sources in the growth media. Additionally, organic acid production rates are higher initially as seen in Figure 3. It has been established experimentally that PNSB are capable of dark-fermentation (Uffen & Wolfe, 1970; Koku et al., 2002; Oh et al., 2004).

Utilization of organic acids

The PNSB are known to consume, as well as produce, organic acids during growth. To investigate organic acid consumption behavior of the model, input fluxes were given for the formate, acetate and lactate.

Table 5 presents the individual effects of adding organic acids (in addition to sucrose) on H_2 production in the case of maximum biomass growth as the objective function. Without organic acids, hydrogen production rate was 0.6783 mmol/h. The production rate was found to increase with increasing initial flux of the organic acids since they could be consumed as supplemental substrates. The rate of hydrogen production was mildly higher for acetic acid compared to lactic acid, and the lowest rate was obtained for formic acid. An experimental hydrogen production study with *R. palustris,* in which both sucrose and several organic acids were utilized could not be found, but the model

results are comparable with the experimental results of Barbosa et al. (2001) who obtained the highest hydrogen production performance with *R. palustris* on acetate and those of Uyar et al. (2009) who obtained highest hydrogen productivities using acetic and lactic acid as substrates to *R. capsulatus*, another PNSB.

Table 5. Individual effects of organic acids on H_2 production

	Hydrogen production rate (mmol/h)		
No acid added		0.6783	
Initial flux of	**Acetate**	**Lactate**	**Formate**
0.001 mmol/h	0.6846	0.6826	0.6825
0.004 mmol/h	0.6952	0.6935	0.6851
0.010 mmol/h	0.7150	0.7130	0.6901

Conclusion

FBA was used to model the hydrogen production metabolic network of *R. palustris* using 148 reconstructed biochemical reactions and 128 compounds, and flux distributions were obtained based on different cases defined for the model. Model results agreed well with previous experimental data. The hydrogen production rate was estimated as 0.68 mmol/h compared to the experimental value of 0.7 mmol/h. The model predicted a substrate conversion efficiency of 56%, close to the experimental value of 53%. Hydrogen was produced by hydrogenase and nitrogenase, with the production rate of hydrogenase higher than that of nitrogenase. The growth rate was the same for two different objective functions, possibly due to the nitrogen limitations.

The capability of the hydrogen production on sucrose by *R. palustris* was assessed with the results of the metabolic model. Maximum hydrogen production was obtained with 10 mmol/h photon flux as the input along with 5 mM sucrose and 2 mM glutamate for the case of maximum biomass growth. At higher photon fluxes, H_2 production rate decreased and PHB production was observed to be antagonistic to H_2 production for both objective functions. The most and least effective organic acid for H_2 production was found as acetic acid and formic acid, respectively. The flux distribution obtained in this work shows that the addition of organic acids to sucrose increases H_2 production.

The results can be exploited for further studies to increase yields of hydrogen production. For example, the pathways for organic acid production can be studied to propose sucrose/organic acid combinations resulting in higher yields. Using FBA, intelligent manipulation of the genome to improve the sucrose metabolism of PNSB offers the potential to obtain insight into a low cost hydrogen production system.

Funding Information

This study was part of project 114M436 supported by the Scientific and Technological Research Council of Turkey (TUBITAK).

Author Contributions

EMDG: methodology, implementation, analysis, draft version. HK: Conceptualization, supervision and administration, reviewing and editing, final manuscript.

Acknowledgements

We thank the Scientific and Technological Research Council of Turkey (TUBITAK) for the support of this work as part of project 114M436. We deeply appreciate the insightful scientific feedback and commentary by Prof. Dr. Meral Yücel.

Credit

This work was based on the M.Sc. thesis studies of the first author, Ezgi Melis Doğan-Güner.

References

Argun, H., Kargi, F., Kapdan, I. K., & Oztekin, R. (2008). Biohydrogen production by dark fermentation of wheat powder solution: Effects of C/N and C/P ratio on hydrogen yield and formation rate. *International Journal of Hydrogen Energy, 33*(7), 1813-1819. https://doi.org/https://doi.org/10.1016/j.ijhydene.2008.01.038

Azbar, N., & Levin, D. B. (2012). State of the art and progress in production of biohydrogen. Bentham Science Publishers.

Azwar, M. Y., Hussain, M. A., & Abdul-Wahab, A. K. (2014). Development of biohydrogen production by photobiological, fermentation and electrochemical processes: A review. *Renewable and Sustainable Energy Reviews, 31*, 158-173. https://doi.org/https://doi.org/10.1016/j.rser.2013.11.022

Barbosa, M. J., Rocha, J. M. S., Tramper J., & Wijffels, R. H. (2001). Acetate as a carbon source for hydrogen production by photosynthetic bacteria. *J Biotechnol, 85*, 25-33. https://doi.org/10.1016/S0168-1656(00)00368-0

Basak, N., & Das, D. (2007). The Prospect of Purple Non-Sulfur (PNS) Photosynthetic Bacteria for Hydrogen Production: The Present State of the Art. *World Journal of Microbiology and Biotechnology, 23*(1), 31-42. https://doi.org/10.1007/s11274-006-9190-9

Caspi, R., Altman, T., Billington, R., Dreher, K., Foerster, H., Fulcher, C. A., Holland, T. A., Keseler, I. M., Kothari, A., Kubo, A., Krummenacker, M., Latendresse, M., Mueller, L. A., Ong, Q., Paley, S., Subhraveti, P., Weaver, D. S., Weerasinghe, D., Zhang, P., & Karp, P. D. (2016). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res, 44*(D1), D471-480. https://doi.org/10.1093/nar/gkv1164

Das, D., & Veziroğlu, T. N. (2001). Hydrogen production by biological processes: a survey of literature. *International Journal of Hydrogen Energy, 26*(1), 13-28. https://doi.org/https://doi.org/10.1016/S0360-3199(00)00058-6

Edwards, J. S., & Palsson, B. O. (2000). The *Escherichia coli* MG1655 in silico metabolic genotype: its definition, characteristics, and capabilities. *Proceedings of the National Academy of Sciences of the United States of America, 97*(10), 5528-5533. https://doi.org/10.1073/pnas.97.10.5528

Eroglu, I., Özgür, E., Eroglu, E., Yücel, M., & Gündüz, U. (2014). Applications of Photofermentative Hydrogen Production. In D. Zannoni & R. De Philippis (Eds.), Microbial BioEnergy: Hydrogen Production (pp. 237-267). Springer Netherlands. https://doi.org/10.1007/978-94-017-8554-9_11

Feist, A. M., & Palsson, B. O. (2010). The biomass objective function. *Current Opinion in Microbiology, 13*(3), 344-349. https://doi.org/https://doi.org/10.1016/j.mib.2010.03.003

Golomysova, A., Gomelsky, M., & Ivanov, P. S. (2010). Flux balance analysis of photoheterotrophic growth of purple nonsulfur bacteria relevant to biohydrogen production. *International Journal of Hydrogen Energy, 35*(23), 12751-12760. https://doi.org/https://doi.org/10.1016/j.ijhydene.2010.08.133

Hallenbeck, P. C., & Liu, Y. (2016). Recent advances in hydrogen production by photosynthetic bacteria. *International Journal of Hydrogen Energy, 41*(7), 4446-4454. https://doi.org/https://doi.org/10.1016/j.ijhydene.2015.11.090

Imam, S., Yilmaz, S., Sohmen, U., Gorzalski, A. S., Reed, J. L., Noguera, D. R., & Donohue, T. J. (2011). iRsp1095: A genome-scale reconstruction of the *Rhodobacter sphaeroides* metabolic network. *BMC Systems Biology, 5*(1), 116. https://doi.org/10.1186/1752-0509-5-116

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2015). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Research, 44*(D1), D457-D462. https://doi.org/10.1093/nar/gkv1070

Keskin, T., & Hallenbeck, P. C. (2012). Hydrogen production from sugar industry wastes using single-stage photofermentation. *Bioresource Technology, 112*, 131-136. https://doi.org/https://doi.org/10.1016/j.biortech.2012.02.077

Klamt, S., Schuster, S., & Gilles, E. D. (2002). Calculability analysis in underdetermined metabolic networks illustrated by a model of the central metabolism in purple nonsulfur bacteria. *Biotechnol Bioeng, 77*(7), 734-751. https://doi.org/10.1002/bit.10153

Koku, H., Eroglu, I., Gunduz, U., Yücel, M., & Turker, L. (2002). Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*. *International Journal of Hydrogen Energy, 27*, 1315-1329. https://doi.org/10.1016/S0360-3199(02)00127-1

Kotay, S., & Das, D. (2008). Biohydrogen as a renewable energy resource—Prospects and potentials. *International Journal of Hydrogen Energy, 33*, 258-263. https://doi.org/10.1016/j.ijhydene.2007.07.031

Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., Miriam, L. L., Pelletier, D. A., Beatty, J. T., Lang, A. S., Tabita, F. R., Gibson, J. L., Hanson, T. E., Bobst, C., Torres y Torres, J. L., Peres, C., Harrison, F. H., Gibson, J., & Harwood, C. S. (2004). Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris. Nature Biotechnology, 22*(1), 55-61. https://doi.org/10.1038/nbt923

McEwan, A. G. (1994). Photosynthetic electron transport and anaerobic metabolism in purple non-sulfur phototrophic bacteria. *Antonie Van Leeuwenhoek, 66*(1-3), 151-164. https://doi.org/10.1007/bf00871637

McKinlay, J. B., & Harwood, C. S. (2010). Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. *Proceedings of the National Academy of Sciences, 107*(26), 11669-11675. https://doi.org/10.1073/pnas.1006175107

McKinlay, J. B. (2014). Systems Biology of Photobiological Hydrogen Production by Purple Non-sulfur Bacteria. In D. Zannoni & R. De Philippis (Eds.), Microbial BioEnergy: Hydrogen Production (pp. 155-176). https://doi.org/10.1007/978-94-017-8554-9_7

Miyake, J., Wakayama, T., Schnackenberg, J., Arai, T., & Asada, Y. (1999). Simulation of the daily sunlight illumination pattern for bacterial photo-hydrogen production. *Journal of Bioscience and Bioengineering, 88*(6), 659-663. https://doi.org/https://doi.org/10.1016/S1389-1723(00)87096-6

Navid, A., Jiao, Y., Wong, S. E., & Pett-Ridge, J. (2019). System-level analysis of metabolic trade-offs during anaerobic photoheterotrophic growth in *Rhodopseudomonas palustris. BMC bioinformatics, 20*(1), 1-16. https://doi.org/10.1186/s12859-019-2844-z

Oh, Y. K., Raj, S. M., Jung-Gyoo, Y., & Park, S. (2011). Current status of the metabolic engineering of microorganisms for biohydrogen production. *Bioresource Technology, 102*(18), 8357-8367. https://doi.org/https://doi.org/10.1016/j.biortech.2011.04.054

Oh, Y. K., Seol, E. H., Lee, E. Y., & Park, S. (2002). Fermentative hydrogen production by a new chemoheterotrophic bacterium *Rhodopseudomonas palustris* P4. *International Journal of Hydrogen Energy, 27*(11-12), 1373-1379.

Ozturk, Y., Yücel, M., Daldal, F., Mandacı, S., Gündüz, U., Türker, L., & Eroğlu, İ. (2006). Hydrogen production by using *Rhodobacter capsulatus* mutants with genetically modified electron transfer chains. *International Journal of Hydrogen Energy, 31*(11), 1545-1552. https://doi.org/https://doi.org/10.1016/j.ijhydene.2006.06.042

Sagir, E., Ozgur, E., Gunduz, U., Eroglu, I., & Yucel, M. (2017). Single-stage photofermentative biohydrogen production from sugar beet molasses by different purple non-sulfur bacteria. *Bioprocess Biosyst Eng, 40*(11), 1589-1601. https://doi.org/10.1007/s00449-017-1815-x

Sasikala, C. H., Ramana, C. H. V., & Rao, P. R. (1995). Regulation of simultaneous hydrogen photoproduction duringgrowth by pH and glutamate in *Rhodobacter sphaeroides* O.U. 001. *International Journal of Hydrogen Energy, 20*(2), 123-126. https://doi.org/https://doi.org/10.1016/0360-3199(94)E0009-N

Schomburg, I., Chang, A., Placzek, S., Söhngen, C., Rother, M., Lang, M., Munaretto, C., Ulas, S., Stelzer, M., Grote, A., Scheer, M., & Schomburg, D. (2013). BRENDA in 2013: integrated reactions, kinetic data, enzyme function data, improved disease classification: new options and contents in BRENDA. *Nucleic acids research, 41*(Database issue), D764-D772.

https://doi.org/10.1093/nar/gks1049

Sparling, R., Carere, C., Rydzak, T., Schellenberg, J., & Levin, D. B. (2012). Thermodynamic and biochemical aspect of hydrogen production by dark fermentation. In N. Azbar & D. B. Levin (Eds.), State of the Art and Progress in Production of Biohydrogen (pp. 160-188). https://doi.org/10.2174/978160805224011201010160

Stephanopoulos, G. N., Aristidou, A. A., & Nielsen, J. (1998). CHAPTER 8 - Metabolic Flux Analysis. 309-351. https://doi.org/https://doi.org/10.1016/B978-012666260-3/50009-1

Uffen R. L., & Wolfe R. S. (1970). Anaerobic growth of purple nonsulfur bacteria under dark conditions. *Journal of Bacteriology, 104*(1), 462-472. https://doi.org/10.1128/jb.104.1.462-472.1970

Uyar, B., Eroglu, I., Yücel, M., & Gündüz, U. (2009). Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. *International Journal of Hydrogen Energy, 34*(10), 4517-4523. https://doi.org/https://doi.org/10.1016/j.ijhydene.2008.07.057

Varma, A., & Palsson, B. O. (1993). Metabolic Capabilities of *Escherichia coli* II. Optimal Growth Patterns. *Journal of Theoretical Biology, 165*(4), 503-522. https://doi.org/https://doi.org/10.1006/jtbi.1993.1203

Varma, A., & Palsson, B. O. (1994). Metabolic Flux Balancing: Basic Concepts, Scientific and Practical Use. *Bio/Technology, 12*(10), 994-998. https://doi.org/10.1038/nbt1094-994

Venkataraman, P. (2009). Applied Optimization with MATLAB Programming. Wiley Publishing.

Vignais, P. M., Colbeau, A., Willison, J. C., & Jouanneau, Y. (1985). Hydrogenase, Nitrogenase, and Hydrogen Metabolism in the Photosynthetic Bacteria. *Advances in Microbial Physiology, 26*, 155-234. https://doi.org/https://doi.org/10.1016/S0065-2911(08)60397-5

Waligórska, M., Seifert, K., Górecki, K., Moritz, M., & Laniecki, M. (2009). Kinetic model of hydrogen generation by *Rhodobacter sphaeroides* in the presence of NH ions. *J Appl Microbiol, 107*(4), 1308-1318. https://doi.org/10.1111/j.1365-2672.2009.04314.x

Zhang, D., Xiao, N., Mahbubani, K. T., del Rio-Chanona, E. A., Slater, N. K. H., & Vassiliadis, V. S. (2015). Bioprocess modelling of biohydrogen production by *Rhodopseudomonas palustris*: Model development and effects of operating conditions on hydrogen yield and glycerol conversion efficiency. *Chemical Engineering Science, 130*, 68-78. https://doi.org/https://doi.org/10.1016/j.ces.2015.02.045

Biotech Studies 31(1), 10-16
http://doi.org/10.38042/ biotechstudies.1069555

BIOTECH STUDIES

RESEARCH PAPER

Effects of calcium concentration, calcium chelators, calcium channel-blockers on *Hsp70a* expression in *Chlamydomonas reinhardtii*

Tuba Sevgi*, Elif Demirkan

Biology Department, Faculty of Arts and Sciences, Bursa Uludag University, 16059, Bursa, Turkey.

How to cite

Sevgi, T. & Demirkan, E. (2022). Effects of calcium concentration, calcium chelators, calcium channel-blockers on *Hsp70a* expression in *Chlamydomonas reinhardtii. Biotech Studies. 31*(1), 10-16. http://doi.org/10.38042/biotechstudies.1069555

Article History
Received 29 September 2021
Accepted 28 January 2022
First Online 07 February 2022

Corresponding Author
Tel.: +90 224 294 29 35
E-mail: tubaavci@uludag.edu.tr

Keywords
Arylsulfatase
Heat shock response
Membrane fluidity

Abstract

In this study, calcium concentration, calcium chelators, and calcium channel blockers that could be effective in triggering the heat shock response in *Chlamydomonas reinhardtii* were investigated. For this purpose, continuously expressed and heat-inducible transformant *C. reinhardtii* strains were used, and heterologously expressed arylsulfatase activities were detected. After a short time of heat shock at 40°C, cultures were shifted to 23°C and different concentrations of calcium (0-1 M $CaCl_2$), EGTA (0-50 mM), BAPTA (0-2 mM), lanthanum (0-300 µM), gadolinium (0-350 µM), and verapamil (0-100 µM) applications were performed. To compare the arylsulfatase activity results at the transcript level, HSP70A expression level was analyzed. Arylsulfatase activity was increased with the increase of the calcium concentration, in the presence of calcium chelators, blockers, and parallel results were obtained in HSP70A expression level. These findings support that both extracellular and intracellular calcium influx is effective in the heat shock response of *C. reinhardtii.*

Introduction

Global warming showed itself in the form of heatwaves in recent years and endangers production efficiency. Each factor that affects production efficiency also affects the human population and welfare (Sung et al., 2003). It is predicted that heat waves will be stronger, long-lasting, and more frequent in the future (Meehl & Tebaldi, 2004). Therefore, it is imperative to understand the heat stress response and sensing mechanism behind generating more heat-tolerant crop plants by genetic engineering.

The heat stress response (HSR) has been studied in detail in bacteria, yeast, flies, and mammals, however, not all concepts have been explained and these may not apply to plant systems. The main reason for this is the plastids, which play a central role in the metabolism of plant cells and contain at least 1300 proteins. Plastids add an extra level of complexity to these eukaryotic cells. Therefore, it is clear that a simpler plant model organism is needed to study HSR (Schroda et al., 2015). *Chlamydomonas reinhardtii* is utilized as a plant model organism since the 1950s (Harris, 2001). Taking advantage of the relatively few players in the protein homeostasis network compared to plants, *C. reinhardtii* is a practical model to work for (Schroda & Vallon, 2009; Schulz-Raffelt et al., 2007).

Increased expression of heat shock protein (HSPs) genes has been used as a marker to study heat shock response in many experiments since the expression of HSPs is a conservative event that occurs in all organisms exposed to heat stress (Rütgers et al., 2017). The expression of genes encoding HSPs is induced when misfolded proteins accumulate in the cell. This was first demonstrated in *Escherichia coli* (Goff & Goldberg, 1985). Also in land plants and *Chlamydomonas* the accumulation of unfolded proteins was shown to trigger HSP gene expression (Kurepa et al., 2003; Sugio et al., 2009; Schmollinger et al., 2013).

Some studies have suggested that the expression of HSPs is due to increased fluidity in the plasma membrane (Gao et al., 2012; Saidi et al., 2009; Suri & Dhindsa, 2008; Wu et al., 2012). The primary heat sensors in the plasma membrane of plant cells were suggested to be calcium channels that open when membrane fluidity increases, thereby allowing the influx of extracellular calcium to trigger the HSR (Saidi et al., 2009, 2010). Saidi et al. (2011) suggested that heat stress in plants is sensed by the entry of extracellular Ca^{2+} through Ca^{2+} channels to the plasma membrane. Regarding this hypothesis, changes in temperature lead the membrane fluidity loss. Ca^{2+} influx activates a calmodulin-dependent kinase, cyclic adenosine monophosphate (cAMP) levels increase in heat stress, thus leading to the activation of cyclic nucleotide-gated calcium channels (CNGC). Then, the heat shock factor (HSF) was activated and mediated the expression of heat stress genes. Stimulation and expression of HSPs allow the cell to restore protein homeostasis while HSF is inactivated, and then the response is weakened. The role of calcium as a secondary messenger in heat shock signal transduction in *Chlamydomonas* has been controversial, insufficient therefore more studies are needed (Gong et al., 1998; Saidi et al., 2009; Gao et al., 2012; Zheng et al., 2012; Schroda et al., 2015).

The most powerful technique for evaluating the effect of a mutation in a promoter is to use a reporter gene whose expression can be detected and quantified a short time after transformation. A handful of reporter genes have been codon optimized in *Chlamydomonas* nuclear genome, including luciferase (Fuhrmann et al., 2004; Shao & Bock 2008), green fluorescent protein (Fuhrmann et al., 1999), xylanase (Rasala et al., 2012), and recently several additional fluorescents (Rasala et al., 2013). However, even with these optimized reporters, signal is low and expensive to detect. Instead heterologously expressed arylsulphatase (ARS), can be detected by simple and sensitive colorimetric assay. Also, almost completely secretes into the surrounding medium by cells lacking a cell wall and allows detectable enzymatic activity in culture media (Hostos et al., 1988; Ohresser et al., 1997). Therefore, ARS was utilized as a strong reporter for quantitative analysis of gene expression in this work.

In this study, calcium concentration, calcium channel blockers (lanthanum, gadolinium, and verapamil), and chelators (EGTA and BAPTA) were used to determine whether calcium affects triggering the heat shock response using inducible *HSP70A* promoter, continuously expressing *β2TUB* promoter and the *ARS* reporter.

Materials and Methods

Strains and culture conditions

C. reinhardtii strain cw15-302 *(arg7-)*, c124, and plasmids pCB412 (*ARG7* [Arginynosuccinate lyase] marker gene-containing plasmid), pJD55 (*β2TUB-ARS* containing plasmid), pCB803 (*HSP70A-ARS* containing plasmid) were kindly provided from M. Schroda (Technical University of Kaiserslautern, Germany) (Schroda et al., 2000), and *C. reinhardtii* nuclear transformation was performed using glass beads method (Kindle, 1990), and HSP70A III-1 and βTub II-32 transformants were used from our previous study as recombinant strains (Sevgi & Demirkan, 2021). *C. reinhardtii* recombinant strain HSP70A III-1 (containing *HSP70A* promoter and *ARS* reporter gene) and *C. reinhardtii* recombinant strain βTub II-32 (containing *β2TUB* promoter and *ARS* reporter gene) were grown in Tris Acetate Phosphate (TAP) medium under continuous light (14 µmol $E.m^{-2}s^{-1}$) on a rotatory shaker at 23°C and 150 rpm (Harris, 2001). Then in total, ~10^8 cells were centrifuged at 3500 rpm for 3 min and the pellet was transferred to 30 mL TAP medium in 100 mL flasks which were adjusted before to 40°C. The pellet was added to the medium, kept at 40°C for 5 min, and immediately shifted to 23°C for calcium applications.

Effect of calcium concentration, calcium ion channel blockers, and calcium chelators

Different concentrations of calcium ($CaCl_2$; 0 mM, 100 mM, 250 mM, 500 mM, 1M), EGTA (triethylene glycol diamine tetraacetic acid) (0 mM, 1 mM, 50 mM), BAPTA (1,2-bis [o-aminophenoxy] ethane-N,N,N',N'-tetraacetic acid) (0 mM, 1 mM, 2 mM), lanthanum (0 µM, 10 µM, 75 µM, 100 µM, 150 µM, 300 µM), gadolinium (0 µM, 100 µM, 150 µM, 250 µM, 300 µM, 350 µM), and verapamil (0 µM, 10 µM, 100 µM) (Sangwan et al., 2002) were used to investigate the effects on heat shock response. Thus, continuously expressed (βTub II-32) and heat-inducible (HSP70A III-1) recombinant *C.reinhardtii* strains were grown at 23˚C with continuous light (CL) until the cell density reached approximately ~10^6 cells/mL. After a short time heat shock, calcium, calcium chelator, and blocker applications were performed. For this purpose, a total of ~10^8 cells were centrifuged at 3500 rpm at room temperature for 3 min and the pellet was added to 30 ml TAP media at 40°C for 5 minutes then again ~10^8 cells were added to 30 mL TAP media containing calcium, calcium chelators, and ion channel blockers at different concentrations. The ARS activities were compared with the control (0mM).

Arylsulfatase enzyme activity assay

Arylsulfatase (ARS) activity was determined according to Ohresser et al. (1997). ARS is the α-naphthol concentration formed in 1 h at 37˚C according to the total amount of chlorophyll (µg α-naphtol/µg chlorophyll h). Chlorophyll concentration was detected according to Porra et al. (1989).

500 µl of culture (~10^8 cells/mL) were pelleted by centrifugation and the supernatant was used for ARS assay according to Ohresser et al. (1997). 500 µl of the reaction mixture contained 400 µl supernatant, 0.4 M glycine-NaOH buffer pH 9.0, 10 mM imidazole, and the

enzyme-substrate 0.3 mM 5-bromo-4 chloro-indolylsulphate (X-SO4). The samples were incubated for 1 h at 37°C with X-SO4 then the reaction was stopped with 500 µl of 4% SDS in 0.2 M Na acetate buffer pH 4.8 and 100 µl of 10 mg/mL tetrazotized-o-dianisidine (Sigma). This compound unites with the α-naphthol released in the reaction, an SDS soluble purple precipitate occurs. The ARS activity was measured at 540 nm within 2 min after the addition of tetrazotized-o-dianisidine, and the activity was expressed as µg naphthol per µg chlorophyll (chl) in the cell pellet per h at 37°C.

RNA extraction and gene expression analysis

HSP70A and *Chlamydomonas* β-subunit-like polypeptide (CβLP) expressions were measured by RT-PCR and compared with ARS activity results. For this purpose, 2×10^7 cells were collected for RNA extraction using the NucleoSpin RNA Plant Kit (Macherey-Nagel, Düren, Germany) by following the manufacturer's instructions. DNA contaminations were removed with Turbo DNase (RNase-free; Ambion, Massachusetts, USA). RT-PCR experiments were perfomed with using a OneTaq One-Step RT-PCR Kit (NEB, Massachusetts, USA) by StepOnePlus™ Real-Time PCR System (Applied Biosystems, Massachusetts, USA) (10 min 48°C, 10 min 95°C; 60 s were set at 65°C for a total of 40 cycles). Samples without a template or reverse transcriptase were always included as control. $HSP70A_{For}$GATCGAGCGCATGGTGC, $HSP70A_{Rev}$TCCATCGACTCCTTGTCCG, $CβLP_{For}$GCCACACCGAGTGGGTGTCGTGCG, $CβLP_{Rev}$CCTTGCCGCCCGAGGCGCACAGCG primers were used. The relative quantification of gene expressions was analyzed by the $2^{-\Delta\Delta Ct}$ method. The protocols of Livak & Schmittgen (2001) was used. *HSP70A* and *CβLP* expressions were measured by RT-PCR and compared with ARS activity results.

Statistical analysis

Data were expressed as mean and standard deviation (SD). Statistical analyses were performed using GraphPad Prism 9.2.0 (Demo Version; GraphPad, San Diego, CA) statistical package program. An independent sample t-test was applied to examine the effect of calcium, calcium chelators, and blockers at the transcript level. The significance was calculated using Student's t-test. A value of $p<0.05$ was considered statistically significant.

Results and Discussion

In this study, the effect of calcium, which is thought to be effective in membrane fluidity and heat shock response, on *HSP70A* expression using *C. reinhardtii* recombinant strains (HSP70A III-1 and βTub II-32) was investigated by using strong promoter (*HSP70A* and *βTub*) and reporter (*ARS*) genes.

Many studies have been performed on perception and response of heat stress in *Chlamydomonas*, plants and other organisms (Morimoto, 1998; Schroda et al., 2000; Schmollinger et al., 2013). Temperature changes may alter the fluidity of biological membranes, which may impair the barrier function of membranes between intercellular compartments and the activity of integral membrane proteins (Saidi et al., 2011). For *Chlamydomonas*, it is still being investigated whether heat shock can be perceived by changes in membrane fluidity, unlike land plants. Some studies in plant cells support that the primary heat sensors in the plasma membrane are calcium channels (Gao et al., 2012; Liu et al., 2003; Suri & Dhindsa, 2008; Saidi et al., 2009; Wu et al., 2012). Results from some studies indicated that calcium accumulating in the cytosol of heat-stressed cells was only derived from extracellular stores (Sangwan et al., 2002; Saidi et al., 2009; Wu et al., 2012), other studies also pointed to intracellular calcium stores as a source (Gong et al., 1998; Zheng et al., 2012). In land plants, the influx of extracellular calcium was found to be substantial as the HSR was diminished when calcium chelators EGTA or BAPTA, or the calcium ion channel blockers lanthanum, gadolinium or verapamil were applied prior to HS (Heat Shock) (Link et al., 2002; Sangwan et al., 2002; Liu et al., 2003; Suri & Dhindsa, 2008; Saidi et al., 2009; Gao et al., 2012; Wu et al., 2012).

As the first step, the possible effect of extracellular calcium on the heat shock response was analyzed in recombinant strains of *C. reinhardtii* (HSP70A III-1 and $β_2$TUB II-32). Secondly, different concentrations of calcium chelators EGTA and BAPTA and the calcium ion channel blockers lanthanum, gadolinium or verapamil were applied to recombinant *C. reinhardtii* strains. The highest ARS activity was obtained in transformant HSP70A III-1 as 1338 µg-αnaphthol/µg chl at 100 mM calcium and the enzyme increased 4.5 times compared to the control. A sharp decrease was observed in ARS activity at concentrations between 100 mM and 150 mM while a slight increase was observed at concentrations between 150 mM-500 mM. This indicates that HSR may have been triggered by opening calcium channels in the cell membrane and allowing calcium flow into the cell. Therefore, it seems that calcium played a role as a second messenger. In the positive control strain $β_2$TUB II-32 ARS activity remained constant as expected (Figure 1). Wu et al. (2012) reported that HS-triggered rapid increases in Ca^{2+} in cytosol is important in mediating downstream HS-related gene expression for the acquisition of thermotolerance in rice. Saidi et al. (2009) suggest that early sensing of mild temperature increments occurs at the plasma membrane of plant cells independently from cytosolic protein unfolding and the heat signal is translated into an effective HSR by way of a specific membrane-regulated Ca^{2+} influx, leading to thermotolerance.

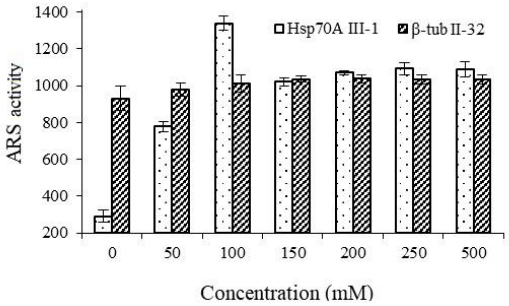

Figure 1. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) of recombinant *C. reinhardtii* strains (HSP70A III-1 and β_2TUB II-32) at different $CaCl_2$ concentrations (0-500 mM). The data presented are mean± SD of three independent experiments. SD, Standard Deviation.

In the studies with Ca chelators and blockers, it was observed that as the EGTA concentration increased, the ARS enzyme activity is also increased in the HSP70A III-1 transformant (Figure 2). When the EGTA concentration reached 50 mM, the amount of ARS increased approximately 5 times compared to control (0mM) (Figure 2a). In BAPTA, this increase is 4 times (Figure 2b). According to ARS activity results, it can be said that the heat stress response was triggered in the presence of calcium chelators, EGTA, and BAPTA. The induction of ARS activity in the presence of EGTA and BAPTA may be due to the insufficient concentration of calcium chelators or the excessive calcium flow released from the intracellular calcium stores. Schmollinger et al. (2013) obtained inconsistent role of extracellular calcium in mediating the HSR with *Chlamydomonas*, while washed cells treated with calcium chelator BAPTA displayed a delayed and less pronounced induction of *HSP* gene expression and reduced thermotolerance under HS, washed cells supplemented with EGTA behaved like controls. Zheng et al. (2012) found that the intracellular calcium (Ca^{2+}) increased rapidly after HS in the Ca^{2+}/calmodulin HS signal transduction pathway. Our results support both the role of extracellular and intracellular calcium utilization in heat stress induction.

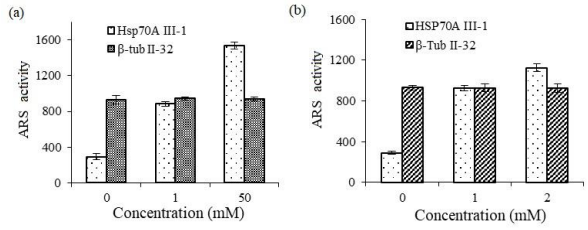

Figure 2. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) of recombinant *C. reinhardtii* strains (HSP70A III-1 and β_2TUB II-32) at different EGTA (0-50 mM) (a), and BAPTA (0-2 mM) concentrations (b). The data presented are mean± SD of three independent experiments.

The effect of different concentrations of calcium channel blockers (lanthanum, gadolinium, and verapamil) on ARS enzyme activity was also investigated. In the presence of lanthanum, ARS activity increased approximately 4-fold for HSP70A III-1 at 75µM concentration (Figure 3a). At 100 µM lanthanum concentration, the activity decreased considerably. When the gadolinium concentration was examined, it was determined that the ARS activity increased 4 times at 150 µM in the HSP70A III-1 transformant (Figure 3b). In the presence of different concentrations of another calcium channel blocker, verapamil, the ARS enzyme activity in the HSP70A III-1 transformant increased 2.5 times at 10 µM compared to the control, while this increase was 4.9 times at 100 µM (Figure 3c). As expected, ARS activity was stable in the transformant β_2TUB II-32 at all concentrations of lockers. It has been reported that the blockers inhibit HS detection/signal transmission (Lancaster & Batchelor, 2000; Saoudi et al., 2004). In our study, it was determined that the response was triggered in the presence of calcium blockers. However, decreases in ARS activity or stability at high concentrations of lanthanum and gadolinium suggest that they act as calcium blockers. It has been stated that the extracellular calcium influx in plants is mediated by the opening of specific calcium channels as a result of increased membrane fluidity at high temperatures (Saidi et al., 2009).

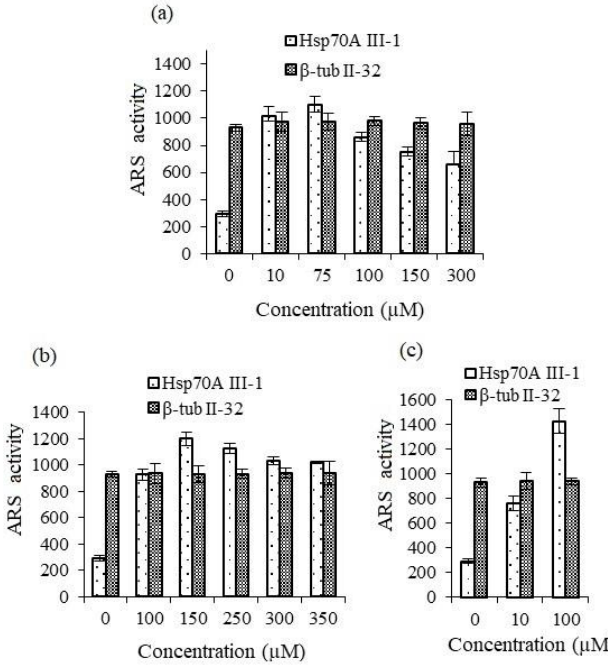

Figure 3. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) of recombinant *C. reinhardtii* strains (HSP70A III-1 and β_2TUB II-32) at different lanthanum (0-300 µM) (a), gadolinium (0-350 µM) (b), verapamil concentrations (0, 10, 100 µM) (c). The data presented are mean± SD of three independent experiments.

All results were evaluated comparatively with RT-PCR. As a result, transcript expressions were in parallel with ARS activities in the samples exposed to different concentrations of EGTA, BAPTA, lanthanum, gadolinium, and verapamil (Figure 4).

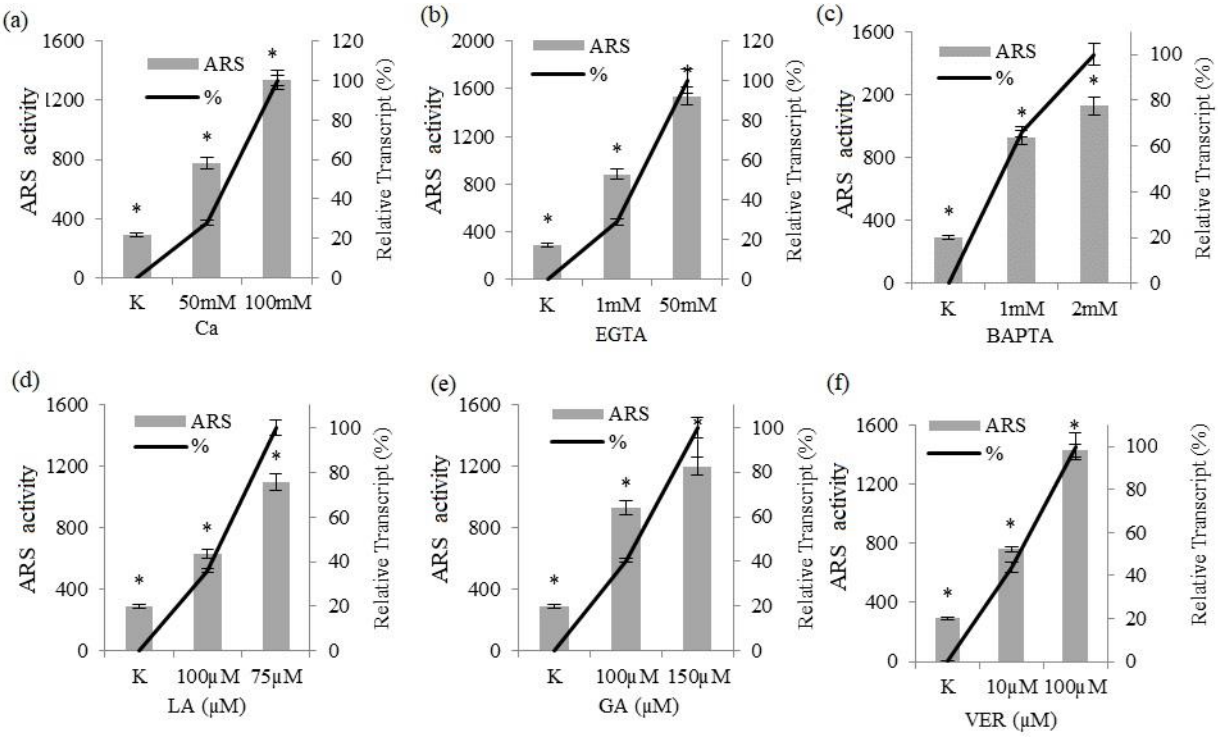

Figure 4. The ARS enzyme activities (µg α-naphtol/µg chlorophyll h) and relative HSP70A expression levels of recombinant C. reinhardtii strain HSP70A III-1 at different calcium concentrations (a), exposure to different concentrations of EGTA (b), and BAPTA (c), lanthanum (d), gadolinium (e), and verapamil (f) as determined by qPCR. The results show the mean of the data from three qPCR replicates and two biological replicates. The highest expression level of the respective transcript was set to 100%. Student's t-test was used for statistical analysis of the samples. *, P< 0.05

Conclusion

This study was aimed to investigate the effect of calcium concentration, calcium channel blockers, and chelators triggering the heat shock response. Our results demonstrated increase in calcium concentration, the presence of calcium chelators and blockers induced the increase in ARS activity. *ARS* induction was also confirmed by qRT-PCR analysis. The increase in *HSP70A* transcript expression levels support that the selective *ARS* reporter gene used in the study is favorable. Overall, our results suggest that both extracellular and intracellular calcium influx was shown to be effective in heat shock response in *C. reinhardtii.* However, further studies investigating the molecular mechanisms are required.

Funding Information

This study is supported by The Scientific and Technological Research Council of Turkey (TUBITAK) under project number 116Z892.

Author Contributions

TS: Data Curation, Formal Analysis, Investigation, Methodology, Writing Original Draft, Funding, Project Administration, Resources. **ED:** Conceptualization, Supervision, Writing, Review and Editing

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknlowledgements

We thank to Michael Schroda, Prof. Dr., Technische Universität Kaiserslautern, Germany for the supply of the strains and the constructs.

References

Fuhrmann, M., Oertel, W., & Hegemann, P. (1999). A synthetic gene coding for the green fluorescent protein (GFP) is a versatile reporter in Chlamydomonas reinhardtii. *The Plant Journal*, *19*(3), 353-361. https://doi.org/10.1046/j.1365-313X.1999.00526.x

Fuhrmann, M., Hausherr, A., Ferbitz, L., Schödl, T., Heitzer, M., & Hegemann, P. (2004). Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene. *Plant molecular biology*, *55*(6), 869-881. https://doi.org/10.1007/s11103-004-2150-6

Gao, F., Han, X., Wu, J., Zheng, S., Shang, Z., Sun, D., & Li, B. (2012). A heat-activated calcium-permeable channel–Arabidopsis cyclic nucleotide-gated ion channel 6–is involved in heat shock responses. *The Plant*

Journal, *70*(6), 1056-1069. https://doi.org/10.1111/j.1365-313X.2012.04969.x

Goff, S. A. & Goldberg, A. L. (1985). Production of abnormal proteins in *E. coli* stimulates transcription of lon and other heat shock genes. *Cell*, 41, 587-595. https://doi.org/10.1016/S0092-8674(85)80031-3

Gong, M., van der Luit, A. H., Knight, M. R., & Trewavas, A. J. (1998). Heat-shock-induced changes in intracellular Ca^{2+} level in tobacco seedlings in relation to thermotolerance. *Plant Physiology*, *116*(1), 429-437. https://doi.org/10.1104/pp.116.1.429

Harris, E. H. (2001). *Chlamydomonas* as a model organism. *Annual review of plant biology*, *52*(1), 363-406. https://doi.org/10.1146/annurev.arplant.52.1.363

Hostos, E. L. de, Schilling, J., & Grossman, A. R. (1989). Structure and expression of the gene encoding the periplasmic arylsulfatase of *Chlamydomonas reinhardtii*. *Molecular and General Genetics MGG*, *218*(2), 229-239. https://doi.org/10.1007/BF00331273

Kindle, K. L. (1990). High-frequency nuclear transformation of *Chlamydomonas reinhardtii. Proceedings of the National Academy of Sciences 87* (3): 1228-1232. https://doi.org/10.1073/pnas.87.3.1228

Kurepa, J., Walker, J. M., Smalle, J., Gosink, M. M., Davis, S. J., Durham, T. L., Sung, D. Y., & Vierstra, R. D. (2003). The small ubiquitin-like modifier (SUMO) protein modification system in Arabidopsis. Accumulation of SUMO1 and _2 conjugates is increased by stress. *J. Biol. Chem*. 278,6862-6872. https://doi.org/10.1074/jbc.M209694200

Lancaster, B., & Batchelor, A. M. (2000). Novel action of BAPTA series chelators on intrinsic K+ currents in rat hippocampal neurones. *The Journal of Physiology*, *522*(2), 231-246. https://doi.org/10.1111/j.1469-7793.2000.t01-1-00231.x

Link, V., Sinha, A. K., Vashista, P., Hofmann, M. G., Proels, R. K., Ehness, R., & Roitsch, T. (2002). A heat-activated MAP kinase in tomato: a possible regulator of the heat stress response. *FEBS letters*, *531*(2), 179-183. https://doi.org/10.1016/S0014-5793(02)03498-1

Liu, H. T., Li, B., Shang, Z. L., Li, X. Z., Mu, R. L., Sun, D. Y., & Zhou, R. G. (2003). Calmodulin is involved in heat shock signal transduction in wheat. *Plant physiology*, *132*(3), 1186-1195. https://doi.org/10.1104/pp.102.018564

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, *25*(4), 402-408. https://doi.org/10.1006/meth.2001.1262

Meehl, G. A., & Tebaldi, C. (2004). More intense, more frequent, and longer lasting heat waves in the 21st century. *Science*, *305*(5686), 994-997. https://doi.org/10.1126/science.1098704

Morimoto, R. I. (1998). Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. *Genes & development*, *12*(24), 3788-3796. https://doi.org/ 10.1101/gad.12.24.3788

Ohresser, M., Matagne, R. F., & Loppes, R. (1997). Expression of the arylsulphatase reporter gene under the control of the nit1 promoter in *Chlamydomonas reinhardtii*. *Current genetics*, *31*(3), 264-271. https://doi.org/10.1093/nar/20.12.2959

Porra, R. J., Thompson, W. A., & Kriedemann, P. E. (1989). Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *975*(3), 384-394. https://doi.org/10.1016/S0005-2728(89)80347-0

Rasala, B. A., Lee, P. A., Shen, Z., Briggs, S. P., Mendez, M., & Mayfield, S. P. (2012). Robust expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide. PLoS ONE, *7*, e43349. https://doi.org/10.1371/journal.pone.0043349

Rasala, B. A., Barrera, D. J., Ng, J., Plucinak, T. M., Rosenberg, J. N., Weeks, D. P., ... & Mayfield, S. P. (2013). Expanding the spectral palette of fluorescent proteins for the green microalga C hlamydomonas reinhardtii. *The Plant Journal*, *74*(4), 545-556. https://doi.org/10.1111/tpj.12165

Rütgers, M., Muranaka, L. S., Schulz-Raffelt, M., Thoms, S., Schurig, J., Willmund, F., & Schroda, M. (2017). Not changes in membrane fluidity but proteotoxic stress triggers heat shock protein expression in *Chlamydomonas reinhardtii*. *Plant, cell & environment*, *40*(12), 2987-3001. Doi: https://doi.org/10.1111/pce.13060

Saidi, Y., Finka, A., Muriset, M., Bromberg, Z., Weiss, Y. G., Maathuis, F. J., & Goloubinoff, P. (2009). The heat shock response in moss plants is regulated by specific calcium-permeable channels in the plasma membrane. *The Plant Cell*, *21*(9), 2829-2843. https://doi.org/10.1105/tpc.108.065318

Saidi, Y., Peter, M., Finka, A., Cicekli, C., Vigh, L., & Goloubinoff, P. (2010). Membrane lipid composition affects plant heat sensing and modulates Ca^{2+}-dependent heat shock response. *Plant Signal. Behav. 5*, 1530-1533. https://doi.org/10.4161/psb.5.12.13163

Saidi, Y., Finka, A., & Goloubinoff, P. (2011). Heat perception and signalling in plants: a tortuous path to thermotolerance. *New Phytologist*, *190*(3), 556-565. https://doi.org/10.1111/j.1469-8137.2010.03571.x

Sangwan, V., Örvar, B. L., Beyerly, J., Hirt, H., & Dhindsa, R. S. (2002). Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. *The Plant Journal*, *31*(5), 629-638. https://doi.org/10.1046/j.1365-313X.2002.01384.x

Saoudi, Y., Rousseau, B., Doussiere, J., Charrasse, S., Gauthier-Rouvière, C., Morin, N., & Job, D. (2004). Calcium-independent cytoskeleton disassembly induced by BAPTA. *European Journal of Biochemistry*, *271*(15), 3255-3264. https://doi.org/10.1111/j.1432-1033.2004.04259.x

Schmollinger, S., Schulz-Raffelt, M., Strenkert, D., Veyel, D., Vallon, O., & Schroda, M. (2013). Dissecting the heat stress response in Chlamydomonas by pharmaceutical and RNAi approaches reveals conserved and novel aspects. *Molecular Plant*, *6*(6), 1795-1813. https://doi.org/10.1093/mp/sst086

Schroda, M., Blöcker, D., & Beck, C. F. (2000). The HSP70A promoter as a tool for the improved expression of transgenes in Chlamydomonas. *The Plant Journal*, *21*(2), 121-131. https://doi.org/10.1046/j.1365-313x.2000.00652.x

Schroda, M., & Vallon, O. (2009). Chaperones and proteases. In *The Chlamydomonas Sourcebook* (pp. 671-729). Academic Press. https://doi.org/10.1016/B978-0-12-370873-1.00027-7

Schroda, M., Hemme, D., & Mühlhaus, T. (2015). The Chlamydomonas heat stress response. *The Plant Journal*, *82*(3), 466-480. https://doi.org/10.1111/tpj.12816

Schulz-Raffelt, M., Lodha, M., & Schroda, M. (2007). Heat shock factor 1 is a key regulator of the stress response in Chlamydomonas. *The Plant Journal*, *52*(2), 286-295. https://doi.org/10.1111/j.1365-313X.2007.03228.x

Shao, N., & Bock, R. (2008). A codon-optimized luciferase from Gaussia princeps facilitates the in vivo monitoring of gene expression in the model alga Chlamydomonas reinhardtii. *Current Genetics*, *53*(6), 381-388. https://doi.org/10.1007/s00294-008-0189-7

Sevgi, T., & Demirkan, E. (2021). Evaluation of the effects of temperature, light and UV-C radiation on HSP70A expression in *Chlamydomonas reinhardtii*. *Turkish Journal of Botany*, *45*: 671-680. https://doi.org/10.3906/bot-2012-43

Sugio, A., Dreos, R., Aparicio, F., & Maule, A. J. (2009). The cytosolic protein response as a subcomponent of the wider heat shock response in *Arabidopsis*. *Plant Cell*, 21, 642-654. https://doi.org/10.1105/tpc.108.062596

Sung, D. Y., Kaplan, F., Lee, K. J., & Guy, C. L. (2003). Acquired tolerance to temperature extremes. *Trends in Plant Science*, *8*(4), 179-187. https://doi.org/10.1016/S1360-1385(03)00047-5

Suri, S. S., & Dhindsa, R. S. (2008). A heat-activated MAP kinase (HAMK) as a mediator of heat shock response in tobacco cells. *Plant, Cell & Environment*, *31*(2), 218-226. https://doi.org/10.1111/j.1365-3040.2007.01754.x

Wu, H. C., Luo, D. L., Vignols, F., & JINN, T. L. (2012). Heat shock-induced biphasic Ca^{2+} signature and OsCaM1-1 nuclear localization mediate downstream signalling in acquisition of thermotolerance in rice (*Oryza sativa* L.). *Plant, Cell & Environment*, *35*(9), 1543-1557. https://doi.org/10.1111/j.1365-3040.2012.02508.x

Zheng, S. Z., Liu, Y. L., Li, B., Shang, Z. L., Zhou, R. G., & Sun, D. Y. (2012). Phosphoinositide-specific phospholipase C9 is involved in the thermotolerance of *Arabidopsis*. *The Plant Journal*, *69*(4), 689-700. https://doi.org/10.1111/j.1365-313X.2011.04823.x

Biotech Studies 31(1), 17-27
https://doi.org/10.38042/biotechstudies.1073521

BIOTECH STUDIES

RESEARCH PAPER

Expression of immune-related gene from African mud catfish *Clarias gariepinus* reared in bioflocs systems after *Aeromonas hydrophilia* infection

Omoniyi Michael Popoola[1,2]*, Ayomide Miracle Oyelade[1], Success Taiwo Torhukerijho[1]

[1]Department of Fisheries and Aquaculture, Federal University of Technology, PMB 704, Akure, Nigeria.
[2]Aquatic Environmental Biotechnology & Nanotechnology Division ICAR-Central Inland Fisheries Research Institute, Indian Council of Agricultural Research, Barrackpore, Kolkata-700 120 West Bengal, India.

How to cite

Popoola, O. M., Oyelade, A. M., & Torhukerijho, S. T. (2021). Expression of immune-related gene from African mud catfish *Clarias gariepinus* reared in bioflocs systems after *Aeromonas hydrophilia* infection. *Biotech Studies*, *31*(1), 17-27. https://doi.org/10.38042/biotechstudies.1073521

Article History
Received 29 December 2021
Accepted 08 February 2022
First Online 14 February 2022

Corresponding Author
Tel.: +234 803 834 9325
E-mail: ompopoola@futa.edu.ng

Keywords
Immune gene
Haematology
Bioflocs
Clarias gariepinus
Carbon nitrogen ratio

Abstract

The influence of various carbon sources as bioflocs on relative immunological gene expression, haematology, growth, and microbial community in *Clarias gariepinus* juvenile culture is investigated in this study. The bioflocs groups (four) were created by daily supplementation with four carbon sources (cassava peel, tapioca, wheat offal, and brewery waste) with a carbon-nitrogen ratio of 20 and the control without carbon addition. The juvenile *Clarias gariepinus* (8.16 ± 0.2 g) was stocked into each bioflocs system and reared for 72 days. The results revealed that the water quality parameter and survival rate differed significantly across the treatments. The microbial community revealed that there were differences in bacterial intensity and diversity among the various culture systems. The haematological parameters between the treatments showed a significant difference $p<0.05$ in the challenged test. qRT-PCR was used to assess immune-related gene expression, and four immune genes (*IL-10*, *TNF-α*, *TGF-β*, *IL-1β*) were shown to be increased. As a result, the bioflocs system can be considered to boost innate immunity and immune-related gene expression. Overall, this research found that using bioflocs technology can help with immunostimulation, and that the effect is independent of the organic carbon utilised to keep the fish alive.

Introduction

An ecologically important and commercially valued fish for sub-Sahara Africa especially Nigerian aquaculture industry is the African catfish, *Clarias gariepinus,* belonging to the family Claridae (Ita, 1980). *Clarias gariepinus* is generally and widely cultivated in ponds because of their ability to survive in captivity, they also occur freely in natural freshwaters in Nigeria (Adebayo & Daramola, 2013). The current demand for fish protein has led to the intensification of aquaculture to make fish available to the growing population, therefore, increasing productivity per unit space is accomplished by increasing the stocking density of fish. Due to limited control over pathogens, health protection and biosecurity are major challenges to production intensification (Xie & Yu, 2007). Attaining sustainability in feed management is also an important aspect of the production intensification of any aquatic organism.

The scientific community's understanding of the genus *Aeromonas* has also evolved. Initially, aeromonads were only recognised as producing systemic disorders in poikilothermic animals. The genus *Aeromonas* is now recognised not only as a significant disease-causing pathogen of fish and other cold-blooded animals, but also as the causative agent for a variety of infectious problems in both immunocompetent and immunocompromised people (Janda & Abbott, 2010). Mesophilic species (*A. hydrophila* and *A. veronii*) infect fish with a similar range of diseases, including motile *Aeromonas septicemia* (hemorrhagic septicemia) in carp, tilapia, perch, catfish, and salmon, and a red sore disease in carp, tilapia,

perch, catfish, and salmon, and ulcerative infections in catfish, cod, carp, and goby (Joseph & Carnahan, 1994; Ture et al., 2018). Over the last decade, mesophilic *Aeromonas* species, most notably *A. hydrophila*, have been connected to massive die-offs and fish kills all over the world, resulting in enormous economic losses. *A. hydrophila* is a pathogenic bacterium that is found all over the world, especially in warm water. They are gram-negative, motile rods with oxidase and catalase activity, as well as fermentative (Sabur, 2006). MAS is caused by the bacterium *A. hydrophila* (motile *Aeromonas septicemia*). This disease affects both farmed and wild fish, however fish in intensive culture systems are more sensitive to the disease condition caused by *A. hydrophila.*

Bioflocs technology is a method of improving water quality by adding extra carbon to the aquaculture system, either from an external source or by increasing the carbon content of the feed. This approach encourages bacterial growth to take up nitrogen, resulting in a faster decrease in ammonium content than nitrification (Hargreaves, 2006). This biotechnological approach has proved efficient in improving water quality and feed usage efficiency compared to conventional practices for farming of Tilapia and *Litopenaeus vannamei* (Avnimelech, 1999; Milstein et al., 2001). The bioflocs rearing system has been developed to implement the use of minimum water exchange and usage in aquaculture ponds, through maintaining adequate water quality within the culture unit as well as producing heterotrophic media and a multiphasic feed source that can serve as food for aquatic organisms (Avnimelech, 1999; Crab et al., 2007; Crab, 2010), invented the bioflocs rearing system to execute minimum water exchange and water utilisation in aquaculture ponds, which improves aquatic creatures' nutritional and reproductive performance (Emerenciano et al., 2013). It has been hypothesised that biofouling could be reduced as a result of the minimum water exchange.

Bioflocs keeps pathogens out of ponds and disease outbreaks from spreading between farms (Crab et al., 2010). Recent studies have evaluated probiotic effects of bioflocs and role in immune response mechanisms in an attempt to demonstrate its benefits to aquatic organisms' health (Wang et al., 2013; Ahmad et al., 2016), suggesting that disease can be prevented in fish using this sustainable rearing system (Wang et al., 2013; Ahmad et al., 2016; Liu et al., 2016). In terms of conferring immunological benefits, C/N ratios in bioflocs improved the innate immune response and antioxidant status in various species of shell and finfishes when challenged with a disease-causing organism (bacteria) (Xu & Pan, 2013; Ekasari et al., 2014; Ahmad et al., 2016), implying that disease can be prevented in fish using this sustainable rearing system. The study aimed at determining how different carbon sources used in bioflocs affect immunological parameters and immune-related gene expression in the *C. gariepinus* challenged with *A. hydrophilia*.

Materials and Methods

Experimental design

Approximately 1000 *Clarias gariepinus* Juvenile (Initial weight 8.21 ± 0.3g) was obtained from the Department of Fisheries and Aquaculture Technology Teaching and Research Farm, the Federal University of Technology Akure and acclimated in a water volume of 100L. Acclimation to experimental conditions was carried for 14 days within the facilities of the institution, and during this period, the juveniles were fed with a commercial diet two times daily (08.00 and 18.00 h) at 3% of their body weight.

T1 (Clearwater), T2 (Cassava peel as a carbon source), T3 (Tapioca as a carbon source), T4 (Wheat offal as a carbon source), T5 (Brewery waste as a carbon source) were the five treatment groups, four bioflocs treatments, and one control in triplicate with a water volume of 500 L. Collecting Pond bottom soil from a reputed fish farm was used to make the inoculum. In glass tanks (5L), inoculum was made by mixing 20g of pond bottom soil with 1 L of well-aerated water containing 10mg L^{-1} ammonium sulphate $(NH)_4SO_4$ and 400 mg L^{-1} of various carbon sources (tapioca, wheat offal, brewery, and cassava peel). For 24 h, the suspension was incubated for the development of microbial growth.

The prepared inoculum was added to the respective experimental groups after floc formation, the tank was aerated for 7 days to ensure optimum floc formation, and carbon sources were added at a rate of 20 g of carbon source per 1 g of TAN as described by (Avnimelech, 1999) who assumed that 20 g of carbon source is required to convert 1 g of TAN, and (Ebeling et al., 2006) to provide an initial substrate and stimulate the growth of all the treatment groups, 100 fish per tank (2 x 1 x 1 m^3) were stocked. Every week, 70% of the water in the control group was replaced with fresh water, whereas there was no water exchange in the bioflocs based groups. Evaporation losses were corrected with dechlorinated fluids to account for the bioflocs' variation and achieve optimal experimental conditions. The experimental fish were fed with commercial diets at 3% of their body weight under continuous aeration using air blower installed at 10 lines (5 l/min per line).

The determination of the required amount of carbon to reduce the total ammoniacal nitrogen was calculated as follows:

Calculation 1: (Carbon Nitrogen (C: N) content in the feed)

$$C: \frac{\text{Kg of feed} \times 0.9(90\%\ \text{dry matter}) \times 0.7(30\%\ \text{of fish assimilation}}{2(\text{Carbon content of the feed} \sim 50\%\ \text{based on dry matter}}$$

$$N: \frac{\text{Kg of feed} \times 0.9(90\%\ \text{dry matter}) \times 0.7(30\%\ \text{of fish assimilation} \times \text{Crude protein of feed}(\%)}{6.25(constant)}$$

Calculation 2: (Adjusting the Carbon Nitrogen Ratio (C: N)

$$C{:}N\ of\ 20{:}1 = (N(Nitrogen\ content\ in\ feed) \times 20) - C(Carbon\ content\ in\ feed) \times 2$$

Haematological indices

Blood samples were obtained by caudal vein-puncture using a syringe and dispensed into a sample bottle containing ethylene diamine tetra-acetic (5 mL EDTA) as an anticoagulant after five experimental fish were taken from each experimental tank and sedated with clove oil (50 mg L^{-1}).

Svobodova et al. (1991) described a method for analysing blood parameters (RBC, HB, WBC, PCV, MCV, MCHC, and MCH). The haematocrit (Ht) was evaluated using the microcentrifuge technique, and the white blood cell (WBC) and red blood cell (RBC) were determined using a haemocytometer. The Ht, HB, and RBC values were used to determine the MCV, MCH, and MCHC.

Microbial community analysis

The APHA (1998) approach was used to characterise the bioflocs and hindgut bacterial populations of the experimental fish under each treatment. 1 mL of the sample was transferred into 9 mL of sterile distilled water in a bottle at the end of the experiment to obtain dilutions 10^{-1}, and 1 mL from the previous dilution was transferred into another 9mL of sterile distilled water to obtain dilutions 10^{-2} and up to 10^{-5}, respectively, using a sterile pipette. After that, 1mL of each sample from dilutions, 10^{-4} and 10^{-5} was put into sterile Petri-dishes. Each plate was then filled with 20 mL of molten sterile nutritional agar that had been cooled to 45 ^{o}C. After gently swirling the plates and allowing them to harden, they were incubated at 37 °C for 24 h. The plates were checked for growing colonies after 24 h. The colonies were counted and their morphological features were recorded. Representative colonies were chosen and sub-cultured on new nutrient agar multiple times until pure cultures were produced. The bacteria were identified using morphological characteristics of the colonies and biochemical assays was performed on each isolate. To establish pure cultures for identification of fungi, representative colonies were chosen and sub-cultured onto fresh potato dextrose agar. Fungal taxonomic identification was used to identify isolates. The hyphae of the fungal isolates were mounted aseptically on a microscopic slide and given a drop of lactophenol cotton blue before being covered with a coverslip. The slide was examined under the microscope with an x40 objective lens.

Challenge test

The experimental fish in different bioflocs system were exposed to pathogenic strain of *A. hydrophilia* (MPSTR 2143), mildly pathogenic strain (Animal care Laboratory Ogere), grown on a brain heart infusion broth (EM Science, Darmstadt Germany) in a shaking bath 27 ^{0}C overnight in the Department of microbiology Federal University of Technology, Akure. The concentration of bacterial suspension was determined by the serial plate count method and diluted to $9.3x10^{5}$ CFU. Prior to challenge test, lethal dose ($LD_{50\text{-}96h}$) of *A. hydrophilia* (MPSTR 2143) against *C. gariepinus* was determined. Fifteen individuals of *C. gariepinus* were distributed in four tanks in duplicate (120 fish) and maintained without feed till 96 h. The fishes were injected intraperitoneally with 1mL of *A. hydrophilia* (MPSTR 2143) suspension with the concentrations: 1×10^{9} $CFUmL^{-1}$, 1×10^{8} $CFUmL^{-1}$, 1×10^{7} CFU/mL and 0 $CFUmL^{-1}$ (control, using 500µL of physiological solution 0.9% NaCl). The percentage of mortality was calculated at 24 h, 48 h, 72 h, and 96 h, respectively as described by Abbott (1925) and the LD_{50} was determined with Probit analysis. For the challenge test, fifty fish were challenged by *A. hydrophila* (10 fish/ group) by intra-peritoneal injection of 0.5 mL of bacterial suspension using 2 ml Insulin syringe at a concentration of 1.7×10^{9} $CFUmL^{-1}$ of bacterial culture/fish for *A. hydrophila* obtained from lethal dose ($LD_{50\text{-}96h}$). Injected fish were observed for up to 14 days for daily monitoring of symptoms. Mortality and survival were monitored and recorded. Survival at the end of 14 days post-infection was calculated using the following formula (Amend, 1981).

$$Relative\ percentage\ survival\ (RPS) = \frac{Number\ of\ surviving\ fishes\ after\ challenge}{Number\ of\ fish\ bathed\ with\ bacteria} \times 100$$

Tissue sampling

After 14 days of being challenged with *A. hydrophila*, liver samples were taken from the various treatments by slicing the fish to reveal the visceral organs. About 50mg of liver was sliced off and placed in 1.5 mL microcentrifuge tubes, which were kept on ice to prevent RNA denaturation owing to temperature changes. The collected materials were transported to the lab, fixed in RNA, and kept at -20 ^{o}C until RNA extraction was completed.

RNA extraction

Total RNA was isolated from liver tissue of *A. hydrophila* treated and control *C. gariepinus* using Trizol reagent. The purity and quantity of isolated total RNA was quantified using Nanodrop™ Thermo Scientific at the 260:280 ratio. After, the extracted RNA was kept at -20 °C till further use.

cDNA synthesis and quantitative Real Time-PCR

Subsequently, complementary DNA (cDNA) was produced according to the manufacturer's procedure using a cDNA synthesis kit (Bio-iScript Rad's cDNA synthesis kit). With *β-actin* and *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a housekeeping gene, specific primers for gene expression were constructed to amplify the targeted genes. The qRT-PCR analysis was performed using the Bio-Rad CFX96 Touch Real time PCR detection equipment and Sso Advanced

Universal SYBR green supermix (Bio-Rad) according to the manufacturer's instructions and modified by Plaza-Diaz et al. (2014) technique.

Data and statistical analysis

For all genes and samples, the Ct was calculated. The comparative Ct method was used to calculate the relative quantitation of *TNF-α, IL-1β*, *IL-10*, and *TGF-β*. The target gene's relative quantification value was standardised to an endogenous control and expressed as 2-Ct relative to the calibrator. Ct is equal to the Ct of the target gene (*TNF-α*, *IL-1β*, *IL-10*, and *TGF-β*) minus the Ct of the endogenous control gene (*GAPDH* and *β-actin*) in this investigation. Ct is equal to the sample's Ct minus the calibrator's Ct. The fold change in expression levels was computed by dividing the treated sample's relative gene expression value by the negative control's relative gene expression value. Statistical analyses were performed with one-way analysis of variance (ANOVA) and Dunnett's test to compare differences between the treatment groups and the negative control group, and the data were tested for normality and heterogeneity of variance using the Kolmogorov-Smirnov test and Levene's test, respectively. Multiple comparisons were taken into account when calculating the p-values. Graphpad Prism version 8.0 was used to visualise the results.

All data were subjected to an analysis of variance (ANOVA) with a significance level of 0.05 (95% confidence), and the findings were given as Mean SE. To look for significant changes between the treatments, Duncan's multiple range tests were utilised. IBM SPSS statistics version 22 for Windows was used to conduct all of the analyses. Prior to statistical analysis, Log 2 Transformation was performed.

Results

The growth performance of *Clarias gariepinus showed* no significant difference ($p<0.05$) in the weight gain, FCR and SGR (Table 1), although a significant difference $p<0.05$ was observed in the relative survival recorded in tapioca-based treatment 76.00±1.50 and lowest in control 50±10.00 (Figure 1).

The haematological parameters (PCV, HB, RBC, WBC, MCH, MCV, and MCHC) of *Clarias gariepinus raised* in bioflocs systems before and after challenge with *A. hydrophilia* is shown in Table 2. The haematological parameters were significantly different $p<0.05$ but tapioca was observed to be the lowest compared to the control and other bioflocs systems. WBC showed no significant difference among the treatments group.

The haematological parameters (PCV, HB, RBC, WBC, MCH, MCV, and MCHC) of *Clarias gariepinus raised* in bioflocs systems before and after challenge with *A. hydrophilia* is shown in Table 2. The haematological parameters were significantly different $p<0.05$ but tapioca was observed to be the lowest compared to the control and other bioflocs systems. WBC showed no significant difference among the treatments group.

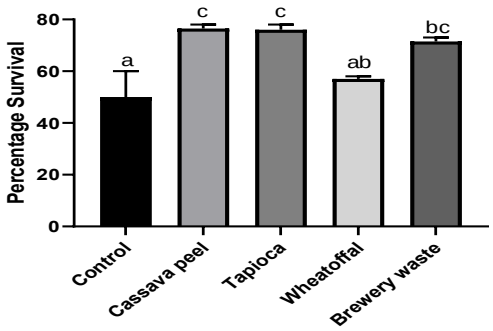

Figure 1. Relative percentage survival of *C. gariepinus in* different treatment groups.

The haematological parameters (PCV, HB, RBC, WBC, MCH, MCV, and MCHC) of *Clarias gariepinus raised* in bioflocs systems before and after challenge with *A. hydrophilia* is shown in Table 2. The haematological parameters were significantly different $p<0.05$ but tapioca was observed to be the lowest compared to the control and other bioflocs systems. WBC showed no significant difference among the treatments group.

Gram-negative bacilli bacterial colonies from the Enterobacteriaceae family were detected, including *Proteus* sp., *Klebsiella* sp., *Shigella* sp., and *Escherichia coli* (Table 3). A group of sporulated gram-positive bacilli linked to lactobacillus and bacteria that are functionally referred to as cocci has been discovered. The presence or absence of the indicated bacterium species in the culture media, as well as heterotrophic bacteria, was a common occurrence.

Colonies from the family Hypocreaceae, which included *Trichoderma* sp., the family Trichomaceae, which included colonies from *Aspergillus* sp. and *Penicillum chysogenum* were among the groups detected (Table 4). A group of filamentous fungi was discovered, including *Fusarium* sp. colonies and the

Table 1. Growth performance of *C. gariepinus juveniles* raised in bioflocs systems

Treatments	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
Initial (g)	8.13±0.48	8.07±0.56	8.18±0.25	8.61±0.01	8.62±0.07
Final (g)	88.45±5.45	81.30±2.20	90.35±3.25	81.70±22.16	77.10±2.20
Weight gain (g)	80.31±5.93	73.23±2.76	82.16±3.50	73.08±22.09	68.47±2.14
SGR	2.98±0.15	2.89±0.12	3.00±0.08	2.76±0.35	2.74±0.03
FCR	0.66±0.05	0.72±0.03	0.64±0.03	0.79±0.24	0.77±0.02

SGR: Specific growth rate, FCR: Feed conversion ratio

Table 2. Haematological Parameters of *C. gariepinus* raised in bioflocs systems

	Treatment	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
HB(g/100ml)	Pre challenge	11.00±0.30^{b}	9.80±0.50^{ab}	9.55±0.15^{a}	10.20±0.20^{ab}	10.20±0.50^{ab}
	Post challenge	12.40±0.30^{b}	10.65±0.65^{a}	11.20±0.50^{ab}	11.15±0.15^{ab}	10.15±0.15^{a}
Ht (%)	Pre challenge	33.00±1.00^{b}	29.50±1.5^{ab}	28.50±0.50^{a}	30.50±0.50^{ab}	30.50±1.50^{ab}
	Post challenge	37.00±1.00^{b}	32.00±2.00^{a}	33.50±1.50^{ab}	33.50±0.50^{ab}	30.50±0.50^{a}
WBC (x103/mm3)	Pre challenge	5250±450.00^{a}	5850±450.00^{a}	6800±300.00^{a}	5150±650.00^{a}	5325±725.00^{a}
	Post challenge	4500.00±300.00^{a}	5725.00±325.00^{a}	5100.00±700.00^{a}	4750.00±250.00^{a}	6250.00±950.00^{a}
RBC	Pre challenge	3.68±0.13^{b}	3.25±0.15^{ab}	3.15±0.05^{a}	3.35±0.05^{ab}	3.38±0.18^{ab}
	Post challenge	4.1±0.10^{b}	3.53±0.23^{a}	3.73±0.18^{ab}	3.73±0.08^{ab}	3.35±0.05^{a}

Values are expressed as mean ± SE. The mean values (n=2) with different superscripts within the same row are significantly different (p<0.05). HB: haemoglobin, Ht: haematocrit, RBC: red blood cell, WBC: white blood cell

saprophytic fungus *Rhizopus* sp. The identification of fungus species and their qualitative characterization to their presence or absence in the culture media was noticed.

Table 3. Characterization of bacteria isolate in biofloc systems

Treatment	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
Bacillus sp.	-	+	+	+	+
Shigella sp.	+	+	-	-	-
Proteus sp.	-	+	+	+	-
Escherichia coli	+	-	+	-	+
Staphylococcus epidermidis	+	-	-	+	-
Staphylococcus aureus	+	-	-	+	+
Klebsiella sp.	+	-	-	-	+

Changes in the expression of all immunological genes investigated as a result of *A. hydrophilla* infection compared to the calibrator sample (Control). When standardised against *GAPDH*, the expression levels of target genes *TNF-α*, *IL-1β*, *TGF-β*, and *IL-10* revealed a considerable down-regulation (Figure 2). There was a substantial difference between the treatments, with cassava peel notably different from the other treatments in terms of *TNF-α* and *IL-1β* expression.

When normalised against *β-actin*, *TNF-α*, and *IL-1β* are down-regulated relative to the calibrator sample, but there is a statistically significant difference (p<0.05) among the bioflocs treatment, but *IL-10* and *TGF-β* are up-regulated with no significant difference among the bioflocs treatment (Figure 3).

Discussion

The non-significant differences in fish growth performance in the treatment groups, Weight gain, SGR, and FCR, could indicate that the bioflocs system did no influence on fish development and that the feed was efficiently utilised by fish produced in the bioflocs system at a Carbon Nitrogen Ratio of 20. (Avnimelech, 2007). There is a sliver of evidence suggesting the biofloc helped to the fish's development and production, which contrasts with the findings of Azim & Little (2008), who found that bioflocs had a low FCR in *Oreochromis niloticus*. Increased turbidity owing to bioflocs, which limits visibility and thus artificial feed intake, could be attributed to the increased FCR and reduced weight gain of fish species in the CP+BFT, WO+BFT, and BW+BFT when compared to the control. When compared with the control, the survival rate of the fish in the bioflocs systems were considerably greater (p<0.05). Several pathogenic species, such as bacteria, fungi, and viruses have been identified as major limiting factors in fish and other aquatic animals' growth. *A. hydrophilia* is the most prevalent and commonly seen bacterial disease in tropical regions, causing serious harm to fish output (Karunasagar et al., 1991). BFT has been demonstrated to boost fish immune responses, but just a few types of research have looked in the resistance of aquatic animals cultivated in bioflocs-based systems to infectious disease, with no promising results thus far. Bioflocs-based systems greatly boosted resistance of *L. vannamei* to infectious myonecrosis virus and *L. rohita* to *A. hydrophila,* according to Ekasari et al. (2014) and Ahmad et al. (2016). This is attributable to their better innate immune responses. Before the bacterial challenge test, there were no significant differences in haematological parameters across the treatments in this study. Although the WBC of the different treatments showed no significant difference to the control, a higher value of WBC was observed in the BFT compared to the control, which could be the result of the destruction of WBC during macrophagosis in the control group, the bioflocs group were able to release more antibody to

Table 4. Characterization of Fungi isolate in biofloc systems

Treatment	Control	Cassava peel	Tapioca	Wheat offal	Brewery waste
Trichoderma viride	+	+	+	-	-
Fusarium sp.	-	+	+	-	-
Aspergillus niger	+	-	+	+	-
Trichoderma sp.	-	-	+	-	-
Aspergillus flavus	-	-	-	+	-
Fusarium oxysporium	-	-	-	+	-
Penicillium chrysogenum	+	-	-	+	+
Rhizopus stolonifer	-	-	-	-	+

counter the effect. Bioflocs have been shown to be an effective way of reducing *A. hydrophila*, a widespread disease-causing bacterium found all over the world, especially in intensive freshwater systems (Saavedra et al., 2004; Zmyslowska et al., 2009). Ulcers, depigmentation, fraying, and reddening of fins are all symptoms of *A. hydrophila* infections, which can be fatal to *C. gariepinus* and result in significant economic losses (FAO, 2016; Kusdarwati et al., 2017). As a result, BFT could be a good way to protect catfish from *A. hydrophila* infection during intensive culture.

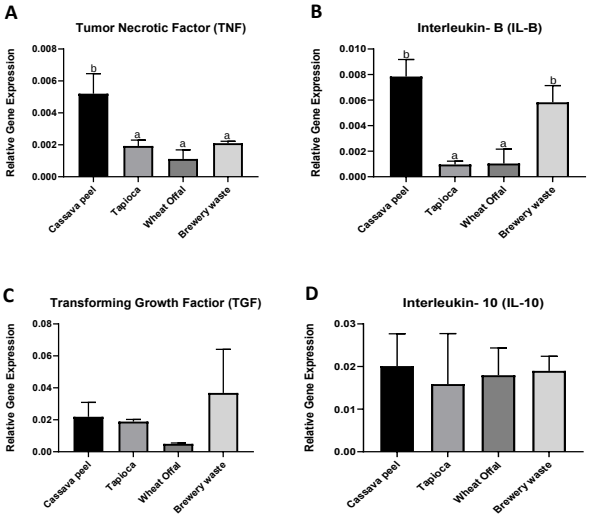

Figure 2. Mean fold change in gene expression + standard error in four treatment groups for four different genes A, B, C, D relative to the calibrator sample normalized against *GAPDH* ($p<0.05$).

Microorganisms such as bacteria and protozoa produce microbial proteins in ponds under aerobic and anaerobic conditions by decomposing organic debris and uneaten feed. According to Reddy and Patrick (1975), the aerobic decomposition process is usually faster than the anaerobic decomposition process because the presence of oxygen speeds up the rate of breakdown. Natural production, nutrient cycling, water quality, and the nutrition of farmed animals are all influenced by microorganisms (Moriarty, 1997; McIntosh et al., 2000). In many cases, the microorganisms identified as being involved in the above process are restricted. So yet, only about a quarter of naturally occurring bacteria have been isolated and characterised (Muyzer et al., 1993). The classification of the groups as microorganisms is common in most BFT culture investigations (Ballester et al., 2010; Ray et al., 2010; Loureiro et al., 2012; Emerenciano et al., 2013). As a result, the formation of a distinct, dominant, uniform, and diverse microbial community with traits including reproductive strategy, small size, short life cycle, and broad tolerance to environmental influences is postulated. These bacteria can also establish themselves as a result of system variables such as carbon supply, initial inoculum, and, as demonstrated in this study, the cultured species' behavior and nutritional habits.

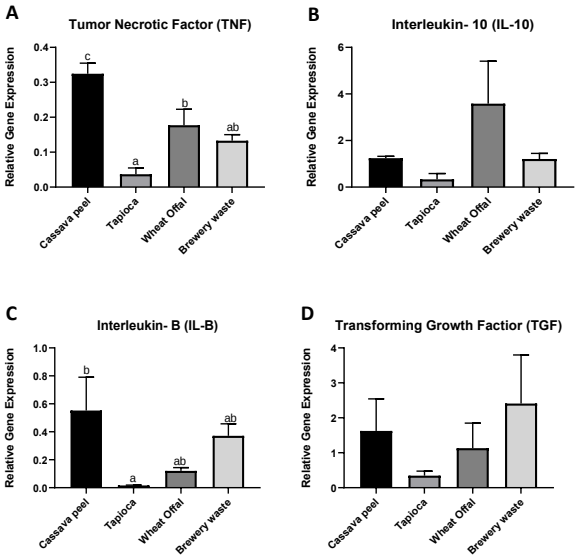

Figure 3. Mean fold change in gene expression + standard error in four treatment groups for four different genes A, B, C, D relative to the calibrator sample normalized against *Actin* ($p<0.05$).

The microbiota in the system has an impact on nutrient dynamics; these were characterised for Enterobacteriaceae, *Bacillus*, *Coccus*, and heterotrophic organisms, which agrees with the findings of Monroy-Dosta et al., (2013) who discovered heterotrophic bacteria such as *Pseudomonas, Bacillus, Vibrios, Enterobacter*, and *Micrococcus* in the microbial community connected with the bioflocs in tilapia culture. In farming settings, this species of bacteria promotes established channels for the elimination of hazardous nitrogen compounds. According to Ebeling et al. (2006), the main routes within the flow of nutrients in BFT culture systems are nitrogen elimination by photoautotrophic algae, nitrogen immobilisation by heterotrophic bacteria of the microbial protein biomass, and nitrogen chemo-autotrophic oxidation in nitrate by nitrifying bacteria. Depending on the type and intensity of the manufacturing system, the relative importance of each one varies. Although heterotrophic bacteria were encouraged to immobilise, the system was dominated by nitrifying bacteria in this study.

The importance of *Bacillus* colonies, which have probiotic properties, in the development of fish culture is underlined because these genera emit a wide range of exoenzymes and polymers that create a hostile environment for pathogenic bacteria (Monroy et al., 2010). Although routes of nitrification and recycling of nitrogen compounds exist under the minimum requirements of nutrients and the management of water quality in optimal ranges for the cultivation of the species, the system is maintained by established bacterial communities (Ayazo-Gene et al., 2019). The results of this study show that the presence of specific

microorganisms varied across treatments, and that a significant difference in CFU of total bacterial and SFU of total fungi could be attributed to differences in the composition of the carbon sources (simple and complex carbohydrate) used in the experiment, allowing us to conclude that the fungi and bacterial communities varied across all treatments. The current microbiota is linked to the system's dynamics, with a higher proportion of enterobacteria and heterotrophic bacteria. The immune system is divided into two parts: innate (non-specific) and adaptive (specific). Innate immunity serves as a barrier against infections, foreign substances, chemical agents, and environmental changes, and fish rely heavily on it. The innate immune system of fish is made up of several different components, each of which serves a specific purpose. Many research has been conducted to determine and understand the behavior of immune-related genes in both normal and pathologic states on *Acipenser dabryanus* (Dabry's sturgeon) (Zhang et al., 2018), on (*Carassius auratus*) (Qihecrucian carp) (Wang et al., 2013, 2016), on *Ctenopharyngodon idella* (grass carp) (Gou et al., 2018), on *Pelteobagrus fulvidraco* (yellow catfish) (Liu et al., 2016), on channel catfish (Prideon et al., 2013), and on *Oncorhynchus mykiss* (rainbow trout) (Yarahmadi et al., 2016). Inadequate research on *C. gariepinus* immune system is a key stumbling block to understanding immune system development, vaccine development, and immune stimulant evaluation. In addition, using marker assisted selection or selective breeding, bacterial resistance strains can be selected. There is still a lack of information about *C. gariepinus* immune-related genes, as well as evaluation of the immune response based on immune genes on the mRNA level following stress induction and bacterial infection. Due to the current state of *C. gariepinus* immune response, it is required to expand research on the expression of immune-related genes following a bacterial challenge. This paper reports on the expression of immune-related genes in the liver (a significant immunological organ in fish) of *C. gariepinus* maintained in bioflocs systems and challenged with *A. hydrophilia*, which will aid in a better understanding of the disease resistance mechanism.

When determining the biological significance of changing gene expression profiles, it's crucial to consider the amount of expression in comparison to the control (Gorgoglione et al., 2013) Relative quantification is a widely used approach for analysing gene expression, and as the name implies, it is an analysis based on the target gene's expression being normalised relative to the expression of a control gene (Kheirelseid et al., 2010). Changes in cytokine gene expression were detected between treatments and between the reference genes employed (Table 5). *TNF-α* and *IL-1β* were observed in the liver to generate a statistically significant increase in the fold among the treatments. In the cascade signaling of pro-inflammatory genes, *TNF-α* is most commonly the first cytokine secreted, which eventually leads to the downstream of *IL-1β* and other chemokines. In parasite and bacterial infections, cytokines such as *IL-1β* and *TNF-α* are key and crucial mediators of pro-inflammatory responses, and they are frequently co-expressed with other macrophage-derived inflammatory mediators such as *IL-1β*. The target genes' fold changes in all treatments, adjusted against *GAPDH*, were significantly lower than in the calibrator sample (Untreated), which could be due to the presence of probiotic bacteria in the bioflocs culture medium. These findings matched those of Xiao et al. (2019), who looked into the effect of *Clostridium butyricum* (CB) diet on yellow catfish (*Pelteobagrus fulvidraco*).

Table 5. Mean fold change in gene expression for four different genes in *C. gariepinus* raised in biofloc systems

Gene	Treatment	*Beta-Actin*	*GAPDH*
TGF-β	Control	1.05±0.74	**6.60±3.53** *
	Cassava peel	0.63±0.92	1.02±0.43
	Tapioca	**2.71±0.58**	0.87±0.07
	Wheat offal	1.24±1.07	1.06±0.11
	Brewery waste	0.08±0.95	1.60±0.84
TNF-α	Control	**3.82±0.83** **	**11.47±1.97****
	Cassava peel	**2.19±0.14***	**3.84±0.35** *
	Tapioca	1.12±0.79	**2.42±0.28**
	Wheat offal	1.27±0.38	1.45±0.80
	Brewery waste	0.89±0.19	**2.57±0.08**
IL-B	Control	0.23±0.76	**7.42±3.55** *
	Cassava peel	1.24±0.67	0.42±0.18
	Tapioca	**6.22±0.23****	**2.64±0.28**
	Wheat offal	**3.32±0.29** **	**3.14±1.47**
	Brewery waste	1.70±0.34	0.02±0.23
IL-10	Control	**6.54±0.23****	**14.19±2.57****
	Cassava peel	**6.85±0.10****	**8.50±0.40** **
	Tapioca	**4.40±1.36** **	**8.00±0.85** **
	Wheat offal	**8.16±0.81****	**8.34±0.37** **
	Brewery waste	**6.78±0.30****	**8.45±0.19** **

The value for the negative control group for each gene and treatment is always 1 and is therefore not shown. Greater than two-fold increases or decreases in gene expression relative to the negative control appear in bold, and statistically significant changes are marked with asterisks: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

The results showed that downregulation of *IL-10* expression in CB treated fish could have resulted in the anti-inflammatory effect of the probiotic administered after bacterial challenge. Probiotics have been shown to be effective against bacterial pathogens in aquaculture species (Chinabut & Puttinaowarat, 2005; Rendueles et al., 2012; Plaza-Diaz et al., 2014). The presence of this probiotic in the bioflocs culture system can increase the organism's tolerance to pathogenic invasion by inducing the secretion of anti-inflammatory cytokines such as IL-10 and TGF-β as standardised against Actin relative to the calibrator sample, the fold change seen in this study was down-regulated (*TNF-α* and *IL-1β*), with the tapioca-based treatment being the most down-regulated among the treatments when compared to the control. This could explain why, in response to the inflammatory response, pro-inflammatory genes were activated more in the tapioca-

based bioflocs than in the other treatments. Other immune-related signaling pathways and enzyme activity production are affected by these inflammatory substances. As a result, *IL-1β* and *TNF-α* are two major pro-inflammatory molecules that can trigger an inflammatory response by controlling the expression of other cytokines (Zhang et al., 2018). It's worth noting that the bioflocs culture technique drastically lowered *TNF-α* and *IL-1β* expression in this study.

The immune response genes *TGF-β* and *IL-10* in *C. gariepinus* were up-regulated in the bioflocs-based treatments, with the exception of the tapioca-based bioflocs system, which was down-regulated compared to a calibrator sample (Untreated). The up-regulation of these anti-inflammatory cytokines shows that the pro-inflammatory response began early in the challenge, since *IL-10* was up-regulated in this study compared to the negative control. *IL-10* regulates the immunological response of the fish by preventing macrophages from releasing pro-inflammatory cytokines such as *TNF-α*, *IL-2*, and *IL-3*. *TNF*, *IL-1β* may have been down-regulated because of this. The regulating effect of *IL-10* on *IL-1β* expression has been found in Indian main carp (*Catla catla*) (Moore et al., 2001; Swain et al., 2011), *IL-10* modulates inflammatory responses and potently inhibits the production of various cytokines including interleukin1 and TNF-α.

TGF-β is a pleiotropic cytokine that regulates inflammatory response onset and resolution. *TGF-β* expression is up-regulated in rainbow trout when parasite pathogens are produced, according to research, and infection with IHNV on rainbow trout expression (Holland et al., 2003; Lindenstrom et al., 2004; Purcell et al., 2004). In this study, the bioflocs system increased *TGF-β* expression in all treatments when compared to the calibrator sample, indicating that the fish grown in the bioflocs system had a higher level of resistance. Although the fold change was normal in magnitude, it was statistically significant between the control and treatment groups.

Conclusion

Due to its numerous advantages, bioflocs technology is one of the most widely used advanced culture methods in shrimp and fish farming. It provides nourishment for the cultured animals and increases the farm's biosecurity while requiring little or no water exchange. This study and its findings have shed light on the impact of bioflocs consumption on *C. gariepinus* immunological performance and gene expression. This provides important information about bioflocs supplementation in feed and its development in culture ponds for the maintenance of optimal water quality parameters, growth performance, and immune gene regulation in *C. gariepinus* grow-out culture systems.

Funding Information

The research work was supported by the Tertiary Education TrustFund (TETFUND) [grant numbers VCPU/TETFund/155C.

Author Contributions

Conceptualization: OMP, Data Curation: AMO, STT, Formal Analysis: AMO, Funding Acquisition: AMO, STT, OMP, Investigation: AMO, STT, Methodology: OMP, AMO, Project Administration: AMO, STT, Resources: AMO, STT, OMP, Supervision: OMP, Visualization: OMP, Writing -original draft: AMO, Writing -review and editing: AMO, STT, OMP.

Acknowledgments

The authors are grateful to the Department of Fisheries and Aquaculture Technology, Department of Microbiology, Federal University of Technology, Akure, and National Center for Genetic Resources and Biotechnology (NACGRAB), Ibadan, for their financial, technical, and logistical support during sampling. We are also grateful to Dr. Oduoye M, and Mr Fred Ali their assistance with field and laboratory work.

References

Abbott, W. S. (1925) A method of computing the effectiveness of an insecticide, *Journal* of *Economic Entomology*, 18, 265–267.

Adebayo, O. O., & Daramola, A. O. (2013). Economic analysis of catfish (*Clarias gariepius*) production in Ibadan metropolis. *Discourse Journal of Agriculture and food sciences*, *1*(7), 128 - 134

Ahmad, I., Verma, A. K., Rani, A. M. B., Rathore, G., Saharan, N. & Gora, A. H. (2016). Growth, non-specific immunity and disease resistance of *Labeo rohita* against *Aeromonas hydrophila* in biofloc systems using different carbon sources. *Aquaculture*, *457*, 61–67. https://doi: 10.1016/j.aquaculture.2016.02.011.

Amend, D. F. (1981). Potency testing of fish vaccines. *Fish biologics: serodiagnostics and vaccines*, 447-454.

APHA (1998). Standard method for the examination of water and wastewater. 20th edition, American Public Health Association, American Waters Works Association and Water Environmental Federation, Washington DC.

Avnimelech, Y. (1999). Carbon and nitrogen ratio as a control element in aquaculture systems. *Aquaculture*, *176*, 227–235. https://doi.org/10.1016/S0044-8486(99)00085-X.

Ayazo-Genes, J., Pertúz-Buelvas, V., Jiménez-Velásquez, C., Espinosa-Araujo, J., Atencio-García, V., & Prieto-Guevara, M. (2019). Describing the planktonic and bacterial communities associated with bocachico *Prochilodus magdalenae* fish culture with biofloc technology. *Revista MVZ Córdoba*, *24*(2), 7209-7217. https://doi.org/10.21897/rmvz.1648.

Azim, M. E. & Little, D. C., (2008). The Biofloc Technology (BFT) in indoor tanks: Water quality, biofloc composition and growth and welfare of Nile tilapia (*Oreochromis niloticus*). *Aquaculture*, *283*, 29-35. https://doi.org/10.1016/j.aquaculture.2008.06.036.

Ballester, E., Abreu, P., Cavalli, R., Emerenciano, M., Abreu, L., & Wasielesky, W. (2010). Effect of practical diets with different protein levels on the performance of *Farfantepenaeus paulensis* juveniles nursed in a zero-exchange suspended microbial flocs intensive system. *Aquaculture Nutrition*, *16*, 163-172. http://dx.doi.org/10.1111/j.1365-2095.2009.00648.x.

Chinabut, S., & Puttinaowarat, S. (2005). The choice of disease control strategies to secure international market access for aquaculture products. *Developments in Biologicals*, *121*, 255–261.

Crab, R., (2010). Bioflocs technology: an integrated system for the removal of nutrients and simultaneous production of feed in aquaculture. PhD thesis, Ghent University. 178 pp.

Crab, R., Avnimelech, Y., Defoirdt, T., Bossier, P., & Verstraete, W., (2007). Nitrogen removal techniques in aquaculture for a sustainable production. *Aquaculture, 270*, 1–14. http://dx.doi.org/10.1016/j.aquaculture.2007.05.006

Crab, R., Lambert, A., Defoirdt, T., Bossier, P., & Verstraete, W., (2010). Bioflocs protect gnotobiotic brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio harveyi*. *Journal of Applied Microbiology, 109*, 1643–1649. https://doi.org/10.1111/j.1365-2672.2010.04791.x

Ebeling, J. M., Timmons, M. B., & Bisogni, J. J. (2006). Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia–nitrogen in aquaculture systems. *Aquaculture*, *257*(1-4), 346-358. https://doi.org/10.1016/j.aquaculture.2006.03.019

Ekasari, J., Azhar, M. H., Surawidjaja, E. H., Nuryati, S., De Schryver, P., & Bossier, P. (2014). Immune response and disease resistance of shrimp fed biofloc grown on different carbon sources. *Fish Shellfish Immunology*, *41*, 332–339. https://doi.org/10.1016/j.fsi.2014.09.004.

Emerenciano, M., Gaxiola, G., & Cuzon, G. (2013) Biofloc technology (BFT): a review for aquaculture application and animal food industry. In: Biomass now – cultivation and utilization (ed. by M.D. Matovic). InTech, Queen's University, Belfast, pp 301–328

Food and Agriculture Organization (FAO) (2016). Cultured Aquatic Species Information Programme *Penaeus Monodon* (Fabricius, 1798). Presence of giant tiger shrimp Penaeus monodon Fabricius, 1798 on the Mexican coast of the Gulf of Mexico. Available from: https://www.researchgate.net/publication/259390783 Presence of giant tiger shrimp Penaeus monodon Fabricius 1798 on the Mexican coast of the Gulf of Mexico [accessed Oct 19 2021].

Gorgoglione, B., Wang, T., Secombes, C. J., & Holland, J. W. (2013). Immune gene expression profiling of proliferative kidney disease in rainbow trout *Oncorhynchus mykiss* reveals a dominance of anti-inflammatory, antibody and T helper cell- like activities. *Veterinary Research, 44*(1), 1-16. https://doi.org /10.1186/1297-9716-44-55.

Gou, C., Wang, J., Wang, Y., Dong, W., Shan, X., Lou, Y., & Gao, Y. (2018). *Hericium caput-medusae* (Bull.:Fr.) Pers. polysaccharide enhance innate immune response, immune-related genes expression and disease resistance against *Aeromonas hydrophila* in grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunology, 72*, 604-610. https://doi.org/10.1016/j.fsi.2017.11.027.

Hargreaves, J. A. (2006). Photosynthetic suspended-growth systems in aquaculture. *Aquaculture Engineering, 34*, 344–363. https://doi.org/10.1016/j.aquaeng.2005.08.009.

Holland, J. W., Gould, C. R., Jones, C. S., Noble, L. R., & Secombes, C. J. (2003). The expression of immune-regulatory genes in rainbow trout, *Oncorhynchus mykiss,* during a natural outbreak of proliferative kidney disease (PKD). *Parasitology*, *126*, S95e102. https://doi.org/10.1017/S0031182003003767.

Ita, E. O. (1980). The review of recent advances in warm water aquaculture research and proposed experimental design for maximizing fish production in Nigerian fish pond. *Lake kainji research institute,* 1-59.

Janda, J. M., & Abbott, S. L. (2010). The Genus *Aeromonas*: Taxonomy, Pathogenicity, and Infection. *Clinical Microbiology Reviews*, *23*(1), 35–73. https://doi.org/10.1128/cmr.00039-09.

Joseph, S. W., & Carnahan, A. (1994). The isolation, identification, and systematics of the motile *Aeromonas* species. *Annual Review of Fish Diseases*. *4*, 315–343. https://doi.org/10.1016/0959-8030(94)90033-7.

Karunasagar, I., Rosalind, G. M., & Karunasagar, I. (1991). Immunological response of the Indian major carps to *Aeromonas hydrophila* vaccine. *Journal of Fish Diseases, 14*, 413–417. https://doi.org/10.1111/j.1365-2761.1991.tb00841.x.

Kheirelseid, E. A, Chang, K. H., Newell, J., Kerin, M. J., & Miller, N. (2010). Identification of endogenous control genes for normalisation of real-time quantitative PCR data in colorectal cancer. *BMC Molecular Biology*, *11*(1), 1-13. https://doi.org/10.1186/1471-2199-11-12.

Kusdarwati, R., Kurniawan, H., & Prayogi, Y. T. (2017, February). Isolation and identification of *Aeromonas hydrophila* and *Saprolegnia* sp. on catfish (*Clarias gariepinus*) in floating cages in Bozem Moro Krembangan Surabaya. In *IOP Conference Series: Earth and Environmental Science*, *55*(1), 012038. IOP Publishing.

Lindenstrøm, T., Secombes, C. J., & Buchmann K. (2004). Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections. *Vetenary Immunology & Immunopathology*, *97*(3-4), 137-48. https://doi:10.1016/j.vetimm.2003.08.016.

Liu, Q. N., Xin, Z. Z., Zhang, D. Z., Jiang, S. H., Chai, X. Y., Li, C. F., Zhou, C. L., & Tang, B. P. (2016). Molecular identification and expression analysis of a *goose-type lysozyme* (*LysG*) gene in yellow catfish *Pelteobagrus fulvidraco*. *Fish and Shellfish Immunology, 58*, 423–428. https://doi.org/10.1016/j.fsi.2016.09.034.

Loureiro, K., Wilson, W., & Abreu, P. (2012). Use of protozoa, rotifers and nematodes as live feed for shrimp cultured in the BFT system. *Atlântica Rio Grande*, *34*(1), 5-12.

McIntosh, D., Samocha, T. M., Jones, E. R., Lawrence, A. L., McKee, D. A., Horowitz, S., & Horowitz, A. (2000). The effect of a bacterial supplement on the high-density culturing of *Litopenaeus vannamei* with low protein diet in outdoor tank system and no water exchange. *Aquacultural Engineering, 21*, 215–227. https://doi.org/10.1016/S0144-8609(99)00030-8.

Milstein, A., Avnimelech, Y., Zoran, M., & Joseph, D. (2001). Growth performance of hybrid bass and hybrid tilapia in conventional and active suspension intensive ponds. *Isreali Journal of Aquaculture-Bamidgeh, 53*, 147–157. http://hdl.handle.net/10524/19037.

Monroy, D., Castro, B., Fernández, P., & Mayorga, R. (2010). Inhibition of *Aeromonas hydrophila* by probiotic strains isolated from the digestive tract of *Pterophyllum scalare*. *Revista Mexicana Ingenieria Química*, *9*(1), 37-42.

Monroy-Dosta, M., De Lara-Andrade, R., Castro-Mejia, J., Castro-Mejia, G., Coelho-Emerenciano, M. (2013). Composición y abundancia de comunidades microbianas asociadas al biofloc en un cultivo de tilapia. *Revista de biología marina y oceanografía*, *48*(3), 511-520. http://dx.doi.org/10.4067/S0718-19572013000300009

Moore, K. W., de Waal Malefyt, R., Coffman, R. I., O'Garra, A. (2001). Interleukin 10 and interleukin 10 receptor. *Annual Review of* Immunology, *19*, 683-765. https://doi.org/10.1146/annurev.immunol.19.1.683

Moriarty, D. J. W. (1997). The role of microorganisms in aquaculture ponds. *Aquaculture*, *151*, 333–349. https://doi.org/10.1016/S0044-8486(96)01487-1.

Muyzer, G., De Waal, E. C., & Uitterlinden, A. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*, *59*(3), 695-700. https://doi.org/10.1128/aem.59.3.695-700.1993

Plaza-Diaz, J., Gomez-Llorente, C., Fontana, L., & Gil, A. (2014). Modulation of immunity and inflammatory gene expression in the gut, inflammatory diseases of the gut and in the liver byprobiotics. *World Journal of Gastroenterology*, *20*(42), 15632–15649. https://dx.doi.org/10.3748%2Fwjg.v20.i42.15632.

Pridgeon, J. W., Klesius, P. H., Dominowski, P. J., Yancey, R. J., & Kievit, M. S. (2013). Chicken-type lysozyme in channel catfish: Expression analysis, lysozyme activity, and efficacy as immunostimulant against *Aeromonas hydrophila* infection. *Fish & Shellfish Immunology*, *35*(3), 680-688. https://doi.org/10.1016/j.fsi.2013.05.018.

Purcell, M. K., Kurath, G., Garver, K. A., Herwig, R. P. & Winton, J. R. (2004). Quantitative expression profiling of immune response genes in rainbow trout following infectious haematopoietic necrosis virus (IHNV) infection or DNA vaccination. *Fish Shellfish Immunology, 17*, 447- 462. https:/doi: 10.1016/j.fsi.2004.04.017. PMID: 15313511.

Ray, A. J., Seaborn, G., Leffler, J. W., Wilde, S.B., Lawson, A., & Browdy, C. L. (2010). Characterization of microbial communities in minimal-exchange, intensive aquaculture systems and the effects of suspended solids management. *Aquaculture 310*, 130–138. https://doi.org/10.1016/j.aquaculture.2010.10.019.

Reddy, K. R., & Patrick, J. W. H. (1975). Effect of alternate aerobic and anaerobic conditions on redox potential, organic matter decomposition and nitrogen loss in a flooded soil. *Soil Biology and Biochemistry*, *7*, 87-94. https://doi.org/10.1016/0038-0717(75)90004-8.

Rendueles, O., Ferri`eres, L., & Fr´etaud, M. (2012). A new zebrafish model of oro-intestinal pathogen colonization reveals a key role for adhesion in protection by probiotic bacterial. *PLoS Pathogens*, *8*(7), e1002815. https://doi.org/10.1371/journal.ppat.1002815.

Saavedra, M. J., Guedes-Novais, S., Alves, A., Rema, P., Tacao, M., Correia, A., & Martinez-Murcia, A., (2004). Resistance to B-Lactam antibiotics in *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus mykiss*). *International Microbiology, 7*, 201–211.

Sabur, M. A. (2006). Studies on the ecology of the pathogenic bacteria Aeromonas hydrophila in indigenous and exotic carps under polyculture condition. *A Ph. D. Dissertation. Department of Aquaculture, Bangladesh Agricultural University, Mymensingh Bangladesh*, *136*.

Svobodova, Z., Pravda, D., & Palackova, J. (1991). *Unified methods of haematological examination of fish*. Research Institute of fish culture and hydrobiology.

Swain, B., Samanta, M., Basu, M., Panda, P., Sahoo, B. R., & Maiti, N. K. (2011). Molecular characterization, inductive expression and mechanism of interleukin 10 gene induction in indian major carp *Catla catla*. *Aquaculture Research, 43, 897–907,* https://doi.org/10.1111/j.1365-2109.2011.02904.x.

Ture, M., Ozcelep, T., Akbulut, B., & Kutlu, I. (2018). Disease of Russian sturgeon (*Acipenser gueldenstaedtii*) caused by *Aeromonas* sp. *Genetics of Aquatic Organisms*, *2*(2), 43-47. https://doi.org/10.4194/2459-1831-v2_2_03

Wang, M., Zhao, X., Kong, X., Wang, L., Jiao, D., & Zhang, H. (2016). Molecular characterization and expressing analysis of the c-type and g-type lysozymes in Qihecrucian carp *Carassius auratus. Fish and Shellfish Immunology, 52*, 210–220. https://doi.org/10.1016/j.aqrep.2019.100198.

Wang, R., Feng, J., Li, C., Liu, S., Zhang, Y., & Liu, Z. (2013). Four lysozymes (one c-type and three g-type) in catfish are drastically but differentially induced after bacterial infection. *Fish Shellfish and Immunology, 35*, 136–145. https://doi.org/10.1016/j.fsi.2013.04.014.

Xiao, Y., Yu, L., Gui, G., Gong, Y., Wen, X., Xia, W., Yang, H., & Zhang, L. (2019). Molecular cloning and expression analysis of interleukin-8 and -10 in yellow catfish and in response to bacterial pathogen infection. *Biomed Research International*, *6*, 1-9 http://dx.doi.org/10.1155/2019/9617659.

Xie, B., & Yu, K. J. (2007). Shrimp farming in China: operating characteristics, environmental impact and perspectives. *Ocean and Coastal Management, 50*, 538–550.

http://dx.doi.org/10.1016/j.ocecoaman.2007.02.006.

Xu, W. J., & Pan, L. Q. (2013). Enhancement of immune response and antioxidant status of *Litopenaeus vannamei* juvenile in biofloc-based culture tanks manipulating high C/N ratio of feed input. *Aquaculture, 412*, 117–124. https://doi.org/10.1016/j.aquaculture.2013.07.017.

Yarahmadi, P., Miandare, H. K., Fayaz, S., & Caipang, C. M. A. (2016). Increased stocking density causes changes in expression of selected stress- and immune-related genes, humoral innate immune parameters and stress responses of rainbow trout *Oncorhynchus mykiss*. *Fish and Shellfish Immunology*, *48*, 43–53. https://doi.org/10.1016/j.fsi.2015.11.007.

Zhang, S., Xu, Q., Boscari, E., Du, H., Qi, Z., Li, Y., Huang, J., Di, J., Yue, H., Li, C., Congiu L, & Wei, Q. (2018). Characterization and expression analysis of g- and c-type lysozymes in Dabry's sturgeon *Acipenser dabryanus. Fish and Shellfish Immunology, 76*, 260–265. https://doi.org/10.1016/j.fsi.2018.03.006.

Zmyslowska, I., Korzekwa, K., & Szarek, J. (2009). *Aeromonas hydrophila* in fish aquaculture. *Journal of Comparative Pathology*, *141*, 313 http://dx.doi.org/10.1016/j.jcpa.2009.08.142.

Biotech Studies 31(1), 28-35
https://doi.org/10.38042/biotechstudies.1103767

BIOTECH STUDIES

RESEARCH PAPER

Evaluating the microbial growth kinetics and artificial gastric digestion survival of a novel *Pichia kudriavzevii* FOL-04

İsmail Gumustop, Fatih Ortakci*

Department of Bioengineering, Faculty of Life and Natural Sciences, Abdullah Gul University, Kayseri, 38080, Turkey.

How to cite

Gumustop, İ. & Ortakci, F. (2022). Evaluating the microbial growth kinetics and artificial gastric digestion survival of a novel *Pichia kudriavzevii* FOL-04. *Biotech Studies. 31*(1), 28-35. http://doi.org/10.38042/biotechstudies.1103767

Article History
Received 22 October 2021
Accepted 05 April 2022
First Online 13 April 2022

Corresponding Author
Tel.: +90 352 224 8800
E-mail: fatih.ortakci@agu.edu.tr

Keywords
Pichia kudriavzevii FOL-04
Probiotic
Bioreactor
Growth kinetics
Acid
Bile

Abstract

Present study aims to explore *Pichia kudriavzevii* FOL-04 (**FOL-04**)'s: i) survival against artificial gastric juice (**AGJ**) and artificial bile juice (**ABJ**), ii) growth kinetics in shake flask (**SF**) and fed-batch trials (**FBT**). Survival of FOL-04 as measured by relative cell density (**RCD**) against AGJ and ABJ was screened at four different pH-levels (control, 3, 2, 1.5) and ox-bile concentrations (control, 0.2%, 1%, 2%), respectively. Growth kinetics was calculated by periodic measurement of OD_{600} in SF (225 rpm, 30°C) or in FBT using exponential feeding regimen where pH, dissolved-oxygen and temperature were controlled at 5.5, 21%, and 30°C, respectively. The doubling-time, maximum specific growth rate, and final cell densities achieved for SF and FBT were 81.7min, 1.67, 11.79 and 170.4 min, 4.75, 37.95, respectively. RCDs calculated were similar for pH=3 and control vs both were significantly higher($p<0.05$) than pH=1.5 and 2 with the latter two pH-levels were not significantly different($p>0.05$). RCDs were similar across control, 0.2%, and 1% ox-bile levels($p>0.05$). However, 2% ox-bile yielded significantly lower RCD ($p<0.05$) compared to all except 1%. FOL-04 is a potential probiotic candidate showing robustness against AGJ and ABJ and remarkable biomass increase was achieved when grown under FBT which could pave the way for developing a yeast-based probiotic using this strain.

Introduction

Yeast is a eukaryotic microorganism that is widely used for the fermentation of alcohol or acids to produce different fermented products such as baking dough, beer, wine, and vinegar. The first use of yeast for fermentation by humans' dates before 8000 BC (Liti, 2015). Some yeast strains have probiotic characteristics that help to prevent certain intestinal disorders. McFarland and Bernasconi (1993) stated that *Saccharomyces boulardii* treatment could inhibit *Clostridioides difficile*, a toxin secreting bacterium that causes nosocomial diarrhea in adults.

Strains of *Pichia kudriavzevii* are widespread in the environment and are often seen in sporadic fermentations (Douglass et al., 2018). The *P. kudriavzevii* strains are also used to manufacture various traditionally fermented foods (Kurtzman, 2011; Smukowski Heil et al., 2018). This species is not under the pathogens list and possesses GRAS status (Bourdichon et al., 2012) due to its safe use over centuries to make fermented foods such as cassava and cacao in Africa, fermented milk in Tibet and Sudan, and maize beverages in Colombia (Bourdichon et al., 2012). It is also utilized as a starter culture for Chinese vinegar and sourdough (De Vuyst et al., 2016; Li et al., 2014). *P. kudriavzevii*'s potential utilization includes probiotic applications (Chelliah et al., 2016) owing to its high stress-tolerance thus displays a growing role in the biotechnology industry such as production of bioethanol (Mukherjee et al., 2017; Radecka et al., 2015), succinic acid, and glycerol (Wang et al., 2001; Xiao et al., 2014).

Shalgam is a non-alcoholic traditional beverage manufactured in Turkey. It is produced by lactic acid fermentation of turnip and red carrot (Karaoglan et al., 2019). Studies showed that predominant microbiota of Shalgam is composed of lactic acid bacteria (LAB) and yeast organisms where *Lactobacillus plantarum* is the most abundant LAB in Shalgam. There are other LAB in Shalgam such as *L. paracasei, L. brevis, and L. fermentum* (Tanguler & Erten, 2012). Also, several health promoting compounds exist in Shalgam, such as anthocyanins and phenolic compounds (Konczak & Zhang, 2004).

Although majority of research on probiotics focused on lactic acid bacteria, there is a growing trend on finding new yeast strains carrying probiotic effects (Hatoum et al., 2012; Kumura et al., 2004; Moslehi-Jenabian et al., 2010; Perricone et al., 2014). As a general approach, most probiotics are evaluated according to their capability to survive and subsequently colonize in the gastrointestinal system. In fact, survival against gastrointestinal conditions and the ability to attach to the intestinal mucosa is crucial to reach the target organ in certain numbers to confer health benefits to the hosts (Ouwehand et al., 2002). In the gastrointestinal tract, firstly, probiotics must pass through the harsh acidic conditions of the stomach that exerts a strong barrier for entry to the gut (Greppi et al., 2017).

To date, relatively few artificial gastric juice and artificial bile juice survival tests, which are the fundamental challenge conditions that probiotics come across when ingested (Sun & Griffiths, 2000; Yetiman et al., 2022; Klaenhammer & Kleeman, 1981; Song et. al., 2003), were explored for *P. kudriavzevii* strains. Also, bioprocess studies targeting *P. kudriavzevii* microbial biomass production characteristics are limited if any. To fill those gaps in the literature i) survival of a novel *P. kudriavzevii* strain FOL-04, isolated from traditional Shalgam, was evaluated under artificial gastric and bile juice conditions, ii) shake flask and fed-batch bioreactor experiments were conducted to explore and compare growth kinetics of *P. kudriavzevii* FOL-04 under two bioprocess conditions. This is the first report describing artificial gastric and bile juice tolerance in addition to microbial growth kinetics of a new *P. kudriavzevii* strain isolated from Shalgam produced in the Southern Anatolia region.

Materials and Methods

Isolation of *P. kudriavzevii* FOL-04

A traditionally fermented lactic acid beverage called Shalgam was purchased from a local store in Southern Anatolia region. After performing serial dilutions of liquid Shalgam sample, Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Merck, Germany) was used to grow yeast strains. The FOL-04 was streak plated and purified for further DNA isolation, PCR, Sanger sequencing, survival against artificial gastric and bile juice, and microbial growth kinetic determinations.

DNA Isolation and PCR

DNA of the yeast sample was isolated with MACHEREY-NAGEL's NucleoSpin® Microbial DNA Mini kit according to yeast DNA extraction protocol. The following primers were used to amplify the 5.8S-ITS rRNA region and D1/D2 domains of the 26S rRNA region:
ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'),
ITS4 (5'-TCCTCCGCTTATTGATATGC-3'),
NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3'),
NL4 (5'-GGTCCGTGTTTCAAGACGG-3'), respectively.

Reagents of PCR amplification (EasyTaq® DNA Polymerase, 10X EasyTaq ® Buffer, 2.5 mM dNTPs, 6X DNA loading dye) was obtained from Transgen Biotech, otherwise specified. A 50 µl of total PCR master mix was prepared by adding 1 µL of templated DNA, forward and reverse primers at 0.2 µM final concentration, EasyTaq® buffer at 1X final concentration, dNTPs at 0.2 µM final concentration, 0.5 µL of EasyTaq® DNA polymerase, and 37.5 µL of nuclease-free water. Thermal cycling started with the first denaturation at 95°C for 5 minutes. Then, it continued with denaturation at 94°C for 30 seconds, annealing at 55°C for ITS1/4, 52.5°C for NL1/4 for 30 seconds. After annealing, samples were extended at 72°C for 2 minutes. Denaturation, annealing, and extension phases were repeated 36 times. Samples were incubated at 72°C for 10 minutes for the last extension, followed by running samples on agarose gel, and the NL amplified DNA fragment was sent for Sanger sequencing. The sequencing results were analyzed at the BLAST at blast.ncbi.nlm.nih.gov (Altschul et al., 1990).

Cultivation in Shake Flasks

Yeast extract-peptone-dextrose (YPD) media, containing 1% yeast extract, 2% peptone, and 2% dextrose (g/L), was used in shake flask cultivations. Firstly, 5 mL of pre-inoculated for 16 hours with the *P. kudriavzevii* YPD media was mixed with 65 mL of YPD media inside the Erlenmeyer flask. Then, the Erlenmeyer flask was incubated at 30°C at 225 rpm in a shaker incubator for 24 hours. Samples were taken every hour to measure optical density at 600 nm wavelength. Doubling time (minutes) was calculated during the exponential phase as follows (Roth, 2006)

$$t_d = \frac{duration \cdot log(2)}{log(finalOD) \cdot log(initialOD)}$$

Where the t_d is doubling time. Maximum specific growth rate (μ_{max}) was calculated by measuring the slope of the steepest linear portion of the growth curve.

Cultivation in Fed-batch Bioreactors

For optimizing *P. kudriavzevii* FOL-04's growth conditions and increase its biomass yield, fed-batch cultivations were performed. Bioreactor cultivations were performed at 700 mL YPD media (50 mL pre-inoculated and 650 ml sterile YPD media). The composition of YPD media was adjusted to 1% yeast

extract, 2% peptone, and 1% dextrose (g/L) to shorten the batch phase. The dissolved oxygen level was set at 25% and controlled with airflow and stirrer cascade. pH was set to 5.5, and the temperature was set to 30°C to achieve the highest biomass (Ndubuisi et al., 2020) during both batch and fed-batch cultivations. Exponential feeding regimen was tested on the growth kinetics of *P. kudriavzevii* FOL-04. A 50% dextrose feed solution was used during fed-batch phase, which started upon arrival of batch fermentation which was evident with no more base consumption in alignment with pH curve flattening. Fed-batch cultivations lasted for around 22 hours, and samples were taken every 2 hours to measure the biomass yield of the *P. kudriavzevii* FOL-04 by measuring its optical density at 600 nm. Doubling time (minutes) was calculated during the exponential phase as follows (Roth, 2006):

$$t_d = \frac{duration \cdot log(2)}{log(finalOD) \cdot log(initialOD)}$$

Where the t_d is doubling time. Maximum specific growth rate (μ_{max}) was calculated by measuring the slope of the steepest linear portion of the growth curve.

Artificial Gastric Juice

To investigate the influence of pH on survival of *P. kudriavzevii* FOL-04, a modified method from Sun & Griffiths (2000) and Yetiman et al. (2022) was applied to prepare artificial gastric juice. First, YPD media was prepared according to manufacturer's instructions and following pH conditions were adjusted using HCl solution: control (no acid supplementation), pH = 1.5, pH = 2, and pH = 3 with each treatment being prepared in six replicates. Each treatment media was autoclaved at 121°C for 15 minutes at 2 atmospheres. Each sterile treatment media was inoculated with fresh culture of *P. kudriavzevii* FOL-04 cells. Each treatment tube was incubated at 30°C under 225 rpm shaking conditions for two days. Then, the optical density of each treatment tube was measured with Shimadzu UVmini-1240 spectrophotometer at 600 nm wavelength. The experiments were performed in six replicates and results were presented as relative cell density ratio (OD_{600} t_{final}/OD_{600} t_0). Statistical analysis of relative cell density ratios were performed with the analysis of variance (ANOVA) and Tukey tests in R programming language (R Core Team, 2020).

Artificial Bile Juice

Artificial bile juice (ABJ) was prepared according to a modified method of Klaenhammer & Kleeman (1981), Song et. al. (2003), and Yetiman et al. (2022). Simply, YPD broth supplemented with following concentrations of ox bile extract (Sigma, Germany): control (no bile), 0.2%, 1%, and 2% by w/v (Yetiman et al., 2022) was autoclaved at 121°C for 15 minutes at 2 atmospheres. Each sterile treatment media was inoculated with fresh culture of *P. kudriavzevii* FOL-04 cells. Each treatment tube was incubated at 30°C under 225 rpm shaking conditions for two days. Then, the optical density of each treatment tube was measured with Shimadzu UVmini-1240 spectrophotometer at 600 nm wavelength. The experiments were performed in six replicates and results were presented as relative cell density ratio (OD_{600} t_{final}/OD_{600} t_0). Statistical analysis of relative cell density ratios were performed with the analysis of variance (ANOVA) and Tukey tests in R programming language (R Core Team, 2020).

Results

PCR Fingerprinting and Sanger Sequencing

After running PCR samples on the agarose gel, both ITS and NL primers amplified PCR products yielded clear bands on pulse field gel electrophoresis. However, non-specific binding was seen on the sample amplified with ITS primer (Figure 1). The sample that was amplified with NL primers had only one clear band on the gel image which shows specific amplicon has been achieved. Therefore, the NL primers amplified DNA fragment of yeast isolate was sent to Sanger sequencing for strain level identification (Sanger et al., 1977).

Figure 1. Gel image of PCR fingerprinting assay of *P. kudriavzevii* FOL-04. There is a non-specifically bonded fragment on ITS.

Sanger sequencing results were analyzed with the Basic Local Alignment Tool (BLAST) (Altschul et al., 1990). BLAST results show that the isolated *P. kudriavzevii* FOL-04 has the highest homology against *P. kudriavzevii* feni92 (KM234470.1) and *P. kudriavzevii* cs280 (KM234470.1) with 98.93% and 58% identity score and query cover score achieved, respectively. BLAST results are represented in a phylogenetic tree to show the phylogenetic distance of species against the *P. kudriavzevii* FOL-04 (Figure 2) (Paradis & Schliep, 2019).

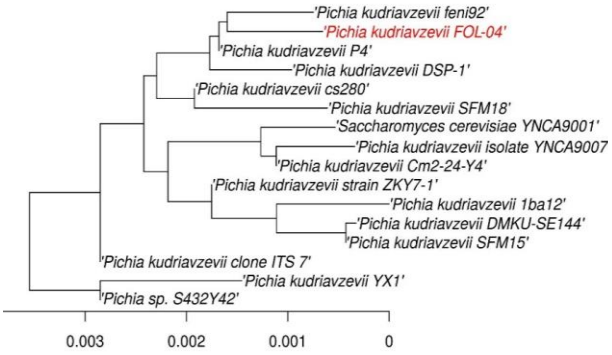

Figure 2. The phylogenetic distance between species and *P. kudriavzevii* FOL-04 (red) based on D1/D2 domains of the 26S rRNA region.

Biomass in Shake Flasks

The biomass yield of *P. kudriavzevii* was observed by measuring optical density values at 600 nm. Results showed that the lag phase he *P. kudriavzevii* strain was around 6 hours and the exponential phase took around 8 hours (Figure 3). During the shake flask cultivation, μ_{max} achieved was 1.67/hour, and the doubling time was calculated as 81.7 minutes.

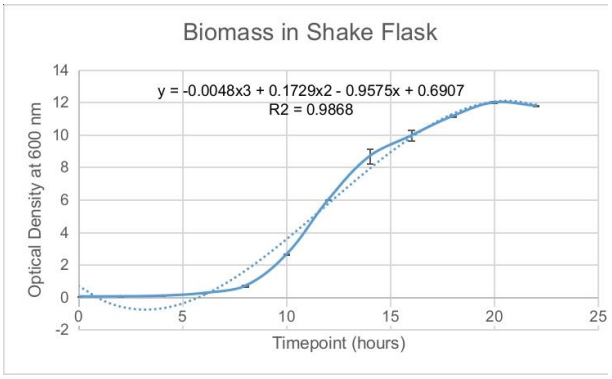

Figure 3. Time vs optical density at 600 nm results of shake flask cultivations.

Biomass in Fed-batch Bioreactor Cultivation

The biomass yield of the *P. kudriavzevii* FOL-04 was measured with the same method that was applied for the shake flask cultivations. Results showed that the lag phase of the *P. kudriavzevii* strain was shortened to 4 hours. The exponential phase took up to 8 hours. Moreover, biomass yield increased after the deceleration phase perhaps due to feeding with 50% dextrose (Figure 4).

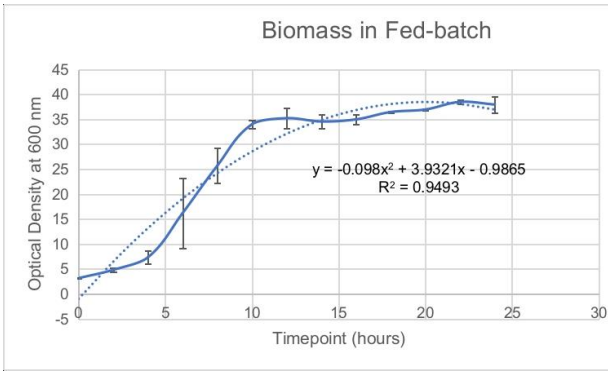

Figure 4. Time vs optical density at 600 nm results of fed-batch bioreactor cultivations.

During the fed-batch cultivation, μ_{max} achieved was 4.75/hour and doubling time calculated was 170.4 min. The first 12 hours of bioreactor cultivation, similar biomass results were achieved. After that, the difference in biomass of the *P. kudriavzevii* strain got bigger in favor of the yeast fed with the exponential feeding regimen. Figure 5 shows that pH was oscillating at 5.4 at the beginning of the batch process. At that time base pump works to increase the pH to 5.5. When the pH exceeds the 5.5 base pump stops to control pH. Base pump restarts to work around 13 h time point to adjust the pH of the vessel. Pre-set dissolved oxygen levels throughout the fed-batch process was adjusted by stirrer and airflow intake through cascade system (Figure 6).

Survival Against AGJ and ABJ

Results of the AGJ tolerance test show that the *P. kudriavzevii* FOL-04 is able to grow in a pH 3 level acidic environment similar to the control environment at pH 6.5. However, the survival of the *P. kudriavzevii* FOL-04 decreases significantly when pH is at or lower than 2. Moreover, Tukey's test results show that there is not a

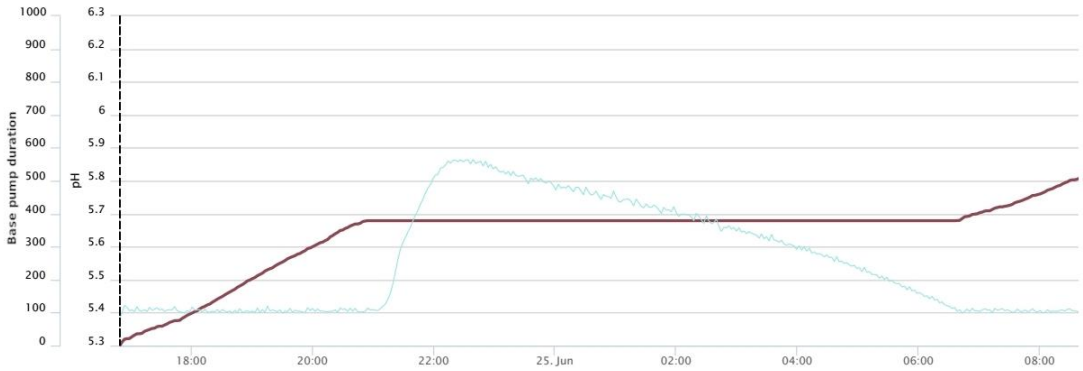

Figure 5. Time vs. pH level (light blue) and base pump duration (purple) during the fed-batch cultivation. X axis shows the time in hours while bioreactors are operating.

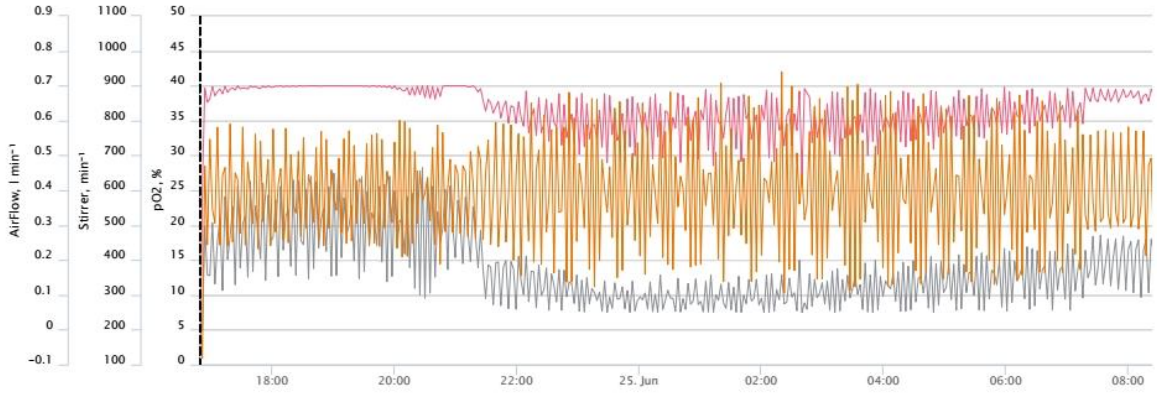

Figure 6. Time vs dissolved oxygen (pO_2) (orange), airflow rate (grey), and stirrer rate (red) during the fed-batch cultivation.

significant difference (p = 0.05) in survival of the *P. kudriavzevii* FOL-04 at pH 2 and pH 1.5 (Table 1, Figure 7).

Table 1. Acid tolerance of *P. kudriavzevii* FOL-04 after 42 hours of incubation. OD_{600} values of AGJ at the onset of incubation were as follows: 0.494 ±0.02 (control), 0.511 ±0.02 (pH 3), 0.470 ±0.09 (pH 2), and 0.550 ±0.02 (pH 1.5). The relative cell density ratio was calculated by dividing final cell densities achieved against initial cell turbidity measured at 600 nm wavelength. Means sharing the same superscript letters are not significantly different than each other (α = .5)

pH	Relative Cell Density Ratio ($OD_{600}\ t_{final}/OD_{600}\ t_0$)	Standard Deviation
1.5	10.4^{cx}	±4.3
2	18.8^{bx}	±10.5
3	34.7^{a}	±9.8
6.5 (Control)	42.2^{a}	±7.7

P. kudriavzevii FOL-04 can easily survive at 0.2% bile salt concentration in YPD media. However, survival of the *P. kudriavzevii* FOL-04 drops significantly when the bile concentration is higher than 1%. In addition, there is not a significant difference in survival of *P. kudriavzevii* FOL-04 observed while bile salt concentration is between 1% and 2% (Table 2, Figure 7).

Table 2. Bile salt tolerance of *P. kudriavzevii* FOL-04 after 42 hours of incubation. OD_{600} values of ABJ at the onset of incubation were as follows: 0.546 ±0.01 (control), 0.593 ±0.01 (0.2%), 0.786 ±0.02 (1%), and 1.026 ±0.06 (2%). The relative cell density ratio was calculated by dividing final cell densities achieved against initial cell turbidity measured at 600 nm wavelength. Means sharing the same superscript letters are not significantly different than each other (α = .5)

Bile Salt Concentration (%)	Relative Cell Density Ratio ($OD_{600}\ t_{final}/OD_{600}\ t_0$)	Standard Deviation
0 (Control)	28.24^{a}	±7.46
0.2	29.18^{a}	±9.51
1	24.47^{ax}	±0.92
2	18.04^{bx}	±1.54

Discussion

Pichia kudriavzevii FOL-04 showed similar growth characteristics during batch cultivation in the bioreactor with a previous study (Ndubuisi et al., 2020). Lag phases

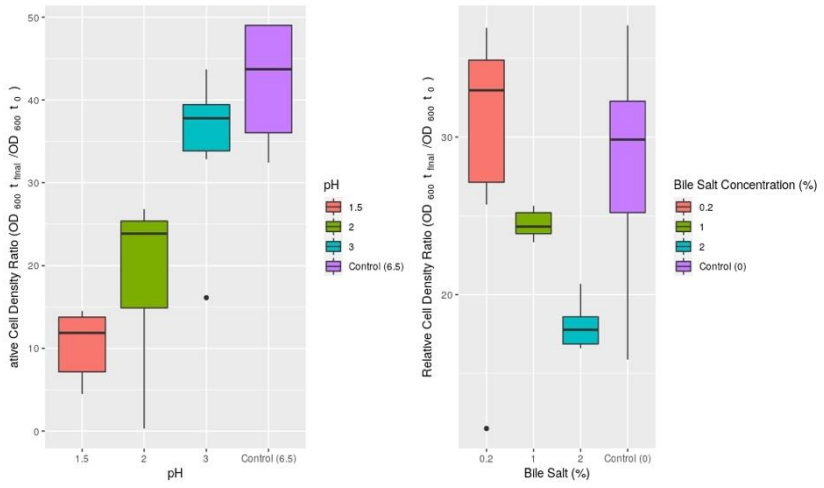

Figure 7. Relative cell density ratios of *P. kudriavzevii* FOL-04 against various pH and bile salt conditions after 42 hours. OD_{600} values of AGJ at the onset of incubation were as follows: 0.494 ±0.02 (control), 0.511 ±0.02 (pH 3), 0.470 ±0.09 (pH 2), and 0.550 ±0.02 (pH 1.5). OD_{600} values of ABJ at the start are following: 0.546 ±0.01 (control), 0.593 ±0.01 (0.2%), 0.786 ±0.02 (1%), and 1.026 ±0.06 (2%).

of both strains last about four hours in the YPD medium. Moreover, their exponential phases took around six hours. Recent studies showed that *P. kudriavzevii* is a viable candidate yeast for biodiesel (Sankh et al., 2013) and ethanol biosynthesis (Díaz-Nava et al., 2017). *P. kudriavzevii* can utilize glycerol, fructose, and glucose as a carbon source. However, *P. kudriavzevii* ITV-S42 lacks the utilization of sucrose and xylose sugars, and it ferments ethanol while sugar concentration is high (Díaz-Nava et al., 2017). A previous study on thermotolerant *P. kudriavzevii* isolated from nuruk, a traditional Korean fermentation starter, showed that *P. kudriavzevii* KCTC17763 provides optimal growth at 30°C in YPD media and synthesizes the highest amount of ethanol (Choi et al., 2017). Moreover, the *P. kudriavzevii* used in the present study performed a slightly longer exponential phase than *P. kudriavzevii* KCTC17763, MBY1358 (Choi et al., 2017), and LC375240 (Ndubuisi et al., 2020).

Bile salt and acid tolerance test results revealed that *P. kudriavzevii* FOL-04 can be a potential probiotic yeast strain. For example, *P. kudriavzevii* FOL-04 is tolerant to bile salts at similar concentrations with other *P. kudriavzevii* strains previously studied, such as M26, M28, M29, M30, M31, O6, G5, G6 (Greppi et al., 2017). These strains survived satisfactorily in pH 2 conditions although *P. kudriavzevii* FOL-04 still survived at pH 2, it showed significant loses in viability compared to pH 3 and pH 6.5. Several *P. kudriavzevii* strains are resistant to elevated temperatures and ethanol conditions, which were previously discovered (Pongcharoen et al., 2018). Chelliah et al. (2016) indicated that an *P. kudriavzevii* strain isolated from traditional Indian food had better survivability than FOL-04 in artificial gastric and bile juice. However, *P. kudriavzevii* OG32, a probiotic strain, possessed remarkably lower survivability in artificial gastric juice compared FOL-04 (Ogunremi, Agrawal, & Sanni, 2015). Chen et al. (2010) reported that *P. kudriavzevii* BY10 and BY15 strains, isolated from raw milk, showed probiotic potential in terms of survivability in ABJ and AGJ. In addition to *P. kudriavzevii* strains, *P. fermentans* BY5 sufficiently survived in ABJ and AGJ to be a potential probiotic strain (Chen et al., 2010). *P. pastoris* X-33 strain has been reported that it can survive in animal feed and gastrointestinal conditions while showing antibacterial activity against *Salmonella* Typhimurium (França et al., 2015). Similarly, *P. guilliermondii* isolated from table olives survived in low pH and high bile salt conditions also possessed antimicrobial activity against *Staphylococcus aureus* ATCC 8702, *Salmonella* Enteridis ATCC 564, and *Listeria monocytogenes* ATCC 19117 (Simões et al., 2021).

Conclusion

A new *Pichia kudriavzevii* FOL-04 was isolated from fermented plant material. We identified this strain using NL primers to amplify the D1/D2 domains of the 26S rRNA region, followed by Sanger sequencing. BLAST analysis against publicly available organisms in NCBI revealed this strain did not completely match with any other *P. kudriavzevii* strains. Artificial gastric juice and artificial bile juice trials were conducted to determine potential survivability of *P. kudriavzevii* FOL-04 against gastrointestinal conditions which revealed that FOL-04 is tolerant to low pH and high ox-bile salt conditions. *P. kudriavzevii* FOL-04 was also processed through shake flask and fed-batch fermentations to determine and compare microbial growth kinetics of this new strain. Fed-batch fermentation trials with 50% dextrose supplementation using exponential feeding regimen provided remarkably higher final biomass yield compared to shake flask experiments as measured by optical density at 600 nm wavelength. *P. kudriavzevii* FOL-04 could be a potential candidate for probiotic yeast strain with promising robustness under harsh AGJ and ABJ conditions also carrying bioprocess compatibility which should lead to further in-depth probiotic characterizations by *in vitro* and *in vivo* trials.

Author Contributions

Conceptualization: FO, Data Curation: FO, IG, Formal Analysis: FO, IG, Funding Acquisition: FO, Investigation: FO, IG, Methodology: FO, Project Administration: FO, Resources: FO, Supervision: FO, Visualization: FO, IG, Writing -original draft: IG, Writing -review and editing: FO, IG.

Acknlowledgements

We would like to thank A.E. Yetiman and A. Keskin for their kind support during the experiments.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I. B., Prajapati, J. B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., & Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*(3), 87–97. https://doi.org/10.1016/j.ijfoodmicro.2011.12.030

Chelliah, R., Ramakrishnan, S. R., Prabhu, P. R., & Antony, U. (2016). Evaluation of antimicrobial activity and probiotic properties of wild-strain *Pichia kudriavzevii* isolated from frozen idli batter. *Yeast*, *33*(8), 385–401. https://doi.org/10.1002/yea.3181

Chen, L.-S., Ma, Y., Maubois, J.-L., He, S.-H., Chen, L.-J., & Li, H.-M. (2010). Screening for the potential probiotic yeast strains from raw milk to assimilate cholesterol. *Dairy Science & Technology*, *90*(5), 537–548. https://doi.org/10.1051/dst/2010001

Choi, D.-H., Park, E.-H., & Kim, M.-D. (2017). Isolation of thermotolerant yeast *Pichia kudriavzevii* from nuruk. *Food Science and Biotechnology*, *26*(5), 1357–1362. https://doi.org/10.1007/s10068-017-0155-6

De Vuyst, L., Harth, H., Van Kerrebroeck, S., & Leroy, F. (2016). Yeast diversity of sourdoughs and associated metabolic properties and functionalities. *International Journal of Food Microbiology*, *239*, 26–34. https://doi.org/10.1016/j.ijfoodmicro.2016.07.018

Díaz-Nava, L. E., Montes-Garcia, N., Domínguez, J. M., & Aguilar-Uscanga, M. G. (2017). Effect of carbon sources on the growth and ethanol production of native yeast *Pichia kudriavzevii* ITV-S42 isolated from sweet sorghum juice. *Bioprocess and Biosystems Engineering*, *40*(7), 1069–1077. https://doi.org/10.1007/s00449-017-1769-z

Douglass, A. P., Offei, B., Braun-Galleani, S., Coughlan, A. Y., Martos, A. A. R., Ortiz Merino, R. A., Byrne, K. P., & Wolfe, K. H. (2018). Population genomics shows no distinction between pathogenic *Candida krusei* and environmental *Pichia kudriavzevii*: One species, four names. *PLOS Pathogens*, *14*(7), e1007138. https://doi.org/10.1371/journal.ppat.1007138

França, R. C., Conceição, F. R., Mendonça, M., Haubert, L., Sabadin, G., de Oliveira, P. D., Amaral, M. G., Silva, W. P. da, & Moreira, Â. N. (2015*). Pichia pastoris* X-33 has probiotic properties with remarkable antibacterial activity against Salmonella Typhimurium. *Applied Microbiology and Biotechnology*, *99*(19), 7953–7961. https://doi.org/10.1007/s00253-015-6696-9

Greppi, A., Saubade, F., Botta, C., Humblot, C., Guyot, J.-P., & Cocolin, L. (2017). Potential probiotic *Pichia kudriavzevii* strains and their ability to enhance folate content of traditional cereal-based African fermented food. *Food Microbiology*, *62*, 169–177. https://doi.org/10.1016/j.fm.2016.09.016

Hatoum, R., Labrie, S., & Fliss, I. (2012). Antimicrobial and Probiotic Properties of Yeasts: From Fundamental to Novel Applications. *Frontiers in Microbiology*, *3*, 421. https://doi.org/10.3389/fmicb.2012.00421

Karaoglan, H. A., Keklik, N. M., & Develi Isıklı, N. (2019). Degradation kinetics of anthocyanin and physicochemical changes in fermented turnip juice exposed to pulsed UV light. *Journal of Food Science and Technology*, *56*(1), 30–39. https://doi.org/10.1007/s13197-018-3434-1

Klaenhammer, T. R., & Kleeman, E. G. (1981). Growth Characteristics, Bile Sensitivity, and Freeze Damage in Colonial Variants of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology*, *41*(6), 1461–1467. https://doi.org/10.1128/aem.41.6.1461-1467.1981

Konczak, I., & Zhang, W. (2004). Anthocyanins—More Than Nature's Colours. *Journal of Biomedicine and Biotechnology*, *2004*(5), 239–240. https://doi.org/10.1155/S1110724304407013

Kumura, H., Tanoue, Y., Tsukahara, M., Tanaka, T., & Shimazaki, K. (2004). Screening of Dairy Yeast Strains for Probiotic Applications. *Journal of Dairy Science*, *87*(12), 4050–4056. https://doi.org/10.3168/jds.S0022-0302(04)73546-8

Kurtzman, C. P. (2011). Chapter 57—Pichia E.C. Hansen (1904). In C. P. Kurtzman, J. W. Fell, & T. Boekhout (Eds.), *The Yeasts (Fifth Edition)* (pp. 685–707). Elsevier. https://doi.org/10.1016/B978-0-444-52149-1.00057-4

Li, P., Li, S., Cheng, L., & Luo, L. (2014). Analyzing the relation between the microbial diversity of DaQu and the turbidity spoilage of traditional Chinese vinegar. *Applied Microbiology and Biotechnology*, *98*(13), 6073–6084. https://doi.org/10.1007/s00253-014-5697-4

Liti, G. (2015). The fascinating and secret wildlife of the budding yeast *S. cerevisiae*. *ELife*, *4*, e05835. https://doi.org/10.7554/eLife.05835

McFarland, L. V., & Bernasconi, P. (1993). *Saccharomyces boulardii*. A Review of an Innovative Biotherapeutic Agent. *Microbial Ecology in Health and Disease*, *6*(4), 157–171. https://doi.org/10.3109/08910609309141323

Moslehi-Jenabian, S., Lindegaard, L., & Jespersen, L. (2010). Beneficial Effects of Probiotic and Food Borne Yeasts on Human Health. *Nutrients*, *2*(4), 449–473. https://doi.org/10.3390/nu2040449

Mukherjee, V., Radecka, D., Aerts, G., Verstrepen, K. J., Lievens, B., & Thevelein, J. M. (2017). Phenotypic landscape of non-conventional yeast species for different stress tolerance traits desirable in bioethanol fermentation. *Biotechnology for Biofuels*, *10*(1), 216. https://doi.org/10.1186/s13068-017-0899-5

Ndubuisi, I. A., Qin, Q., Liao, G., Wang, B., Moneke, A. N., Ogbonna, J. C., Jin, C., & Fang, W. (2020). Effects of various inhibitory substances and immobilization on ethanol production efficiency of a thermotolerant *Pichia kudriavzevii*. *Biotechnology for Biofuels*, *13*(1), 91. https://doi.org/10.1186/s13068-020-01729-5

Ogunremi, O. R., Agrawal, R., & Sanni, A. I. (2015). Development of cereal-based functional food using cereal-mix substrate fermented with probiotic strain – *Pichia kudriavzevii* OG32. *Food Science & Nutrition*, *3*(6), 486–494. https://doi.org/10.1002/fsn3.239

Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: An overview of beneficial effects. *Antonie van Leeuwenhoek*, *82*(1), 279–289. https://doi.org/10.1023/A:1020620607611

Paradis, E., & Schliep, K. (2019). ape 5.0: An environment for modern phylogenetics and evolutionary analyses in R. *Bioinformatics*, *35*, 526–528. https://doi.org/10.1093/bioinformatics/bty633

Perricone, M., Bevilacqua, A., Corbo, M. R., & Sinigaglia, M. (2014). Technological characterization and probiotic traits of yeasts isolated from Altamura sourdough to select promising microorganisms as functional starter cultures for cereal-based products. *Food Microbiology*, *38*, 26–35. https://doi.org/10.1016/j.fm.2013.08.006

Pongcharoen, P., Chawneua, J., & Tawong, W. (2018). High-temperature alcoholic fermentation by new thermotolerant yeast strains *Pichia kudriavzevii* isolated from sugarcane field soil. *Agriculture and Natural Resources*, *52*(6), 511–518. https://doi.org/10.1016/j.anres.2018.11.017

R Core Team. (2020). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Radecka, D., Mukherjee, V., Mateo, R. Q., Stojiljkovic, M., Foulquié-Moreno, M. R., & Thevelein, J. M. (2015). Looking beyond Saccharomyces: The potential of non-conventional yeast species for desirable traits in bioethanol fermentation. *FEMS Yeast Research*, *15*(6). https://doi.org/10.1093/femsyr/fov053

Roth V. (2006). Doubling Time Computing. Retrieved October 13, 2021, from http://www.doubling-time.com/compute.php

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, *74*(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463

Sankh, S., Thiru, M., Saran, S., & Rangaswamy, V. (2013). Biodiesel production from a newly isolated *Pichia kudriavzevii* strain. *Fuel*, *106*, 690–696. https://doi.org/10.1016/j.fuel.2012.12.014

Simões, L. a., Cristina de Souza, A., Ferreira, I., Melo, D. s., Lopes, L. a. a., Magnani, M., Schwan, R. f., & Dias, D. r. (2021). Probiotic properties of yeasts isolated from Brazilian fermented table olives. *Journal of Applied Microbiology*, *131*(4), 1983–1997. https://doi.org/10.1111/jam.15065

Smukowski Heil, C., Burton, J. N., Liachko, I., Friedrich, A., Hanson, N. A., Morris, C. L., Schacherer, J., Shendure, J., Thomas, J. H., & Dunham, M. J. (2018). Identification of a novel interspecific hybrid yeast from a metagenomic spontaneously inoculated beer sample using Hi-C. *Yeast*, *35*(1), 71–84. https://doi.org/10.1002/yea.3280

Tanguler, H., & Erten, H. (2012). Occurrence and growth of lactic acid bacteria species during the fermentation of shalgam (salgam), a traditional Turkish fermented beverage. *LWT - Food Science and Technology*, *46*(1), 36–41. https://doi.org/10.1016/j.lwt.2011.10.026

Wang, Z., Zhuge, J., Fang, H., & Prior, B. A. (2001). Glycerol production by microbial fermentation: A review. *Biotechnology Advances*, *19*(3), 201–223. https://doi.org/10.1016/S0734-9750(01)00060-X

Song, S.-H., Cho, Y.-H., & Park, J. (2003). Microencapsulation of *Lactobacillus casei* YIT 9018 using a Microporous Glass Membrane Emulsification System. *Journal of Food Science*, *68*(1), 195–200. https://doi.org/10.1111/j.1365-2621.2003.tb14139.x

Sun, W., & Griffiths, M. W. (2000). Survival of bifidobacteria in yogurt and simulated gastric juice following immobilization in gellan–xanthan beads. *International Journal of Food Microbiology*, *61*(1), 17–25. https://doi.org/10.1016/S0168-1605(00)00327-5

Xiao, H., Shao, Z., Jiang, Y., Dole, S., & Zhao, H. (2014). Exploiting *Issatchenkia orientalis* SD108 for succinic acid production. *Microbial Cell Factories*, *13*(1), 121. https://doi.org/10.1186/s12934-014-0121-4

Yetiman, A. E., Keskin, A., Darendeli, B. N., Kotil, S. E., Ortakci, F., & Dogan, M. (2022). Characterization of genomic, physiological, and probiotic features *Lactiplantibacillus plantarum* DY46 strain isolated from traditional lactic acid fermented shalgam beverage. *Food Bioscience*, *46*, 101499. https://doi.org/10.1016/j.fbio.2021.1014

Biotech Studies 31(1), 36-44
http://doi.org/10.38042/biotechstudies.1103695

BIOTECH STUDIES

RESEARCH PAPER

Isolation and characterization of alkane hydrocarbons-degrading *Delftia tsuruhatensis* strain D9 from petroleum-contaminated soils

Ayşe Eren, Kemal Güven*

Molecular Biology and Genetics Department, Faculty of Science, Dicle University, Diyarbakır, 21280, Turkey.

How to cite

Eren, A. & Güven, K. (2022). Isolation and characterization of alkane hydrocarbons-degrading *Delftia tsuruhatensis* strain D9 from petroleum-contaminated soils. *Biotech Studies. 31*(1), 36-46. http://doi.org/10.38042/biotechstudies.1103695

Article History
Received 30 October 2021
Accepted 06 April 2022
First Online 14 April 2022

Corresponding Author
Tel.: +90 412 241 10 00/3064
E-mail: kemalg@dicle.edu.tr

Keywords
Alkane hydrocarbon-degrading bacterium
Oil pollution
16S rRNA gene sequence analysis
GC-MS analysis
Delftia

Abstract

A bacterial strain from petroleum-contaminated soil in south-eastern Turkey was isolated and characterized to determine the potential of alkane hydrocarbon biodegradation. Phenotypic characteristics and the sequence analysis of the 16S rRNA gene revealed that the strain D9 is a member of the *Delftia* genus and most similar to *Delftia tsuruhatensis* (100%). The optimum pH and temperature values for the growth of *D. tsuruhatensis* strain D9 were found to be 9.0-10.0 and 35°C, respectively. The strain was found to grow in some single, medium and long-chain hydrocarbons such as decane, hexadecane, and squalene, tested by short-time incubation in basal medium (BM) in the presence of 1% hydrocarbon concentrations under optimum conditions. After incubation for 3 days, 65% of the single hydrocarbon hexadecane was degraded by the *D. tsuruhatensis* strain D9, revealed by GC-MS analysis. The biodegradation of petroleum hydrocarbons by *D. tsuruhatensis* strain D9 isolated and characterized in the present study shows that it can be a good candidate in the bioremediation process.

Introduction

Petroleum hydrocarbons are well known to cause pollution over the world and oil spills cause a great danger to various ecosystems (Head et al., 2006; Emtiazi et al., 2009). Due to petroleum being an important resource of the global economy with several million tons of crude oil, its transport, manipulation, and exploitation can lead to accidental spills (Malatova 2005; Crone & Tolstoy, 2010; Mapelli et al. 2017). When petroleum enters the natural environment, all biotic communities are exposed and affected due to the oil hydrocarbons and toxic fumes (Peterson et al., 2003; Fingas, 2011). Several techniques are possible for hydrocarbon pollution removal: Physical, chemical, or biological (Costes & Druelle, 1997; Chu & Kwan, 2003; Hamme et al., 2003; Koma et al., 2003; Parales & Haddock, 2004; Pieper et al., 2004; Malatova, 2005; Mittal & Singh, 2009). Mechanical, evaporation, dispersion, burying, and washing are the main technologies commonly used for soil remediation, although these technologies are expensive and can result in insufficient decomposition of contaminants (Medina-Bellver et al., 2005). Bioremediation using the microorganisms degrading hydrocarbons has major advantages to remove contaminants from environments (Adams et al., 2015) which is regarded as a productive, environmental-friendly, and low-cost technology (Liu et al., 2014). Bioremediation, which is the process of using microorganisms to detoxify or remove pollutants due to their diverse metabolic capabilities is a developing method also for the elimination and degradation of pollutants arising from the petroleum industry (Medina-Bellver et al., 2005). Recently, a wide variety of microorganisms from oil-contaminated soils, which can degrade hydrocarbons within petroleum were studied. Among microorganisms including, bacteria, archaea, and fungi, the bacteria are

the most plentiful and significant in ecosystems (Acer et al., 2020). To date, more than 79 genera of bacteria have been identified that can degrade hydrocarbons in crude oil (Tremblay et al., 2017) including *Acinetobacter*, *Achromobacter*, *Alkanindiges*, *Arthrobacter*, *Alteromonas*, *Dietzia*, *Burkholderia*, *Enterobacter*, *Mycobacterium*, *Marinobacter*, *Kocuria*, *Pseudomonas*, *Streptococcus*, *Streptobacillus*, *Staphylococcus*, and *Rhodococcus* which play significant roles in the destruction of petroleum hydrocarbons (Margesin. et al., 2003; Chaerun et al., 2004; Jin et al., 2012; Nie et al., 2014; Varjani & Upasani, 2016; Acer et al., 2016; Sarkar et al., 2017; Varjani, 2017; Xu et al., 2017; Xu et al., 2018; Acer et al., 2020). In recent studies, *Delftia* species have been reported to degrade various contaminants such as chlorobenzene degraded by *D. tsuruhatensis* (Ye et al., 2019), Dimethylphenols degraded by *Delftia* sp. LCW (Vásquez-Piñeros et al., 2018) and diesel oil degraded by *Delftia* sp. NL1 (Lenchi et al., 2020). The *Delftia,* described in the year 1999 within the class Betaproteobacteria, is a group that contains only five characterized species: *Delftia acidovorans* (Wen et al., 1999), *D. tsuruhatensis* (Shigematsu et al., 2003), *D. lacustris* (Jørgensen et al., 2009), *D. litopenaei* (Chen et al., 2012) and most recently identified *D. deserti* (Li et al., 2015). *Delftia* spp. are known to have a wide geographic distribution in marine and fresh-water, rhizosphere, soil, plants, and clinical samples (Wen et al., 1999).

However, there are few studies regarding the degradation of petroleum hydrocarbons by various *Delftia* sp. strains. They report different effectiveness and range of degradable hydrocarbons suggesting that there is still a pool of unexplored information. Therefore, more studies are needed on the degradation of petroleum hydrocarbons by *Delftia* sp. In the current study, we isolated and identified *D. tsuruhatensis* strain D9 from petroleum-contaminated soils and investigated its potential for petroleum hydrocarbon biodegradation. It is expected that the result of this work will contribute towards improving biodegradation efforts of petroleum-contaminated sites.

Materials and Methods

Collection of Samples

For the isolating bacteria which degrade petroleum-hydrocarbons, a soil sample was collected from petroleum-contaminated soil around the petrol well at areas of Southern Raman (237. petroleum station, Batman).

The Basal medium (BM) consisted, per liter: 5.0 mL phosphate buffer, 1.0 mL solution of calcium chloride, 3.0 mL solution of magnesium sulfate, 1.0 mL solution of ferric chloride, as well as 1.0 mL mineral elements at trace levels which contain $ZnSO_4.H_2O$, $MnSO_4$ and $(NH_4)_6MoO_{24}.4H_2O$. 1% crude oil was filtered (0.2 mm pore size) and then transferred into the medium as the sole carbon source to get the energy to determine the bacteria biodegrading the petroleum hydrocarbons. The crude oil used in the experiments was provided from a petroleum-contaminated site. 1 g of the soil samples were transferred into 100 mL BM composed of 1% crude oil and samples were incubated in a shaker water bath at 30°C at 120 rpm for 5 days. After incubation, 1 mL aliquots were taken from each sample and transferred to freshly prepared BM containing 1% of crude oil. Following two subculture cycles, the bacterial cells were cultivated on Nutrient agar at 30°C for 5 days. Phenotypically different colonies on the plates were transferred to a fresh Nutrient agar.

Morphological, Biochemical, and Physiological Characteristics

Aspect, color, consistency, elevation, shape, size, and surface, etc. characteristics were used for phenotypical characterization of selected colonies. Gram staining to confirm the gram reaction was investigated using a light microscope according to the Dussault method (Dussault, 1955). Motility was determined by the hanging drop method. For the presumptive identification, data of phenotypic characteristics from the conventional methods such as catalase, citritase, indole, oxidase, and urease activity, utilization of starch, and gelatine were used.

Antibiotic Resistance

The antimicrobial susceptibility profiles of the isolate were determined using the antibiotic disk diffusion method. Antibiotic multi-disks were comprised of ampicillin (10 µg), fucidic acid (10 µg), lincomycin (15 µg), neomycin (10 µg), novobiocin (5 µg), kanamycin (5 µg), chloramphenicol (30 µg), nystatin (100 units), gentamicin (10 µg), tilmicosin (15 µg), streptomycin (10 µg), penicillin (2,10 units), bacitracin (10 units), tetracycline (30 µg). Following the inoculation of bacterial *D. tsuruhatensis* strain D9 on Nutrient agar in Petri dishes, the antibiotic disks were replaced on Nutrient agar. The agar plates were placed into an incubator at 30°C for 48 h. Following incubation, the evaluations were made by measuring the diameters of the zone of inhibition.

Phylogenetic Analysis

The final isolate namely *D. tsuruhatensis* strain D9 chosen as the petroleum hydrocarbon degrader were identified using a gene sequence of 16S rRNA. The 16S rRNA sequence analysis of the isolate was conducted by BM Laboratory system (Technocity/Ankara). Universal 16S rDNA primers (27 F, 5-AGAGtttGAtcAtGGctcAG-3 and 1492 R, 5-tAcGG ttAccttGttAcGActt-3) were applied for bacterial identification through PCR technique. PCR conditions was designed as 95°C for 5 min; 40 cycles at 95°C for 45 s, 57°C for 45 s, 72°C for 60 s, and final step at 72°C for 5 min. 1.5% of Agarose gel was applied for detecting amplified amplicon of 16S rDNA fragment which eluted (ExoSAP-IT™ PCR Product Cleanup Reagent, ThermoFisher Scientific, USA), sequenced by

ABI 3730XL Sanger Sequencing Equipment and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA).The 16S rRNA gene sequence for *D. tsuruhatensis* strain D9 was determined (*D. tsuruhatensis* strain D9:1405). The GenBank database using a BLAST search (http://www.ncbi.nlm.nih.gov/blast/) was used to compare the sequences. The 16S rRNA gene similarities were retrieved from the database, determining the most closely related strains. A phylogenetic tree was obtained by using the software package CLC Sequence Viewer 8 (Qiagen, Denmark).

Growth of the Strain in the Medium Supplemented with Hydrocarbons

The bacterial cells grown overnight in nutrient broth liquid medium was washed several times with BM to determine the growth in BM supplemented with hydrocarbons (hexane, heptane, octane, decane, pentadecane, hexadecane, squalene, and toluene; all of these hydrocarbons were obtained from Sigma-Aldrich, St. Louis, MO, USA). The bacterial strain was cultivated in 25 mL BM containing 1% hydrocarbons in 100 mL Erlenmeyer flasks and incubated at pH 7.0, 30°C, and 120 rpm for 3 days in hydrocarbons. After incubation, bacterial growth was measured by using a spectrophotometer at 600nm. Each data point represents the mean of at least three experiments.

Analysis of Degradation of Hexadecane by the Strain

The degradation activities of alkane hydrocarbons by *D. tsuruhatensis* strain D9 were determined by Gas Chromatography-Mass Spectrometry (GC-MS). Pre-cultured bacterial strain incubated for at least 12 hours at 30°C was transferred to 25 mL of BM plus with 1% hydrocarbon hexadecane in a 100 mL flask. After incubation, the bacterial cells were removed and the remained hexadecane in the medium was used for calculating the degradation ratio of the hydrocarbon tested. The remaining hexadecane fractions were analyzed by GC-MS with a flame ionization detector (Hewlett Packard, Wilmington, USA; FID; HP 6850) and also equipped with a PONA quartz capillary column (100 m x 0.250 mm [inner diameter, i.d.] x 0.50 m). Helium was utilized as a carrier gas to perform split injections. The increase of column temperature was from 60°C to 320°C with a rate of 3°C per minute. The injector and detector temperatures used were 300°C and 350°C, respectively. Individual hexadecane fraction was determined, using an authentic standard for matching the retention time (70.528 min).

Results and Discussion

Collection of Samples

For the isolation of alkane hydrocarbon-degrading *D. tsuruhatensis* strain D9 strain, soil samples were collected from petroleum-contaminated soil around the petrol well at areas of Southern Raman (237. petroleum station, Batman).

Biochemical, Physiological, and Morphological Characteristics

The results of the biochemical and morphological tests on the isolated *D. tsuruhatensis* strain D9 were used for identification which is presented in Table 1. As can be seen from Figure 1, the cells are rod-shaped and gram-negative. *D. tsuruhatensis* strain D9 is aerobic and able to utilize oxidase, catalase, starch hydrolysis, citrate, urease, as well as positive for indole test and negative gelatine test.

Lenchi et al. (2020) found that *Delftia* sp. NL1 strain was gram-negative, not-motile, rod-shaped, oxidase-positive, and catalase-negative. Moreover, several other studies on *Delftia* strains showed that they are gram-negative, motile, rod-shaped, catalase, and oxidase-positive (Carro et al., 2017; Jørgensen et al., 2009; Shigematsu et al., 2003).

The time-course experiments showed that the growth of *D. tsuruhatensis* strain D9 was observed to be maximum up to 8 h, while the growth is constant from 12 h to 48 h, followed by the death phase up to 72 h (Figure 2).

Table 1. The phenotypical characteristics of the *Delftia tsuruhatensis* strain D9 in comparison with other *Delftia* species

Characteristic	*D. tsuruhatensis* strain D9	*Delftia* sp. strain NL1	*D. tsuruhatensis* strain T7^T	*D. lacustris* strain 332^T	*D. rhizosphaerae* strain RA6T	*D. acidovorans* strain ACM 489
Cell shape	R	R	R	R	R	R
Gram staining	-	-	-	-	-	-
Motility	+	-	+	+	ND	+
Oxygen requirement	A	ND	ND	ND	A	A
Growth temperature (°C)	20-40	20-50	10-40	3-37	4-30	4-41
Optimum Growth temperature (°C)	35	30	35	25	28	30
Growth pH	5.0-10.0	5.0-9.0	5.0-9.0	5.0-10.0	6.0-10.0	ND
Optimum growth pH	9.0-10.0	8.0	7.0	6.0-7.0	7.0	ND
Starch hydrolysis	+	-	-	-	-	-
Gelatin hydrolysis	-	-	ND	+	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	-	+	+	+	+
Citrate	+	ND	+	+	ND	ND
Urease	+	ND	+	ND	-	ND
Indole	+	ND	ND	ND	-	+
NaCl resistance (%)	3	2	ND	0.1	1	0.5-1.5
References	This study	(Lenchi et al., 2020)	(Shigematsu et al., 2003)	(Jørgensen et al., 2009)	(Carro et al., 2017)	(Wen, 1999)

+ = positive; - = negative; ND= no data available; R= rod; A= aerobic.

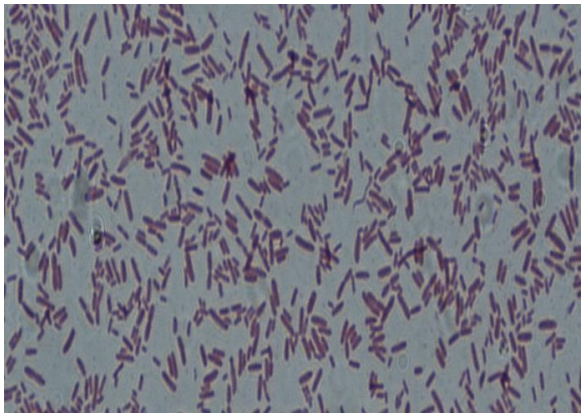

Figure 1. The Gram-staining of *D. tsuruhatensis* strain D9 observed under a light microscope (×1000 magnification).

To deterimine optimum pH and temperature values, pH, *D. tsuruhatensis* strain D9 was cultivated in Nutrient broth for 24 h, after which bacterial growth was measured by using a spectrophotometer at 600nm.

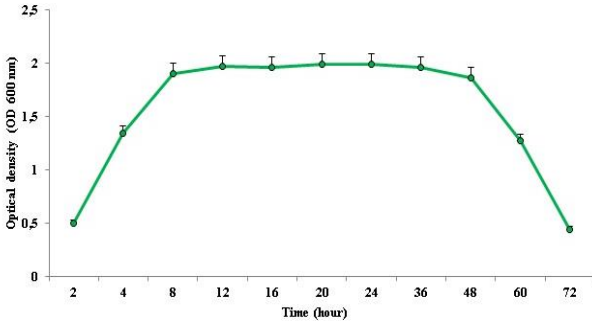

Figure 2. Effects of cultivation time on the growth of *D. tsuruhatensis* strain D9. The cells were incubated at 30°C, pH 7.0 for 72 h.

The range of growth for *D. tsuruhatensis* strain D9 was determined to be between 20°C and 40°C with an optimum of 35°C (Figure 3a). Furthermore, *D. tsuruhatensis* strain D9 grew at pH ranging from 5.0 to 10.0 with a wide optimum value of pH 9.0-10.0 (Figure 3b). *D. tsuruhatensis* strain D9 was also found to tolerate NaCl up to 3%. In a previous study by Shigematsu et al. (2003), strain *D. tsuruhatensis* strain T7^T also grew at 10-40 °C, with 35°C of optimum growth. Similarly, the range of pH for growth was from 5.0 to 9.0, with an optimum value of pH 7.0. Jørgensen et al. (2009) reported that cells *D. lacustris* strain 332^T grow between 3 and 37°C, with optimum growth at 25°C, as well as observing growth at a pH range of 5.0 to 10.0, with an optimum of pH 6.0-7.0. In a recent study, optimal growth for the strain *Delftia sp.* NL1 strain was observed at 30°C and pH 8.0 (Lenchi et al., 2020). The reason of different and such a high optimum pH for *Delftia tsuruhatensis* strain D9 compared to other strains described above is probably due to different culture media utilised with different initial pH and composition, as bacteria tend to release acidic products into their environment, which can interfere with their growth by changing the pH of the culture media.

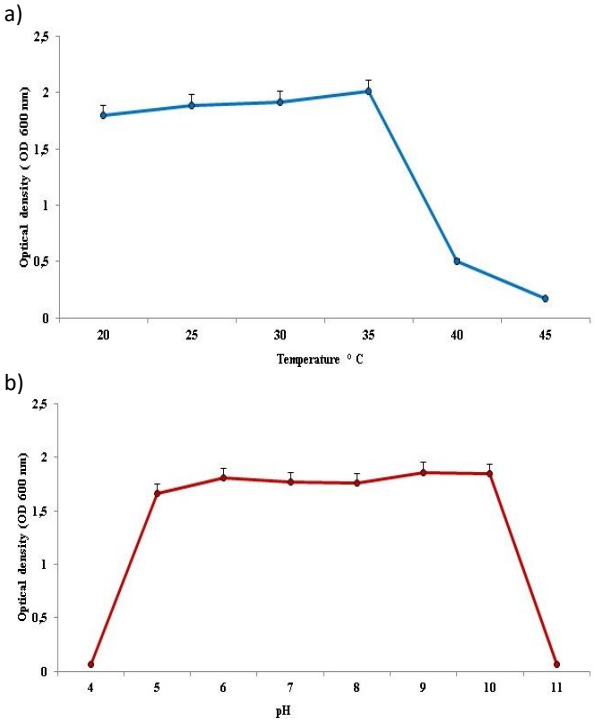

Figure 3. Effects of temperature (a) and pH (b) on the growth of *D. tsuruhatensis* strain D9. The bacterial strain was cultivated in Nutrient broth for 24 h, after which the growth was measured by using a spectrophotometer at 600nm.

Antibiotic Resistance

In Table 2, the effects of various antibiotics on *D. tsuruhatensis* strain D9 were determined by measuring the zone (mm) of inhibiton of antibiotics. *D. tsuruhatensis* strain D9 was resistant to ampicillin, gentamicin, streptomycin, penicillin, bacitracin, fusidic acid, novobiocin, lincomycin, neomycin, kanamycin, nystatin, however, it was semi-sensitive to chloramphenicol, tilmicosin, sensitive to tetracycline. There have been several studies on the effects of antibiotics on *Delfitia* sp. For example, Lenchi et al. (2020) reported that strain *Delftia* sp. NL1 was resistant to ampicillin, but it was sensitive to fusidic acid, gentamicin, and kanamycin. *Delftia tsuruhatensis* strain TR1180 was also found to be resistant to ampicillin, gentamicin, tetracycline and streptomycin, but sensitive to chloromphenicol (Cheng et al., 2021).

Table 2. Antibiogram results of *D. tsuruhatensis* strain D9

Antibiotics	*D. tsuruhatensis* strain D9 (mm: zone of inhibition)
Novobicin (5 µg)	0
Fucidic acid (10 µg)	0
Kanamycin (5 µg)	0
Bacitracin (10 units)	0
Gentamicin (10 µg)	0
Tilmicosin (15 µg)	13
Ampicillin (10 µg)	0
Streptomycin (10 µg)	0
Chloramphenicol (30 µg)	12
Lincomycin (15µg)	0
Penicillin G (2 units)	0
Tetracycline (30 µg)	33
Neomycin (10 µg)	0
Penicillin G (10 units)	0
Nystatin (100 units)	0

Phylogenetic Analysis

The sequence analysis of the 16S rRNA gene indicated that *D. tsuruhatensis* strain D9 belonged to the genus *Delftia*. Following the phylogenetic analysis, similarity calculations revealed that the closest relative of *D. tsuruhatensis* strain D9 was *Delftia* sp. NLl. As can be seen in Figure 4, the constructed phylogenetic dendrogram shows *D. tsuruhatensis* strain D9 identified as *D. tsuruhatensis* strain D9 (100%) (GenBank accession number: MT374262).

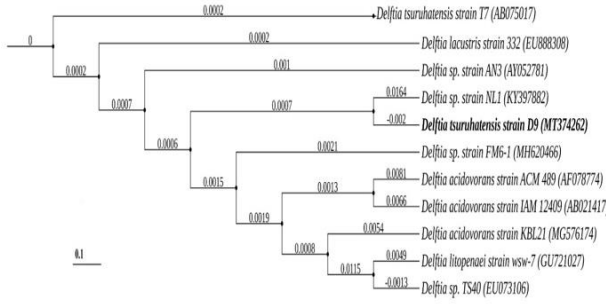

Figure 4. The 16S gene sequence analysis of the *D. tsuruhatensis* strain D9.

Growth of the Bacterial Strain in Various Hydrocarbons

Figure 5 shows that *D. tsuruhatensis* strain D9 uses alkane hydrocarbons to grow. For studying the growth of alkane-degrading bacteria, a basal mineral salt medium (BM) modified from Nie et al. (2010) was used. *D. tsuruhatensis* strain D9 was found to biodegrade and grow in medium and long-chain alkanes including decane, hexadecane, and squalene, rather than the short-chain alkanes. Hexadecane was found to be the most preferred hydrocarbon to grow effectively. In a previous study, *Delftia tsuruhatensis* B7 strain was also found to grow in crude oil and hexadecane (Roy et al., 2014). Moreover, *Delftia lacustris* strain LZ-C was grown in the mineral salt medium with PAHs as sole carbon source including naphthalene, 2-methylnaphthalene, anthracene, phenanthrene, pyrene, naphthol, benzene, phenol or toluene (Wu et al., 2016).

GC Analysis of Degradation of Hexadecane by *D. tsuruhatensis* strain D9

The gas chromatography analysis of hexadecane degradation by the strain incubated at a growth temperature of 30°C in BM with 1% (v/v) hexadecane for 3 days under constant shaking is shown in Figure 6b, compared to abiotic control in Figure 6a. The average hexadecane (1%) degradation rate was found to be as much as 65% after 3 days incubation time.

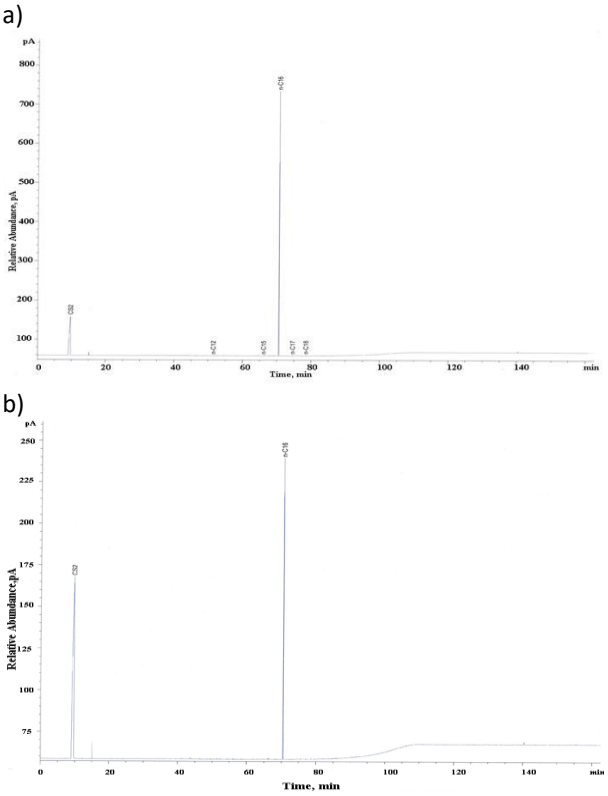

Figure 6. GC Analysis of hexadecane degradation. (a) Abiotic control of 1% hexadecane. (b) After degradation of 1% hexadecane by *D. tsuruhatensis* strain D9.

Oil hydrocarbons have been the most dangerous pollutants in the environment, and seem to continue to be a major energy and chemical source (Peterson et al.,

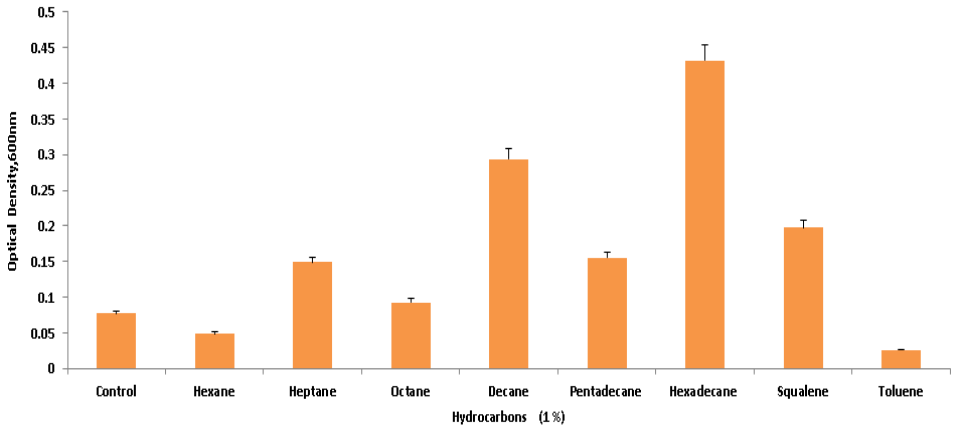

Figure 5. Growth of *D. tsuruhatensis* strain D9 at 1% concentration of different hydrocarbon sources. Each data represents the mean of at least 3 different experiments.

2003; Macaulay & Rees, 2014; Khan et al., 2018). It has been more than a century that the biodegradation of hydrocarbons by microbial activity has been recognized (Muthukamalam et al., 2017). In many studies, petroleum hydrocarbon-degrading bacteria have been described from various environments including oil-contaminated soils and waste-water (Cappello et al., 2012; Jurelevicius et al., 2012). The capacity of native bacterial populations is well known to mineralize hydrocarbons in crude oil-contaminated soil sites (Ojo, 2006). Wang et al. (2011) studied *Dietzia* sp. determining that bacteria use n-alkanes (C_6-C_{40}) as a carbon source. Throne-Holst et al. (2007) determined that *Acinetobacter* strain can use carbon sources in the range of decane and tetracontane long-chain n-alkanes. Razak et al. (1999) isolated *Acinetobacter* sp. degrading more than 60% crude oil in 15 days. In another study, diesel-oil alkanes were degraded an average of 58.1% by *Acinetobacter baumannii* in 10 days incubation (Nkem, 2016). Among the various groups of bacteria, *Pseudomonas* species and strains were found to be the most potent oil-degrading bacteria, having a wide distribution at the petroleum contaminated sites. Zhang et al. (2011) found that the strain *Pseudomonas* DQ8 degraded crude oil, n-alkanes, and polycyclic aromatic hydrocarbons (PAH). *Pseudomonas aeruginosa* was found to be a degradation of 49.93% of 0.5% diesel oil at the end of 20 days incubation period (Panda et al., 2013). *P. aeruginosa* had shown 34.4% of diesel oil degradation in 10 days (Simaria et al., 2015). Godini et al. (2018) found that three strains, namely *Staphylococcus arlettae* (strain 3), *Pseudomonas* sp. (strain 8), *Pseudomonas* sp. (strain 5) utilized crude oil containing various compounds, as carbon and energy source. In a recent study, GC-MS analysis of the hydrocarbon compounds indicated that the studied crude oil possessed alkanes in the range of C_{20}-C_{44}. *Enterobacter* species ALK-23, *Bacillus methylotrophicus* ALK16 and *Alcaligenes* species ALK-14 were found to degrade maximally 5.59%, 8.11%, and 11.65%, respectively (Dwivedi et al.,2019). There have also been some studies on the use of members of *Delftia* genus in bioremediation processes. Liu et al. (2002) found that *Delftia* sp. strain (named AN3) for its sole carbon, nitrogen, as well as energy sources utilizes aniline or acetanilide. *D. tsuruhatensis* AD9 obtained from the soil site near a textile dyeing plant were also found to degrade aniline (Geng et al., 2009). Similarly, several *Delftia* strains degrading aniline were also reported (Kahng et al., 2000; Urata et al., 2004; Sheludchenko et al., 2005; Xiao et al., 2009).

Moreover, phenanthrene degrading *D. acidovorans* strain was collected and characterized from soil contaminated with PAH (Vacca et al., 2005). Moreover, *D. lacustris* strain LZ-C was found to degrade compounds such as naphthalene and 2-methylnaphthalene (Wu et al., 2016). In recent studies, *Delftia* species have been reported to degrade various contaminants such as chlorobenzene degraded by *D. tsuruhatensis* (Ye et al., 2019), dimethylphenols degraded by *Delftia* sp. LCW (Vasquez-Pineros et al., 2018). Most recently, more than 66.76% of diesel oil was found to be degraded by *Delftia* sp. NL1 in only 7 days (Lenchi et al., 2020).

Conclusion

A wide variety of microorganisms including bacteria that can degrade petroleum hydrocarbons were isolated from oil-contaminated soils. Bacteria are the most dominant microorganisms determined in microbial ecology that can degrade petroleum hydrocarbons. Among bacteria, *Delfitia* is recently found to be the most common genera that are capable of degrading hydrocarbons. A bacterial strain designated as D9 from petroleum-contaminated soil was isolated and characterized by phenotypic characteristics and 16S rRNA gene sequence analysis, which was found to be most similar to *Delftia tsuruhatensis* (100%). The optimum pH and temperature values for the growth of *D. tsuruhatensis* strain D9 were found to be 9.0-10.0 and 35°C, respectively. Although *D. tsuruhatensis* strain D9 was found to grow in some single, medium and long-chain hydrocarbons, it should be emphasized that *D. tsuruhatensis* strain D9 is a very effective degrader of hexadecane as much as 65% in a short incubation time (3 days). Further studies on *D. tsuruhatensis* strain D9 and microbial consortium studies with the related strains may provide a good advantage in the bioremediation process of oil-contaminated soil.

Funding Information

This study, a part of Ayse EREN's Ph.D. project, was supported by Dicle University Scientific Research Projects Coordination Unit (Project number: FEN.19.014).

Author Contributions

AE: Conceptualization, Data Curation, Formal Analysis, Visualization, and Writing-original draft; KG: Funding Acquisition, Project Administration, Resources, Data Curation, Formal Analysis, Investigation, Methodology, Writing-review, and editing.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

References

Adams, G.O., Fufeyin, P.T., Okoro, S.E., Ehinomen, I. (2015). Bioremediation, biostimulation and bioaugmention: a

review. *International Journal of Environmental Bioremediation & Biodegradation*, 3, 28-39. https://doi.org/ 10.12691/ijebb-3-1-5

Acer, Ö., Güven, K., Bekler, F.M., Gül-Güven, R. (2016). Isolation and characterization of long-chain alkane-degrading *Acinetobacter* sp. BT1A from oil-contaminated soil in Diyarbakır, in the Southeast of Turkey. *Bioremediation Journal*, 20(1), 80-87. https://doi.org/10.1080/10889868.2015.1096898

Acer, Ö., Güven, K., Poli, A., Donato, P.D., Leone Buono, L., Gül Güven, R., Nicolaus, B., Finore, I. (2020). *Acinetobacter mesopotamicus* sp. nov., petroleum-degrading bacterium, isolated from petroleum-contaminated soil in Diyarbakir, in the Southeast of Turkey. *Current Microbiology*, 77(2) ,3192-3200. https://doi.org/10.1007/s00284-020-02134-9

Chaerun, S. K, Tazaki, K., Asada, R., Kogure, K. (2004). Bioremediation of coast areas 5 years after the Nakhodka oil spill in the Sea of Japan: isolation and characterization of hydrocarbon-degrading bacteria. *Environment International,* 30(7), 911-922. https://doi.org/10.1016/j.envint.2004.02.007

Chen, W.M., Lin, Y.S., Sheu, D.S., Sheu, S.Y. (2012). *Delftia litopenaei* sp. nov., a poly-β-hydroxybutyrateaccumulating bacterium isolated from a fresh water shrimp culture pond. *International Journal of Systematic and Evolutionary Microbiology,* 62, 2315-2321. https://doi.org/10.1099/ijs.0.037507-0

Cheng, C., Zhou, W., Dong, X., Zhang, P., Zhou, K., Zhou, D., ... & Ying, J. (2021). Genomic Analysis of *Delftia tsuruhatensis* Strain TR1180 Isolated From A Patient From China With In4-Like Integron-Associated Antimicrobial Resistance. *Frontiers in cellular and infection microbiology*, 11: 663933. https://doi.org/10.3389/fcimb.2021.663933

Crone, T. J., & Tolstoy, M. (2010). Magnitude of the 2010 gulf of mexico oil leak. *Science* ,330-634. https://doi.org/10.1126/science.1195840

Costes, J.M., & Druelle, V. (1997). Polyciclic aromatic hydrocarbons in the environment: The rehabilitation of old industrial sites. *Oil and Gas science and Technology*, 52, 425-440. http://dx.doi.org/10.2516/ogst:1997051

Chu, W., & Kwan, C.Y. (2003). Remediation of contaminated soil by a solvent/surfactant system. *Chemosphere*, 53(1), 9-15.http://dx.doi.org/10.1016/S0045-6535(03)00389-8

Carro, L., Mulas, R., Pastor-Bueis, R., Blanco, D., Terrón, A.,Gonzalez-Andres, F., Peix, A. (2017). *Delftia rhizosphaerae* sp. nov. İsolated from the rhizosphere of Cistus ladanifer. *International Journal of Systematic and Evolutionary Microbiology,* 67, 1957-1960. https://doi.org/10.1099/ijsem.0.001892

Cappello, S., Santisi, S., Calogero, R., Hassanshahian, M., Yakimov, M.M. 2012. Characterization of oil-degrading bacteria isolated from bilge water. *Water Air and Soil Pollution,* 223, 3219-3226. https://doi.org/10.1007/s11270-012-1103-y

Dwivedi, A., Chitranshi, S., Gupta, A., Kumar, A., Lal B.J. (2019). Assessment of the petroleum oil degradation capacity of indigenous bacterial species isolated from petroleum oil-contaminated soil. *International Journal of Environmental Research*, 13, 735-746. https://doi.org/10.1007/s41742-019-00210-y

Dussault, H.P. (1955). An improved technique for staining red halophilic bacteria. Journal of *Bacteriol*, 70, 484-485. https://doi.org/10.1128/jb.70.4.484-485.1955

Emtiazi, G., Saleh, T., Hassanshahian, M. (2009). The effect of bacterial glutathione s-transferase on morpholine degradation. *Biotechnology Journal,* 4, 202-205. https://doi.org/10.1002/biot.200800238

Fingas, M.F. (2011). Oil spill science and technology: prevention, response, and clean up, 1st. ed. GPP. Elesvier Inc, Burlington, MA 01803, USA.

Geng, L., Chen, M., Liang, Q., Liu, W., Zhang, W., Ping, S., Lu, W., Yan, Y., Wang, W, Takeo, M., Lin, M. (2009). Functional analysis of a putative regulatory gene, tadR, involved in aniline degradation in *Delftia tsuruhatensis* AD9. *Archives of Microbiology,* 191, 603-614. https://doi.org/10.1007/s00203-009-0488-5

Godini, K., Samarghandi , M.R, Zafari , D., Rahmani, A.R., Afkhami, A., Arabestani, M.R. (2018). Isolation and identification of new strains of crude oil degrading bacteria from Kharg Island, Iran. *Petroleum Science and Technology*, 869-874. https://doi.org/10.1080/10916466.2018.1447961

Hamme, D.J, Singh, A., Ward, O.P. (2003). Recent Advances in Petroleum Microbiology. *Microbiology and Molecular Biology Reviews*. 67(4), 503-549. https://journals.asm.org/doi/full/10.1128/MMBR.67.4.503-549.2003

Head, I.M., Jones, D.M., & Roling, W.F. (2006). Marine microorganisms make a meal of oil. *Nature Reviews Microbiology*, 4,173-182. https://doi.org/10.1038/nrmicro1348.

Jin, H.M., Kim, J.M., Lee H. J., Madsen, E. L., Jeon, C.O. (2012). Alteromonas as a keyagent of polycyclic aromatic hydrocarbon biodegradation in crude oil-contaminated coastal sediment. *Environmental Science and Technology*. 46(14), 7731-7740. https://doi.org/10.1021/es3018545

Jørgensen, N.O., Brandt, K.K, Nybroe, O., Hansen, M. (2009). *Delftia lacustris* sp. nov., a peptidoglycan-degrading bacterium from fresh water, and emended description of *Delftia tsuruhatensis* as a peptidoglycan-degradingbacterium. *Intrnational Journal of Systematic and Evolutionary Microbiology,* 59(9), 2195-2199. https://doi.org/10.1099/ijs.0.008375-0

Jurelevicius, D., Cotta, S.R., Peixoto, R., Rosado, A.S., Seldin, L. (2012). Distribution of alkane-degrading bacterial communities in soils from King George Island, Maritime Antarctic. *European Journal of Soil Biology,* 51, 37-44. https://doi.org/10.1016/j.ejsobi.2012.03.006

Koma, D., Hasumi, S., Motoki, K. (2003). Biodegradation of n-alkylcyclohexanebyco-oxidation via multiples path ways *Acinetobacter* sp. ODDK71. *Journal of Bioscience and Bioengineering*, 95, 641-644. http://dx.doi.org/10.1016/S1389-1723(03)80178-0

Khan, M.A.I., Biswas, B., Smith, E., Naidu, R., Megharaj, M. (2018). Toxicit yassessment of fresh and weathered petroleum hydrocarbons in contaminated soil-a review. *Chemosphere*, 212,755-767. https://doi.org/10.1016/j.chemosphere.2018.08.094

Kahng, H.Y., Kukor, J.J., Oh, K.H. (2000). Characterization of strain HY99, a novel microorganism capable of aerobic and anaerobic degradation of aniline. *FEMS Microbiology Letters,* 190 (2), 215-221. https://doi.org/10.1111/j.1574-6968.2000.tb09289.x

Liu, H., Xu, J., Liang, R., Liu, J. (2014). Characterization of the medium-and long-chain n-alkanes degrading *Pseudomonas aeruginosa strain* SJTD-1 and its alkane hydroxylase genes. PLoS ONE 9(8): e105506. https://doi.org/10.1371/journal.pone.0105506

Lenchi, N., Kebbouche-Gana, S., Servais, P., Lamine Gana, M., Llirós, M. (2020). Diesel biodegradation capacities and biosurfactant production in saline-alkaline conditions by *Delftia* sp NL1, isolated from an Algerian oil field. *Geomicrobiology Journal*, 37 (5):454-466. https://doi.org/10.1080/01490451.2020.1722769

Li, C.T., Yan, Z.F., Chu, X., Hussain, W.D., Yunus, Z., Hozzein, W.N., Li, W.J., Abaydulla G. (2015). *Delftia deserti* sp. nov., isolated from a desert soil sample. *Antonie Van Leeuwenhoek*, 107,1445-1450. http://dx.doi.org/10.1007/s10482-015-0440-4

Liu, Z., Yang, H., Huang, Z., Zhou, P., Liu S.J. (2002). Degradation of aniline by newly isolated, extremely aniline tolerant *Delftia* sp. AN3. *Applied Microbiology and Biotechnology,* 58(5),679-682. http://dx.doi.org/10.1007/s00253-002-0933-8

Mapelli, F., Scoma, A., Michoud, G., Aulenta, F., Boon, N., Borin, S., Kalogerakis, N., Daffonchio, D. (2017). Biotechnologies for marine oil spill cleanup: Indissoluble Ties with Microorganisms. *Trends in Biotechnology*, 35(9),860-870. https://doi.org/10.1016/j.tibtech.2017.04.003

Malatova, K. (2005). Isolation and characterization of hydrocarbon degrading bacteria from environmental habitats in western New York State. Rochester Institute of Technology. Rochester. https://scholarworks.rit.edu/cgi/viewcontent.cgi?referer=&httpsredir=1&article=9044&context=theses

Mittal, A., & Singh, P. (2009). Isolation of hydrocarbon-degrading bacteria from soils contaminated with crude oil spills. *Indian Journal of Experimental Biology*, 47(9), 760-765. https://pubmed.ncbi.nlm.nih.gov/19957890/

Medina-Bellver, J.I., Marın, P., Delgado, A., Rodríguez-Sánchez, A., Reyes, E., Ramos, J.L., Marqués, S. (2005). Evidencefor in Situ Crude Oil Biodegradation after the Prestige Oil Spill. *Environmental Microbiology*, 7,773-779. http://dx.doi.org/10.1111/j.1462-2920.2005.00742.x

Margesin, R., Labbe, D., Schinner, F., Greer, C. W, Whyte, L.G. (2003). Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Applied and Environmental Microbiology,* 69(6), 3085-3092. http://dx.doi.org/10.1128/AEM.69.6.3085-3092.2003

Macaulay, B.M., Rees, D. (2014). Bioremediation of oilspills: a review of challenges for research advancement. *Annals of Environmental Science*, 8, 9-37. https://openjournals.neu.edu/aes/journal/article/view/v8art2

Muthukamalam, S., Sivagangavathi, S., Dhrishya, D., Rani, S.S. (2017). Characterization of dioxygenasesandbiosurfactantsproducedbycrudeoildegradingsoilbacteria. *Brazilian Journal of Microbiology,* 48(4), 637-647. http://dx.doi.org/10.1016/j.bjm.2017.02.007

Nie, M., Yin, X., Ren, C., Wang, Y., Xu, F., & Shen, Q. (2010). Novel rhamnolipid biosurfactants produced by a polycyclic aromatic hydrocarbon-degrading bacterium Pseudomonas aeruginosa strain NY3. Biotechnology advances, 28(5), 635-643. http://dx.doi.10.1016/j.biotechadv.2010.05.013

Nie, Y., Liang, J.L, Fang, H., Tang, Y.Q, Wu, X.L. (2014). Characterization of a Cyp153 alkane hydroxylase gene in a gram-positive *Dietzia* sp. DQ12- 45-1b and its team role with alkw1 in alkane degradation. *Applied Microbiology and Biotechnology*,98,163-173. http://dx.doi.org/10.1007/s00253-013-4821-1

Nkem, B.M., Halimoon, N., Yusoff, F.M., Johari, W.L.W., Zakaria, M. P., Medipally, S.R., Kannan, N. (2016). Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of Cellulosimicrobium cellulans and *Acinetobacter baumannii* from tarball at Terengganu beach, Malaysia. *Marine Pollution Bulletin*,107(1), 261-268. http://dx.doi.org/10.1016/j.marpolbul.2016.03.060

Ojo, O.A. (2006). Petroleum hydrocarbon utilization by native bacterial population from a wastewater canal Southwest Nigeria. *African Journal of Biotechnology,* 5(4), 333-337. https://www.ajol.info/index.php/ajb/article/view/137800

Peterson, C.H., Rice, S.D., Short, J. W., Esler, D., Bodkin, J.L., Ballachey, B.E., Irons, D.B. (2003). Long-termecosystem response to the exxon valdez oil spill. *Science*, 302, 2082-2086. https://doi.org/10.1126/science.1084282

Parales, P.E., & Haddock, J.D. (2004). Biocatalytic degradation of pollutants. *Current Opinion in Biotechnology*, 15(4),374-379. http://dx.doi.org/10.1016/j.copbio.2004.06.003

Pieper, D.H., Dos Santos, V.M., Golyshin, P.N. (2004). Genomic and mechanistic insight into the biodegradation of organic pollutant. *Current Opinion in Biotechnology*, 15, 215-224. http://dx.doi.org/10.1016/j.copbio.2004.03.008

Panda, S.K., Kar, R.N., Panda, C.R. (2013). Isolation and identification of petroleum hydrocarbon degrading microorganisms from oil contaminated environment. *İnternational Journal of Environmental Sciences*, 3(5),1314-1321.https://doi.org/10.6088/ijes.2013030500001

Razak, C.N.A., Wang, W., Rahman, S.H.S.A., Basri, M., Salleh, A.B. (1999). Isolation of the crude oil degrading marine *Acinetobacter sp.* Ell. *Acta Scientific Biotechnology*,19 (3):213-223. https://doi.org/10.1002/ABIO.370190307

Roy, A. S., Baruah, R., Borah, M., Singh, A. K., Boruah, H. P. D., Saikia, N., ... & Bora, T. C. (2014). Bioremediation potential of native hydrocarbon degrading bacterial strains in crude oil contaminated soil under microcosm study. *International Biodeterioration & Biodegradation*, *94*, 79-89. http://dx.doi.org/10.1016/j.ibiod.2014.03.024

Shigematsu, T., Yumihara, K., Ueda, Y., Numaguch, M., Morimura, S., Kida, K. (2003). *Delftiatsuruhatensis* sp. nov., a terephthalate-assimilating bacterium isolated from activated sludge. *International Journal of Systematic and Evolutionary,* 53(5), 1479-1483. https://doi.org/10.1099/ijs.0.02285-0

Sarkar, P., Roy, A., Pal, S., Mohapatra, B., Kazy, S.K, Maiti, M.K, Sar, P. (2017). Enrichment and characterization of hydrocarbon-degradingbacteria from petroleum refinery waste as potent bioaugmentation agent for in

situ bioremediation. *Bioresource Technology,*242:15-27. https://doi.org/10.1016/j.biortech.2017.05.010

Simaria, C., Pant, G., Sibi, G. (2015). Characterizationandevaluation of polycyclic aromatic hydrocarbon (Pah) degrading bacteria isolated from oil contaminated soil. *Applied Microbiology: Open Access*, 1:1. https://doi.org/10.4172/2471-9315.1000104

Sheludchenko, M.S., Kolomytseva, M.P, Travkin, V.M., Akimov, V.N., Goloveva, L.A. (2005). Degradation of aniline by *Delftia tsuruhatensis* 14S in batch and continuous processes. *Applied Biochemistry and Microbiology*, *41*(5), 465-468. https://doi.org/10.1007/s10438-005-0083-8

Tremblay J., Yergeau, E., Fortin, N., Cobanli, S., Elias, M., King, T.L., Lee, K., Greer, C.W. (2017). Chemical dispersants enhance the nactivity of oil-and gas condensate-degrading marine bacteria. *The ISME Journal*, 11, 2793-2808. https://doi.org/10.1038/ismej.2017.129

Throne-Holst, M., Wentzel, A., Ellingsen, T.E., Kotlar, H.K., Zotchev, S.B. (2007). Identification of novel genes involved in long-chain n-alkane degradation by *Acinetobacter* sp. strain DSM 17874. *Applied and Environmental Microbiology,* 73(10),3327-3332. https://doi.org/10.1128/AEM.00064-07

Urata, M., Uchida, E., Nojiri, H., Omori, T., Obo, R., Miyaura, N., Ouchiyama, N. (2004). Genes involved in aniline degradation by *Delftia acidovorans* strain 7N and its distribution in the natural environment. *Bioscience*, *Biotechnology*, and *Biochemistry,* 68(12), 2457-2465. https://doi.org/10.1271/bbb.68.2457

Varjani, S.J., & Upasani, V.N. (2016). Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM 5514. *Bioresource Technology,* 222,195-201. https://doi.org/10.1016/j.biortech.2016.10.006

Varjani, S.J. (2017). Microbial degradation of petroleum hydrocarbons. *Bioresource technology*, 223, 277-286. https://doi.org/10.1016/j.biortech.2016.10.037

Vásquez-Piñeros, M.A., Martínez-Lavanchy P.M, Jehmlich, N., Pieper D.H, Rincón C.A, Harms, H., Junca, H., Heipieper, H.J. (2018). *Delftia* sp. LCW, a strain isolated from a constructed wetland shows novel properties for dimethylphenol isomers degradation. *BMC Microbiology*, 18:108. https://doi.org/10.1186/s12866-018-1255-z

Vacca, D., Bleam, W., Hickey, W.(2005). Isolation of soil bacteria adapted to degrade humic acid-sorbed phenanthrene. *Applied and Environmental Microbiology,* 71, 3797-380. https://doi.org/10.1128/AEM.71.7.3797-3805.2005

Wu, W., Huang, H., Ling, Z., Yu, Z., Jiang, Y., Liu, P., Li, X. (2016). Genome sequencing reveals mechanisms for heavy metal resistance and polycyclic aromatic hydrocarbon degradation in *Delftia lacustris* strain LZ-C. *Ecotoxicology*, 25, 234-247. https://doi.org/10.1007/s10646-015-1583-9

Wang, X.B, Chi, C.Q., Nie, Y., Tang, Y.Q., Tan, Y., Wu, G, Wu, X.L. (2011). Degradation of petroleum hydrocarbons (C_6-C_{40}) and crude oil by a novel *Dietzia* strain. *Bioresource Technology,* 102: 7755-7761. https://dx.doi.org/10.1016/j.biortech.2011.06.009

Wen, A., Fegan, M., Hayward, C., Chakraborty, S., Lindsay, I.S. (1999). Phylogeneticrelation ships among members of the Comamonadaceae ,and description of *Delftia acidovorans* (den Dooren de Jong 1926 and Tamaoka et al. 1987) gen. nov., comb. nov. *International Journal of Systematic Bacteriology,* 2, 567-576. https://doi.org/10.1099/00207713-49-2-567

Xiao, C., Ning, J., Hai Yan, H., Sun, X.D, Hu, J. (2009). Biodegradation of aniline by a newly isolated *Delftia* sp. XYJ6. *Chinese Journal of Chemical Engineering*,17(3), 500-505. https://doi.org/10.1016/S1004-9541(08)60237-2

Xu, X., Zhai, Z., Li, H., Wang, Q., Han, X., Yu, H. (2017). Synergeticeffect of bio-photocatalytichybrid system: g-C3N4, and *Acinetobacter*, sp. JLS1 for enhanced degradation of C16 alkane. *Chemical Engineering Journal,* 323, 520-529. https://doi.org/10.1016/j.cej.2017.04.138

Xu, X., Liu, W., Tian, S., Wang, W., Qi, Q., Jiang, P., ... & Yu, H. (2018). Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis. *Frontiers in microbiology*, 9, 2885. https://doi.org/10.3389/fmicb.2018.02885

Ye, J.X., Lin, T.H., Hu, J.T., Poudel, R., Cheng, Z.W., Zhang, S.H., Chen, J.M., Chen, D.Z. (2019). Enhancing chlorobenzene biodegradation by *Delftia tsuruhatensis* using a water-silicone oil biphasic system. *International Journal of Environmental Research and Public Health,*16(9), 1629. https://doi.org/10.3390/ijerph16091629

Zhang, Z., Hou, Z., Yang, C., Ma, C., Tao,F., Xu, P. (2011). Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. *Bioresource Technology*, 102(5), 4111-4116. https://doi.org/10.1016/j.biortech.2010.12.064

BIOTECH STUDIES
VOL 31 ISSUE 1 JUNE 2022 ISSN 2687-3761 E-ISSN 2757-5233

CONTENTS