GAME THEORETICAL ANALYSIS OF COOPERATION AND CHEATING AMONG LIPASE PRODUCING *YARROWIA LIPOLYTICA* SUB-CULTURES

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GAME THEORETICAL ANALYSIS OF COOPERATION AND CHEATING AMONG LIPASE PRODUCING *YARROWIA LIPOLYTICA* SUB-CULTURES

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ABSTRACT

GAME THEORETICAL ANALYSIS OF COOPERATION AND CHEATING AMONG LIPASE PRODUCING *YARROWIA LIPOLYTICA* SUB-CULTURES

Game theory is a mathematical tool for understanding *social dilemmas* and solving conflicts of interest between agents with set of *strategies* and goal of maximizing personal interests (*payoffs*). Among microorganisms, different phenotypes, such as complete or partial failure in exoenzyme production, can be regarded as strategies in a game where growth rates of cells represent fitnesses of each strategy. Consequently, the productivity of the population is determined by the interplay between the exoenzyme producer "Cooperator" and the exoenzyme non-producer "Cheater" sub-cultures. Among non-conventional yeasts, *Yarrowia lipolytica* can efficiently produce extracellular lipases to degrade hydrophobic substrates such as fats and fatty acids. In this thesis a game theoretical model is constructed to analyze the evolutionary dynamics and the lipase productivity of the *Y. lipolytica* population in terms of structured mathematical models. Fermentation model results indicate that fed-batch mode simultaneously promoted the Cheating and the lipolytic activity compared to batch mode which signifies the importance of the specific productivity independent from sub-culture frequencies. The evolutionary stability of the "Cooperator" and the "Cheater" sub-cultures are analyzed with a "stable state finder algorithm". Two distinct game regimes were identified. When the lipase production cost is low, Cooperators dominate the population. At intermediate and higher costs, Cooperators coexist with Cheaters. These regimes correspond to *"No-Conflict"* and *"Snowdrift"* regimes respectively. The evolutionary stable states of the "multi-player fermentation game" is affected by the initial conditions. At higher initial cell densities equilibrium frequency of the Cooperators decreases. Cooperators benefit from higher initial olive oil concentrations and consequently the lipolytic activity increases. Also, Cheaters benefit from higher initial oleic acid concentrations and as a result the lipolytic activity decreases. The methodology of finding the Evolutionary stable state can also be applied to scenarios where different species are considered as players. In growing biotechnological literature, co-cultures are becoming prominent examples of such scenarios.

ÖZET

LİPAZ ÜRETEN *YARROWIA LIPOLYTICA* ALT KÜLTÜRLERI ARASINDAKİ İŞBİRLİĞİ VE HİLEKARLIĞIN OYUN TEORİSİ İLE ANALİZİ

Oyun teorisi, sosyal ikilemleri anlamakla birlikte, *stratejileri* ve kişisel çıkarları (*ödüller*) en üst düzeye çıkarma hedefi olan taraflar arasındaki çıkar çatışmalarını çözmek için kullanılan matematiksel bir araçtır. Ekzoenzim üretimindeki tam veya kısmi kusurlar gibi farklı fenotipler, hücrelerin büyüme oranlarının her stratejinin uygunluğunu temsil ettiği bir oyunda stratejiler olarak kabul edilebilir. Sonuç olarak, popülasyonun üretkenliği, ekzoenzim üreticisi "İşbirlikçi" ile ekzoenzim üretici olmayan "Hileci" alt kültürleri arasındaki etkileşim tarafından belirlenir. Geleneksel olmayan mayalar arasında, *Yarrowia lipolytica*, yağlar ve yağ asitleri gibi hidrofobik substratları parçalamak için hücre dışı lipazları verimli bir şekilde üretebilir. Bu tezde, *Y. lipolytica* popülasyonunun evrimsel dinamiklerini ve lipaz üretkenliğini yapılandırılmış matematiksel modeller açısından analiz etmek için oyun teorisi modeli oluşturulmuştur. Fermantasyon modeli sonuçları, kesikli fermentasyona kıyasla, kesikli beslemenin, alt kültür frekanslarından bağımsız olarak Hilekarlığı ve lipolitik aktiviteyi aynı anda desteklediğini göstermesi özgül üretkenliğin önemini belirtirmiştir. "İşbirlikçi" ve "Hileci" alt kültürlerinin evrimsel kararlılığı "kararlı durum bulucu algoritması" ile analiz edilmiştir. İki farklı oyun rejimi belirlenmiştir. Lipaz üretim maliyeti düşük olduğunda, İşbirlikçiler popülasyona hakimdir. Orta ve daha yüksek maliyetlerde, İşbirlikçiler Hilecilerle bir arada bulunur. Bu rejimler sırasıyla *"No-Conflict"* ve *"Snowdrift"* rejimlerine tekabül etmektedir. "Çok oyunculu fermantasyon oyununun" evrimsel kararlı durumları, başlangıç koşullarından etkilenir. Başlangıç hücre yoğunluğunun daha yüksek olduğu durumda, İşbirlikçilerin denge frekansı azalır. İşbirlikçiler başlangıçtaki yüksek zeytinyağı konsantrasyonlarından yararlanır ve sonuç olarak lipolitik aktivite artar. Ayrıca, Hileciler başlangıçtaki yüksek oleik asit konsantrasyonlarından yararlanır ve sonuç olarak lipolitik aktivite azalır. Evrimsel kararlı durumu bulma metodolojisi, farklı türlerin oyuncu olarak kabul edildiği senaryolara da uygulanabilir. Büyüyen biyoteknolojik literatürde, ortak kültürler bu tür senaryoların öne çıkan örnekleri haline geliyor.

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LIST OF SYMBOLS/ABBREVIATIONS

f_{Cheat}	Cheater's fitness
f_{Coop}	Cooperator's fitness
P_{Cheat}	Cheater's payoff
P_{Coop}	Cooperation's payoff
x_{Cheat}	Frequency of the cheater
x_{Coop}	Frequency of the cooperator
x_{max}	Maximum initial frequency
x_{min}	Minimum initial frequency
$\emptyset$	Weighted average fitness
m	Number of players with minimum initial frequency
n	Number of players
N	Number of participants
P	Cheater's payoff against cheater
R	Cooperation's payoff against cooperation
S	Cooperation's payoff against cheater
T	Cheater's payoff against cooperation

k_{11}	Maximum adsorption constant related to lipase in aqueous phase
k_{12}	Saturation adsorption constant related to lipase in aqueous phase
k_{1m}	Intracellular lipase synthesis maximum rate coefficient
k_{1s}	Intracellular lipase synthesis saturation constant
k_{es}	Lipase excretion saturation constant
k_i	Intracellular lipase synthesis inhibition coefficient
k_m	Lipase transport rate coefficient
k_{oh}	Olive oil hydrolysis parameter
k_s	Maintenance coefficient
k_{so}	Substrate transport rate coefficient
k_{so1}	Transport saturation constant for substrate
k_μ	Lipase transport saturation constant
Y_{SS}	Oleic acid/olive oil yield coefficient

Y_{SX}	Substrate/biomass yield coefficient
μ_0	Basic growth rate
μ	Specific growth rate
μ_{Cheat}	Specific growth rate of the cheater
μ_{Coop}	Specific growth rate of the cooperator
μ'_{max}	Maximum specific growth rate
c	Cost of cooperation
k	Half saturation constant
p	Total cell density
q	Proportionality constant
r	Reward parameter
β	Maintenance coefficient
δ	Privileged share
δS	Excretion function with active transport
δS_{Coop}	Cooperator's excretion function with active transport
Lip_{Aq}	Aqueous lipase
Lip_{Ext}	Extracellular lipase
Lip_{Int}	Intracellular lipase
S_{Cheat}^{OA}	Intracellular oleic acid of the cheater
S_{Coop}^{OA}	Intracellular oleic acid of the cooperator
S_{Ext}^{OA}	Extracellular oleic acid
S_{Ext}^{OO}	Extracellular olive oil
S_{Int}^{OA}	Intracellular oleic acid
X_{Cheat}	Biomass of the cheater
X_{Coop}	Biomass of the cooperator
X	Biomass
EGT	Evolutionary game theory
ESS	Evolutionary stable strategy
NE	Nash equilibrium
NPD	N-person prisoner's dilemma

NSD	N-person snowdrift game
ODE	Ordinary differential equation
PD	Prisoner’s dilemma
SD	Snowdrift game

1. INTRODUCTION

1.1. Game Theory and Evolutionary Dynamics

Game theory is a mathematical tool for understanding and solving conflicts of interest between agents [1]. The agents are considered to be players with set of strategies and goal of maximizing personal interests (*Payoffs*). The game problem is distinguished from the standard optimization problem since the best strategy choice depends on strategy choices made by other players [1]. The optimal choice of strategies and the game's outcome is studied under the solution concepts such as, min-max, Nash solution, Stackelberg solution and Pareto optimality [2–5].

1.1.1. 2- Person Games

The game theory is especially useful to explain social dilemmas. Social dilemmas occur where the optimal behavior of an individual does not align with the optimal outcome for the group [6]. The dilemma can be explained by a game where 2 players engage in a pairwise interaction by selecting either to "Cooperate" or to "Cheat". The payoffs of the strategy choices are represented in matrix form (payoff matrix) in Table 1.1.

Since the game is symmetrical, we can analyze decisions of one player at a time. There are 4 possible games depending on the payoff parameters.

No Conflict: If R > T , S > P, Cooperate is the dominant strategy and the only stable equilibrium is mutual cooperation. This is a game of No Conflict. There is no problem in explaining cooperation here [7].

Table 1.1. Pairwise representation of social dilemmas. Letters represent the pay-offs for the focal player

		Opponent: Coop	Opponent: Cheat
Focal Player	Coop	R	S
	Cheat	T	P

Prisoner's dilemma: If T > R > P > S, Cheat is a dominant strategy and the only stable equilibrium is mutual Cheating. Mutual cooperation would give a higher payoff to both players. The problem with cooperation here is to explain how to escape from the inefficient stable equilibrium of mutual Cheating. Spatial and stochastic effects or iteration of the game can lead to evolution of cooperation [8–10].

Snowdrift: If T > R > S > P, "mutual Cooperation" is better than "Cooperating while the other player Cheats", but "Cheating while the other player cooperates" is better than "mutual Cooperation", and "mutual Cheating" is the worst possible outcome. This is an anti-coordination game, with two asymmetric equilibria with pure strategies and one symmetric equilibrium in mixed strategies. The problem is to explain how to increase the number of Cooperators and thus the average fitness of the population [11–13].

Stag Hunt (Coordination): If R > P > T , S , "mutual Cooperation" is better than "mutual Cheating", and both "mutual Cheating" and "mutual Cooperation" give better results than lack of coordination. This is a coordination game, with two symmetric equilibria with pure strategies. It has received little attention in evolutionary biology. The problem with cooperation here is to shift from the risk-dominant equilibrium "mutual Cheating" to the payoff-dominant equilibrium "mutual Cooperation".

1.1.2. N-Person Games

Social dilemmas, can be understood and studied by 2-person games without difficulty, however in nature, they generally occur among populations encompassing more than 2 individuals. Therefore, a transition from 2-person games with pairwise interaction to N-person games with collective action is required [6].

In the N-person games the lack of cooperation leads to impairment of production of a public good. A *public good* is defined as non-excludable (members cannot be excluded from consuming it) and non-rivalrous (the consumption by one, won't diminish the availability to another). Generally, public goods are not purely non-rivalrous and non-excludable but they are approximated to be. For instance, production of exoenzymes in microorganisms are in fact *common goods* [6].

N participants are divided into two types of players in the *N-Person Prisoner's dilemma* (NPD): There are i Cooperators that pay a contribution c, and $N - i$ Cheaters that doesn't pay any contribution. The contribution sum is multiplied by a reward factor r (> 1) and then shared among all participants. Accordingly, the payoffs for the Cheater and the Cooperator are

$$P_{Cheat}(i) = \frac{rci}{N} \tag{1.1}$$

and

$$P_{Coop}(i) = \frac{rci}{N} - c = P_{Cheat}(i) - c. \tag{1.2}$$

respectively. Assuming that, in a population with randomly assigned roles, the probability of finding j Cooperators among N individuals (including self) is $f_j(x)$, where x is the frequency of Cooperators. The fitness of the individual player utilizing the Cheater strategy is

$$W_{Cheat} = \sum_{j=0}^{N-1} f_j(x) \frac{rcj}{N}, \tag{1.3}$$

And the individual fitness of the individual player utilizing the Cooperation strategy is

$$W_{Coop} = \sum_{j=0}^{N-1} f_j(x) \left[\frac{rc(j+1)}{N} - c \right] = W_D + \frac{rc}{N} - c, \quad (1.4)$$

If $r/N > 1$, there is no social dilemma, Cooperating is the only ESS since it dominates Cheating. Else if $r/N < 1$, Cheating is the only ESS since it dominates Cooperation; the average fitness becomes zero at this state which is smaller than the average fitness of the former state $(r-1)c$.

In the *N-person Snowdrift game* (NSD) the cost of cooperation is distributed among the Cooperators. Therefore, the payoffs for Cheating and Cooperation are, respectively

$$P_{Cheat}(i) = \begin{cases} rc & \text{if } 1 \le i \le N-1 \\ 0 & \text{if } i = 0 \end{cases} \quad (1.5)$$

and

$$P_{Coop}(i) = rc - \frac{c}{i} \text{ for } 1 \le i \le N. \quad (1.6)$$

Where there are i Cooperators in the population and the benefit of cooperation $rc = b$ in units of cost c. In this game, Cooperators will be in coexistence with Cheaters in infinite and well-mixed populations. Unlike NPD the cost is non-linear decreasing function in NSD. Unless N is large, the NSD and NPD behave differently. Even more dramatic effects can be observed when there is non-linearity in the benefit rather than the cost [14].

1.1.3. Evolutionary Game Theory

Evolutionary game theory (EGT) is the application of classical Game theory to evolving populations in biology. EGT analyzes evolutionary stability of strategies and specifies dynamics for the population where the fitness of an organisms depends both its own strategy and strategies of others [8,11,15]. Aspects of living organisms such as evolution of cooperation [9,13,16], sex ratios [11], selection of biochemical pathways [17] and biofilms [18] were analyzed by EGT and furthermore, it can be used for the classification in data analysis [19].

1.1.4. Nash Equilibria and Evolutionary Stable Strategies

In the classical Game Theory, the strategies with their corresponding payoffs are apparent to and consciously chosen by the players. The game's solution is termed as the "Nash equilibrium" where no player does better by adopting some other strategy than the current one [20]. In Evolutionary Game Theory however, players inherit the strategies from their predecessors and the awareness of the game is not needed. The payoffs are defined by reproductive successes. The strategy that will be stable under natural selection is called the *Evolutionary stable strategy* (ESS). The ESS can be roughly defined as an uninvadable strategy when it is adopted by the majority of the population [21]. An ESS is a modified or refined version of a Nash equilibrium.

Let the payoff for playing strategy S against T strategy represented by P(S,T). The strategy pair (S,S) is a Nash equilibrium if and only if for both players, for any strategy S:

$$P(S,S) \geq P(T,S)$$

For an ESS two conditions must be specified for a strategy S to be an ESS. For all $S \neq T$, either

$$P(S,S) > P(T,S) \text{ or}$$

$$P(S,S) = P(T,S) \text{ and } P(S,T) > P(T,T)$$

Table 1.2. Pairwise *Prisoner's dilemma* payoff table with numerical payoffs

	Cooperate	Cheat
Cooperate	3, 3	1, 4
Cheat	4, 1	2, 2

Prisoner's dilemma

Table 1.3. Pairwise *Harm thy neighbor* payoff table with numerical payoffs

	A	B
A	2, 2	1, 2
B	2, 1	2, 2

Harm thy neighbor

Nash equilibria and the Evolutionary Stable Strategy may coincide perfectly for simple games such as *Prisoner's dilemma* where the (Cheat) strategy is an ESS and (Cheat, Cheat) is a Nash equilibrium. In some games there may be Nash equilibria that are not Evolutionary Stable Strategies. In harm thy neighbor, since players don't do better by switching their strategies, both (A,A) and (B,B) are Nash equilibria. However, since B can neutrally invade "A predominated population", only B is an ESS.

Table 1.4. Pairwise *Chicken (Snowdrift) game* payoff table with numerical payoffs

	Swerve	Stay
Swerve	0, 0	-1, +1
Stay	+1, -1	-20, -20

Chicken (Snowdrift) game

Consider the Chicken (*Snowdrift)* game in Table 1.4. The game has two pure strategy Nash equilibria, (Stay, Swerve) and (Swerve, Stay) and a mixed strategy Nash equilibrium where the players play Swerve with the probability p and play Stay with the probability 1-p. ESS of the game depends whether players are able to distinguish their role as "row or column player". If such uncorrelated asymmetry exists, pure strategy Nash equilibria are ESSes. If players aren't able to distinguish their roles, uncorrelated asymmetry does not exist, both players must choose the same strategy and the mixed Nash equilibrium will be ESS [11]. Therefore, the term ESS can be applied individually to a mixed strategy. A

mixed ESS may be equivalent to a population state where mixture of pure strategies coexists. While the ESS refers to the strategy itself, *Evolutionary stable state* (ES State) refers to population-wide balance where the population can restore its genetic composition after a small disturbance [22].

1.1.5. Replicator Dynamics

In evolutionary games, the competition aspect (the game) together with the natural selection (replicator dynamics), contributes to an understanding of co-evolution, group selection and ecological dynamics [8]. The replicator dynamics shows the growth rate of an organism by taking account of the difference between the average payoff of the organism and the average payoff of the population as a whole. The stable states of a given population is equivalent to the attractor states of the replicator equations. The general form of the replicator equation is:

$$\dot{x}_i = x_i[f_i(x) - \emptyset(x)] \tag{1.7}$$

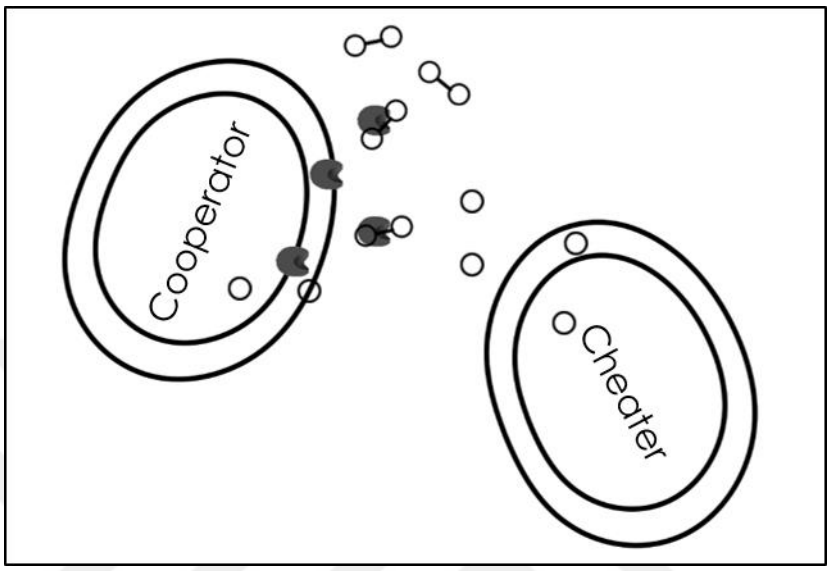
$$\emptyset(x) = \sum_{j=1}^{n} x_j f_j(x) \tag{1.8}$$

Where x_i is the frequency of the type i in the population, $x = (x_i, \dots, x_n)$ is the type distribution vector, $f_i(x)$ is the frequency dependent fitness of type i and $\emptyset(x)$ is the weighted average fitness in the population of the n types.

1.2. Cooperation and Cheating

Different phenotypes, such as complete or partial failure in exoenzyme production, can be regarded as strategies in an evolutionary game where growth rates of cells represent fitnesses of each strategy. While epigenetic and genetic states correspond to strategies, gene silencing and mutations corresponds to switches between strategies [23]. Optimization and analysis of biotechnological setups can be done with the EGT, where the productivity of the population is determined by the interplay between the exoenzyme producer and the exoenzyme non-producer sub-cultures [23]. Although maximization of

exoenzyme production is desired, exoenzyme production is often decreased by the non-producing subpopulation of microorganisms [24,25]. Throughout the study, we refer exoenzyme producers as the Cooperators and the exoenzyme non-producers as the



Cheaters. Cheaters can benefit from the products released by the activity of enzymes produced by Cooperators (Figure 1.1.).

Figure 1.1. Interplay between microbial cells for the exoenzyme secretion. Complex substrates are indicated by linked circles. Cooperating cells ('Coop') secrete exoenzyme (black sliced circles) to release growth substrate (single circles). Cheater cells ('Cheat') do not produce exoenzymes yet benefit from the growth substrate

An example of this phenomenon is observed in *SUC* genes encoding the yeast invertase. Some strains of *S. cerevisiae* carry only non-functional *SUC2* gene as the only *SUC* gene [26]. Moreover, there are species that doesn't carry any *SUC* genes like *S. italicus* [27]. These species benefit from the invertase produced by other *Saccharomyces* species. When artificially generated, wild-type *S. cerevisiae* coexist with the cells in which the *SUC2* gene is knocked out [28].

In Greig and Travisano's study on *S. cerevisiae* (2003), they have measured Cheater's fitness relative to Cooperator in a sucrose-rich agar plate: In order to control the role of density, the plates were inoculated with cell numbers ranging from well-separated (108 cells per plate) to densely touching layers ($1.6x10^9$ cells per plate) [28]. In every plate the invertase producer "Cooperator" and a non-producer "Cheater" strain were started with

equal numbers of cells. After incubating 3 days at 30°C, the final cell numbers were counted. Finally, the relative fitness of the Cheater was calculated. As a result the relative fitness the Cheater has increased with the increasing initial total cell density (Figure 1.2.) [28].

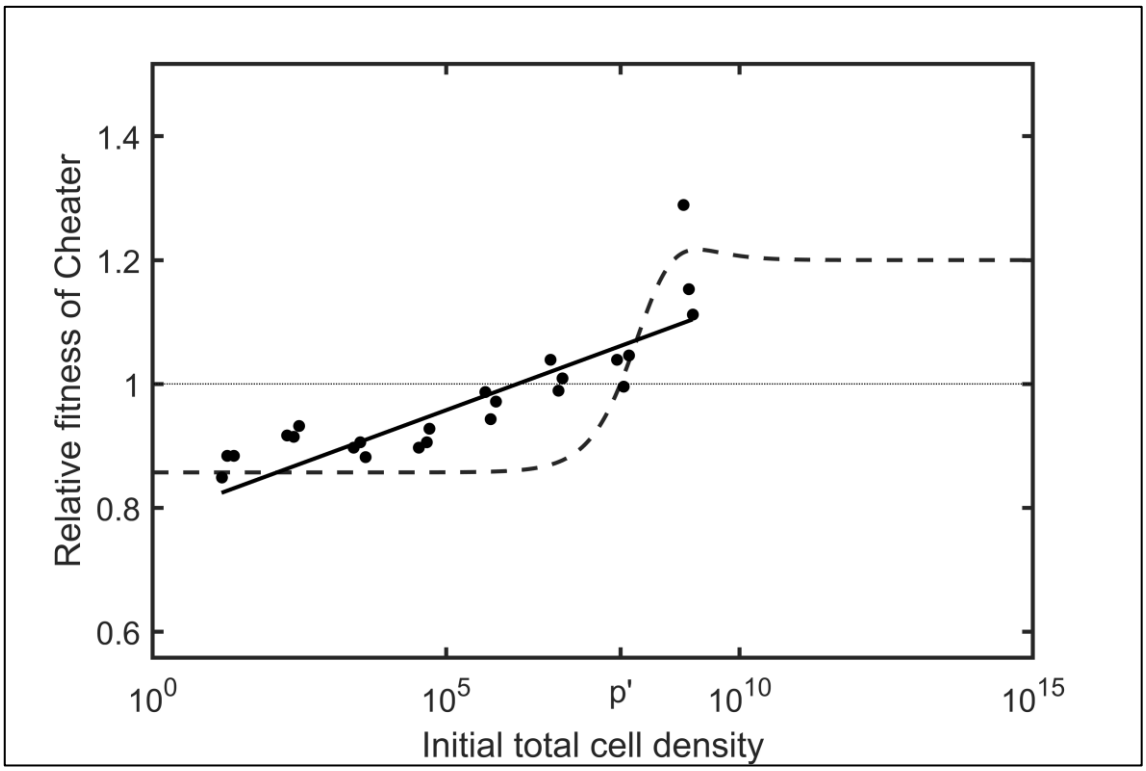


Figure 1.2. Relative fitness of Cheater at varying levels of Initial total cell density. Points and their line of best fit represents experimental data from Greig and Travisano, 2003 [28]. Dashed curve is the model representation of Equation 1.10 as described in Schuster et al., 2010 [23] in which, the fitness of Cooperators and Cheaters are equal at ρ'. Parameter values are: $\mu_0 = 0.2/\text{h}$, $\mu' max = 0.4/\text{h}$, $k = 2$ mM, $q = 8 \times 10^{-9}$ mM, $c = 0.1/\text{h}$, $\delta = 1$ mM, $x_{Coop} = 0.5$, which leads p' to $= 10^{7.97}$

They, concluded that this phenomenon corresponds to Prisoner's dilemma by fitting the data into straight line (Figure 1.2.) and concluded that in high densities Cheaters invade closely Cooperating populations by exploiting the enzyme produced by a Cooperating neighbor. However, later on again with *S. cerevisiae*, Gore et al., 2009 [29] showed that even in well-mixed cultures a coexistence between the Cooperator and the Cheater can be established (Figure 1.3a., Figure 1.3b.). In the study of Gore et al., 2009 [29], competition experiments were conducted between the wildtype Cooperator and the invertase knockout

"Cheater" strain. Limited histidine concentrations in the media creates a handicap for the histidine auxotroph wildtype Cooperator strain, allowing the manipulation of the "cost" of cooperation. They have showed that, Cooperator, decreased in fraction when in majority (Figure 1.3a.top.) and increased in fraction when in minority (Figure 1.3a.bottom.). Moreover, regardless of the initial composition of the population (fractions), equilibrium state can be reached within experimental timescales in histidine limited conditions (Figure 1.3b.). Finally, as the cost of cooperation increases (decrease of histidine concentration), both the equilibrium fraction of Cooperators as well as their mean growth rate decreases (Figure 1.3c.).

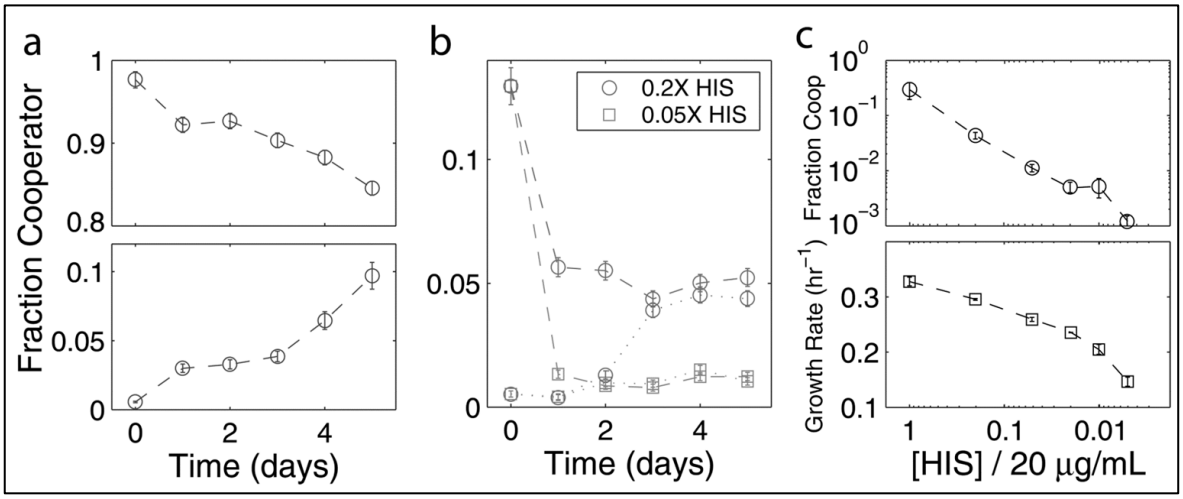


Figure 1.3. Coexistence between the Cooperator and the Cheater strains, as represented in Gore et al., 2009 [29]

A theoretical analysis of Cooperation and Cheating for biotechnological purposes were studied by Schuster et al., 2010 [23]. In their model, they have considered exponentially growing populations with the basic growth rate μ_0 when the desired growth substrate is absent. Cells utilize growth substrate S which is at the surface of a given cell. The dependence of growth rate on desired substrate S is represented with the Monod equation.

$$\mu'(S) = \frac{\mu'_{max} \cdot S}{k + S} \tag{1.9}$$

k and μ'_{max} represents half saturation constant and maximum growth rate respectively. Since most enzymes are located at the vicinity or periplasmic space of the cell, the growth

substrate captured by the enzyme-producing cell even in the well-mixed systems. This "privileged share" has an effect on the concentration of growth substrate and denoted as δ. The frequency of Cooperators and total cell density are denoted as x_{Coop} and ρ respectively. The cost of the enzyme production is represented with c, in growth rate. And q is the proportionality constant relating Cooperators to growth substrate. Relative fitness of Cheaters was calculated with the following equation,

$$\frac{\mu_{Cheat}}{\mu_{Coop}} = \frac{\mu_0 + \mu'(q\rho x_{Coop})}{\mu_0 - c + \mu'(q\rho x_{Coop} + \delta)}, \tag{1.10}$$

where the specific growth rate of the Cheater cell, and the Cooperator cell are represented as μ_{Cheat} and μ_{Coop} respectively. Using the experimental data from Greig and Travisano, 2003 [28], relative fitness of Cheaters were plotted against the initial cell density where the fraction of Cooperators and Cheaters are equal ($x_{Coop} = 0.5$) (Figure 1.2.).

With theoretical analysis, Schuster et al., 2010, demonstrates that secretion of exoenzymes is *Snowdrift game* under wide range of physiological conditions as experimentally demonstrated in *S. cerevisiae* by Gore et al., 2009 (

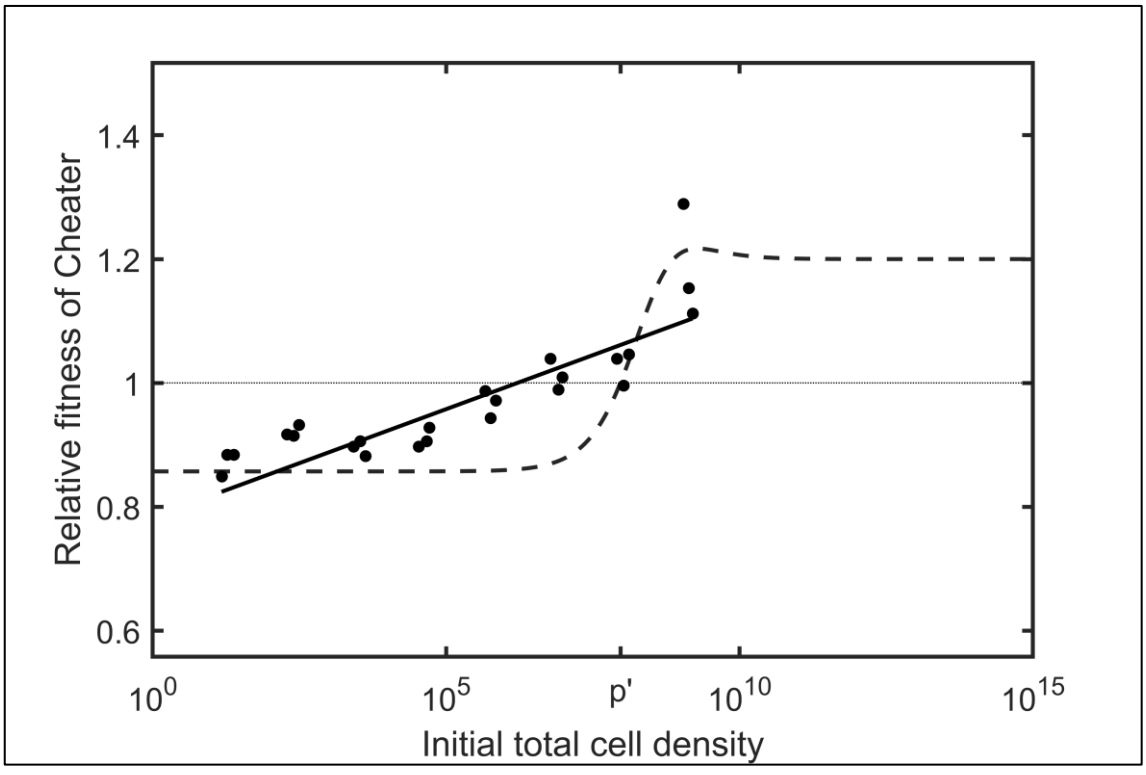


Figure 1.2.) [29]. The cooperation occurs when the benefit from producing exoenzymes exceeds the cost of its production and its accounted with the privileged share δ by Schuster et al., 2010 [23]. For instance, when exoenzymes are bound to cell-wall or periplasmic space or if the medium is not well mixed, Cooperators may retain a part of the growth substrate [28,30]. Schuster et al., 2010 notes that cost of exoenzyme production and secretion must be considered or estimated for biotechnological applications. Further investments are worth making if the cost of exoenzyme production allows for the cooperation.

1.3. Extracellular Lipase from *Yarrowia lipolytica*

Among non-conventional yeasts, *Yarrowia lipolytica* is a model organism used for the study of protein secretion, substrate degradation and biolipid production [31]. It can efficiently degrade hydrophobic substrates such as fats and fatty acids, produce biotechnologically valuable products such as extracellular enzymes, organic acids and aroma compounds and has a great potential for biodegradation and bioremediation [32]. Extracellular enzymes secreted by *Y. lipolytica*, including lipases, alkaline or acid proteases, phosphatases, RNase and inulinase could be used in the food, pharmaceutical, detergent industries [33].

As serine hydrolases, lipases, otherwise known as triacylglycerol acylhydrolases catalyze the hydrolysis of the ester bond of tri-, di-, and monoglycerides of long chain fatty acids into fatty acids and glycerol [31].

The *LIP2* is the major gene responsible for all of the extracellular lipase activity of *Y. lipolytica* whose expression is induced by olive oil and oleic acid and repressed by glucose [34]. As the first step of triglycerides catabolism, *Y. lipolytica* produces intracellular, cell-bound and extracellular lipases [35,36]. The production of lipase by *Y. lipolytica* is growth associated and mainly cell-bound at the beginning of cultivation. The lipase releases after about 50 percent of the substrate (glucose or olive oil) is consumed and reaches its maximum concentration at the late stationary phase (Figure 1.4.) [36].

Since the *Y. lipolytica* extracellular lipase is biocatalyst for biotechnological processes, food, pharmaceutical and environmental industries are interested in large scale production

of the enzyme [37]. Large scale production processes requires development and validation of mathematical models for optimization and process control [38].

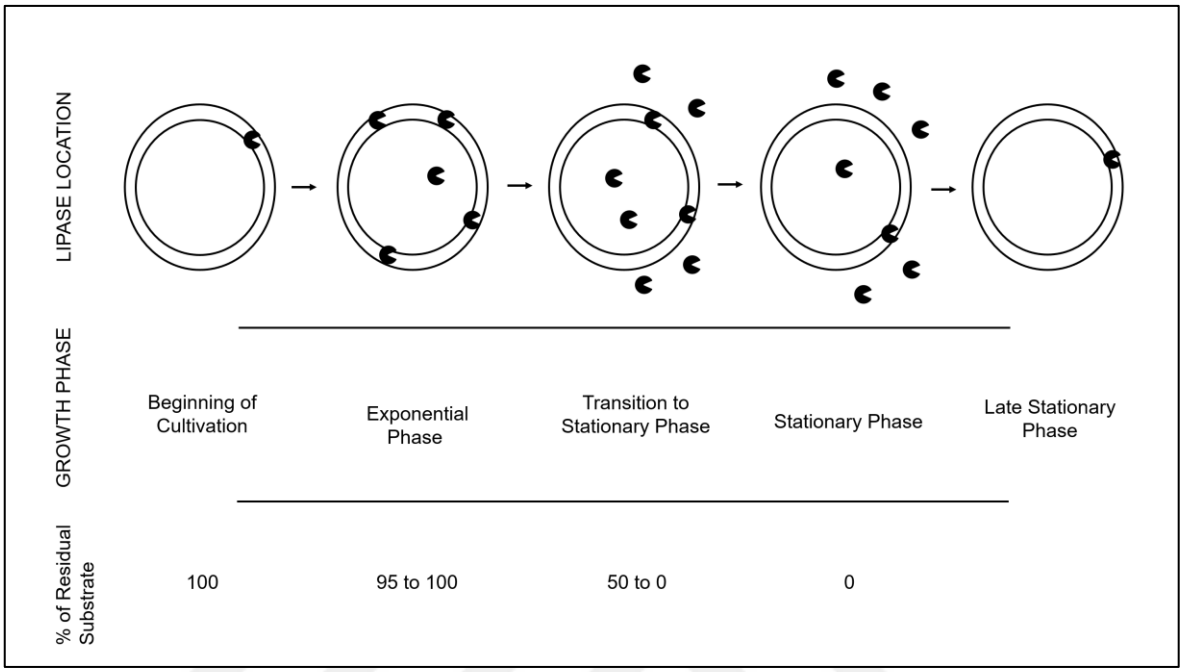


Figure 1.4. Lipase location, growth phase and substrate consumption as represented in Pereira-Meirelles, 2000 [36]. At the beginning of cultivation, a basal activity of lipase (sliced circles) is present. Next, at exponential phase lipase stays mostly cell-bound and then it is released through transitioning to and during stationary phase, until late stationary phase where only a basal lipase activity remains

1.3.1. Mathematical Model for Lipase Production in *Y. lipolytica*

In bioreactors, the effects of different process parameters on the microorganism are represented by kinetic models [39]. *Y. lipolytica* batch and continuous lipase production was modeled using simple structured mathematical models in which the state variables and parameters were estimated [40]. The growth is expressed by Monod's equation as a function of the intracellular concentration of substrate (oleic acid),

$$\mu = \frac{\mu_{max} + S_{Int}^{OA}}{k_{ss} + S_{Int}^{OA}}, \tag{1.11}$$

where S_{Int}^{OA} refers to intracellular oleic acid and k_{ss} is intracellular substrate saturation constant. Accordingly, mass balance equation is expressed as,

$$\frac{dX}{dt} = \mu \cdot X. \tag{1.12}$$

Transportation of oleic acid towards the cell is expressed as active transport with saturation and then it is consumed, transformed and stored by the cell. The state variable S_{Int}^{OA} (Intracellular oleic acid) and S_{Ext}^{OA} (Extracellular oleic acid) is expressed as,

$$\frac{dS_{Int}^{OA}}{dt} = \frac{k_{so} \cdot S_{Ext}^{OA}}{\mathrm{k}_{so1} + S_{Ext}^{OA}} - (Y_{SX} \cdot \mu + \beta) - \mu \cdot S_{Int}^{OA} \tag{1.13}$$

and

$$\frac{dS_{Ext}^{OA}}{dt} = -\frac{k_{so} \cdot S_{Ext}^{OA}}{\mathrm{k}_{so1} + S_{Ext}^{OA}} \cdot X \tag{1.14}$$

respectively. Where k_{so} is substrate transport rate coefficient, k_{so1} is transport saturation constant for substrate, Y_{SX} biomass/substrate yield coefficient and β is maintenance coefficient.

Lipase production is considered to be induced by extracellular oleic acid and it is expressed by the ratio substrate/biomass. Accordingly, state variable Lip_{Int} (Intracellular lipase) is expressed as,

$$\frac{dLip_{Int}}{dt} = \frac{k_{1m} \cdot \frac{S_{Ext}^{OA}}{X}}{k_{1s} + \frac{S_{Ext}^{OA}}{X} + k_i \cdot \left(\frac{S_{Ext}^{OA}}{X}\right)^2 \cdot Lip_{Int},} - \frac{k_m \cdot Lip_{Int}}{k_s + Lip_{Int}} \cdot \frac{1}{k_\mu + \mu} \cdot \delta S - \mu \tag{1.15}$$

where k_{1m} is maximum rate coefficient for intracellular lipase synthesis, k_{1s} is intracellular lipase synthesis saturation constant and k_i is intracellular lipase synthesis inhibition coefficient.

The lipase excretion is expressed with saturation limited active transport. Internal substrate consumption is necessary but can be neglected. Accordingly, the state variable Lip_{Ext} (Extracellular lipase) is expressed as,

$$\frac{dLip_{Ext}}{dt} = \frac{k_m \cdot Lip_{Int}}{k_s + Lip_{Int}} \cdot \frac{1}{k_\mu + \mu} \cdot X \cdot \delta S, \tag{1.16}$$

where k_m is lipase transport rate coefficient, k_s is lipase transport saturation constant, k_μ is lipase transport saturation constant and δS (Equation 1.18) is a function that accounts for excretion that is described as active transport with saturation. The adsorption of extracellular lipase in the oleic acid-water interphase is expressed with saturation coupled adsorption law. Accordingly, the state variable Lip_{Aq} (Aqueous lipase) is expressed as,

$$Lip_{Aq} = \frac{Lip_{Ext}}{1 + \frac{k_{11} \cdot S_{Ext}^{OA}}{k_{12} + S_{Ext}^{OA}}}, \tag{1.17}$$

where k_{11} is maximum adsorption constant related to lipase in aqueous phase and k_{12} is saturation adsorption constant related to lipase in aqueous phase.

Finally, δS function is expressed as below equation, where k_{es} is lipase excretion saturation constant.

$$\delta S = \frac{S_{Int}^{OA}}{k_{es} + S_{Int}^{OA}}, \tag{1.18}$$

Table 1.5. *Y. lipolytica* mathematical model parameters

$Y_{XS} = 0.86\ g\ g^{-1}$	$k_{1m} = 2.5\ U\ mg^{-1}\ h^{-1}$	$k_{1s} = 0.08\ g\ g^{-1}$	$k_i = 12\ g\ g^{-1}$
$k_s = 0.25\ U\ \ mg^{-1}$	$k_{so} = 0.153\ g\ g^{-1}\ h^{-1}$	$K_{so1} = 0.135\ g\ l^{-1}$	$\mu_{max} = 0.253\ h^{-1}$
$k_{ss} = 0.01\ g\ g^{-1}$	$k_{11} = 0.50$	$k_{12} = \ 0.19\ g\ l^{-1}$	$k_{es} = 10^{-7} g\ g^{-1}$

$k_\mu = 0.075\ h^{-1}$	$\beta = 0$		

1.4. Aim of the Thesis

The aim of our proposed thesis is to construct a game theoretical model to analyze the evolutionary dynamics and the lipase productivity of the *Y. lipolytica* population using structured mathematical models. In particular, necessary mathematical tools will be developed in order to determine the equilibrium frequencies of the lipase producer and the non-producer (Cooperator and Cheater) sub-cultures. Ultimately, significance of evolutionary pressures in the fermentation setups and possible improvements in terms of lipase productivity will be analyzed.

As mentioned in Chapter 1.2., *S. cerevisiae* exhibits producer, non-producer sub-cultures and experimental studies has shown that increase in initial cell density has benefitted Cheater sub-cultures (Figure 1.2.). There were no records for producer and non-producer sub-cultures for *Y. lipolytica* in the literature. However, like extracellular invertase produced by *S. Cerevisiae*, the extracellular lipase produced by *Y. lipolytica* is "*Public good*". Therefore, the following hypothesis is proposed. Higher initial population densities will benefit Cheater sub-cultures and consequently inhibit lipase production.

2. MATERIALS AND METHODS

2.1. Multi-Player Fermentation Game

Multi-player fermentation game is played between the Cooperator and the Cheater, corresponding to lipase "producer" wildtype and the lipase "non-producer" mutant sub-cultures of *Y. lipolytica* respectively. The produced lipase is assumed to be in periplasmic space and can hydrolyze the extracellular olive oil (substrate). The product of the periplasmic hydrolysis is captured by the cell, thus acts as a privileged share, while the product of the extracellular hydrolysis is equally transported by both sub-cultures. The medium is assumed to be a well-mixed fermentation setup running with either batch or fed-batch mode. The main substrates are olive oil and oleic acid.

2.1.1. Deriving Players

The Multi-player fermentation game consist of common (e.g. extracellular substrate, extracellular enzyme) and sub-culture specific (e.g. intracellular substrate, intracellular enzyme, biomass) state variables (Figure 2.1.). Previous mathematical model of *Y.lipolytica* (Equations 1.11-1.18) [40] is modified to derive one more sub-culture, namely a "lipase non-producing mutant". Together with wild-type, the sub-culture specific state variables are duplicated. Meanwhile, common state variables are kept intact. The following ODE set represents the mathematical model of the Multi-player fermentation Game.

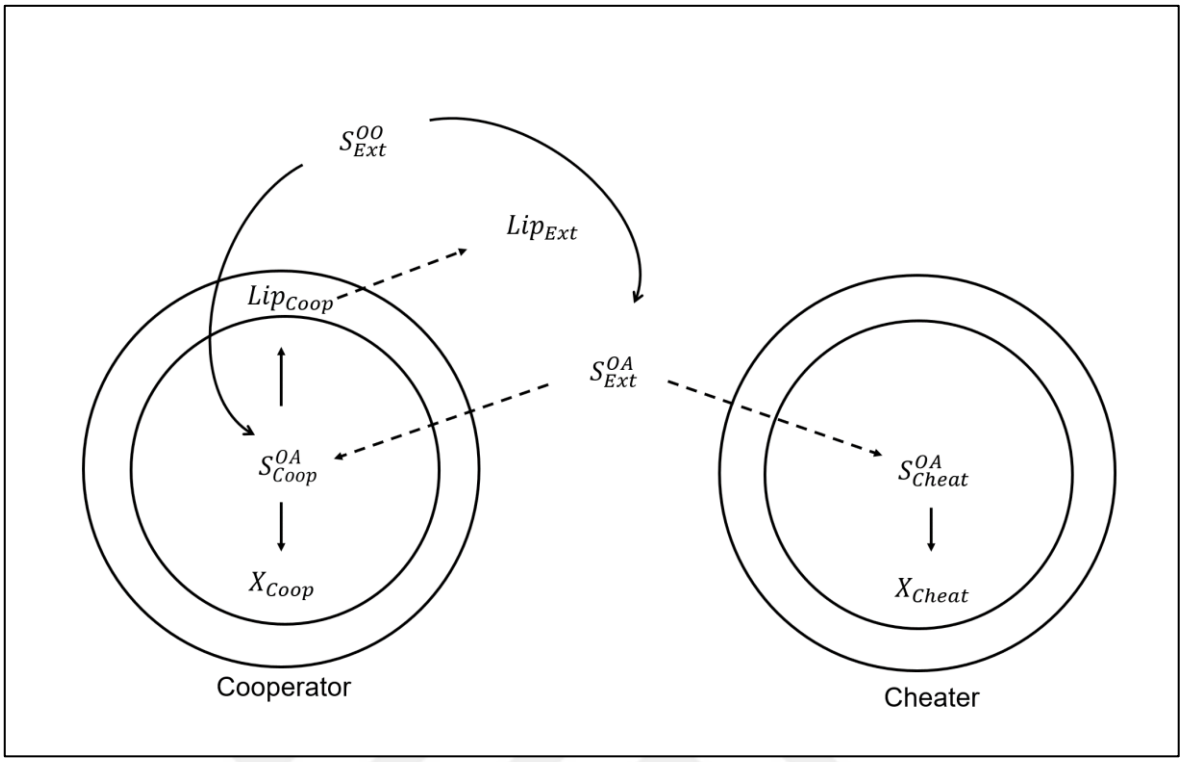


Figure 2.1. Model representation of Multi-player fermentation game. Oil is being hydrolyzed both at the extracellular and the periplasmic space by the extracellular lipase Lip_{Ext} and the periplasmic lipase Lip_{Coop}, resulting in extracellular oleic acid S_{Ext}^{OA} and intracellular oleic acid S_{Coop}^{OA} respectively. While both types of cells use intracellular oleic acid for the cell growth, only the Cooperator produce lipase which then is excreted into the extracellular space

The growth rates, biomasses and intracellular substrates of "Cooperator" and the "Cheater" are described separately. The growth rates of the Cooperator and the Cheater are,

$$\mu_{Coop} = \frac{\mu_{max} + S_{Coop}^{OA}}{k_{ss} + S_{Coop}^{OA}} \tag{2.1}$$

and

$$\mu_{Cheat} = \frac{\mu_{max} + S_{Cheat}^{OA}}{k_{ss} + S_{Cheat}^{OA}} \tag{2.2}$$

respectively, where S_{Coop}^{OA} and S_{Cheat}^{OA} refers to intracellular substrates and k_{ss} is intracellular substrate saturation constant. Accordingly, mass balance equations are expressed as,

$$\frac{dX_{Coop}}{dt} = \mu_{Coop} \cdot X_{Coop} \tag{2.3}$$

and

$$\frac{dX_{Cheat}}{dt} = \mu_{Cheat} \cdot X_{Cheat}. \tag{2.4}$$

Transportation of oleic acid towards the cell is expressed as active transport with saturation and then it is consumed, transformed and stored by the cell. The state variable S_{Coop}^{OA} and S_{Cheat}^{OA} is expressed as,

$$\frac{dS_{Coop}^{OA}}{dt} = \frac{k_{so} \cdot S_{Ext}^{OA}}{k_{so1} + S_{Ext}^{OA}} - \left(Y_{SX} \cdot \mu_{Coop} + \beta\right) - \mu_{Coop} \cdot S_{Coop}^{OA} \tag{2.5}$$

and

$$\frac{dS_{Cheat}^{OA}}{dt} = \frac{k_{so} \cdot S_{Ext}^{OA}}{k_{so1} + S_{Ext}^{OA}} - \left(Y_{SX} \cdot \mu_{Cheat} + \beta\right) - \mu_{Cheat} \cdot S_{Cheat}^{OA} \tag{2.6}$$

respectively, where k_{so} is substrate transport rate coefficient, k_{so1} is transport saturation constant for substrate, Y_{SX} biomass/substrate yield coefficient and β is maintenance coefficient. The extracellular oleic acid S_{Ext}^{OA} is a common state variable therefore, in which the oleic acid transport rate is described for each sub-culture as,

$$\frac{dS_{Ext}^{OA}}{dt} = -\frac{k_{so} \cdot S_{Ext}^{OA}}{k_{so1} + S_{Ext}^{OA}} \cdot X_{Coop} - \frac{k_{so} \cdot S_{Ext}^{OA}}{k_{so1} + S_{Ext}^{OA}} \cdot X_{Cheat}. \tag{2.7}$$

Lipase production and transportation is described for the sole lipase producer Cooperator sub-culture and assumed to be induced by extracellular oleic acid and it is expressed by the ratio substrate/biomass. Accordingly, state variable Lip_{Coop} is expressed as,

$$\frac{dLip_{Coop}}{dt} = \frac{k_{1m} \cdot \frac{S_{Ext}^{OA}}{X_{Coop} + X_{Cheat}}}{k_{1s} + \frac{S_{Ext}^{OA}}{X_{Coop} + X_{Cheat}} + k_i \cdot \left(\frac{S_{Ext}^{OA}}{X_{Coop} + X_{Cheat}}\right)^2} - \frac{k_m \cdot Lip_{Coop}}{k_s + Lip_{Coop}} \cdot \frac{1}{k_\mu + \mu_{Coop}} \cdot \delta S_{Coop} - \mu_{Coop} \cdot Lip_{Coop}, \quad (2.8)$$

where k_{1m} is maximum rate coefficient for intracellular lipase synthesis, k_{1s} is intracellular lipase synthesis saturation constant and k_i is intracellular lipase synthesis inhibition coefficient. Finally, state variable Lip_{Ext} is expressed as

$$\frac{dLip_{Ext}}{dt} = \frac{k_m \cdot Lip_{Coop}}{k_s + Lip_{Coop}} \cdot \frac{1}{k_\mu + \mu_1} \cdot X_{Coop} \cdot \delta S_{Coop} \quad (2.9)$$

where k_m is lipase transport rate coefficient, k_s is lipase transport saturation constant, k_μ is lipase transport saturation constant and δS_{Coop} is a function that accounts for excretion that is described as active transport with saturation

$$\delta S_{Coop} = \frac{S_{Coop}^{OA}}{k_{es} + S_{Coop}^{OA}}. \quad (2.10)$$

2.1.2. Oil Hydrolysis in Extracellular Space

In order to create a social dilemma, an extracellular olive oil state variable needs to be added to the *Y. lipolytica* model. The olive oil hydrolysis is described as a first order kinetics [41]. The product of the extracellular hydrolysis, extracellular oleic acid is described as,

$$\frac{dS_{Ext}^{OA}}{dt} = Y_{SS} \cdot k_{oh} \cdot S_{Ext}^{OO} \cdot Lip_{Ext} \quad (2.11)$$

and accordingly, reduction in extracellular olive oil is described as,

$$\frac{dS_{Ext}^{OO}}{dt} = -k_{oh} \cdot S_{Ext}^{OO} \cdot Lip_{Ext} \tag{2.12}$$

The main compound of virgin olive oil is oleic acid (65 to 85 percent) [42]. Accordingly, it is assumed that the oil hydrolysis yields 70 percent oleic acid and represented as yield coefficient Y_{SS}. k_{oh} is the olive oil hydrolysis parameter and its value has been estimated as 0.5 [41].

2.1.3. Oil Hydrolysis in Periplasmic Space

While olive oil is being hydrolyzed at extracellular space, the lipase-producing wildtype can perform cell-bound oil hydrolysis at periplasmic space. Resulting intracellular oleic acid is described as,

$$\frac{dS_{Coop}^{OA}}{dt} = Y_{SS} \cdot k_{oh} \cdot S_{Ext}^{OO} \cdot Lip_{Coop} \tag{2.13}$$

and accordingly, reduction in extracellular olive oil is described as,

$$\frac{dS_{Ext}^{OO}}{dt} = -k_{oh} \cdot S_{Ext}^{OO} \cdot Lip_{Coop} \cdot X_{Coop}. \tag{2.14}$$

2.1.4. Estimation of Lipase Production Cost

The lipase production cost c implemented as reduction in intracellular substrate per unit lipase produced and described as,

$$\frac{dS_{Coop}^{OA}}{dt} = -c \cdot Lip_{Coop} \cdot S_{Coop}^{OA}, \tag{2.15}$$

2.2. Stable State Finder Algorithm

Lipase production metabolism with many players, is a dynamical system with set of various ordinary differential equations. In order to find the equilibrium frequencies of the player types we need to find the attractors of the system which corresponds to the Evolutionary stable states. The attractors are dependent on the initial frequencies of the player types. Here, the attractors of the system are found by analyzing different sets of initial frequencies where among n players, m players start with minimum frequency x_{min} (1 percent throughout the study) and $n-k$ players start with the maximum frequency x_{max} which is calculated with the following equation,

$$x_{max} = \frac{1-(x_{min} \cdot m)}{n-m} \tag{2.16}$$

Each arrangement of initial frequencies is used as initial condition and ODE is solved for the specified time. If the end frequencies have differed from the initial frequencies than the end frequencies are used as initial conditions for the next ODE set. If the end frequencies are the same as the input frequencies than it is concluded that the ODE is reached to the stable state. The algorithm is implemented with MATLAB programming language version R2021a and presented in the Appendix A (Algorithm. A.1).

2.2.1. Validation of the Stable State Finder Algorithm.

In order to validate the stable state finder algorithm, the population dynamics of the social dilemma games (*Prisoner's dilemma*, *Snowdrift*, *Coordination*, *No-conflict*) were simulated with the replicator dynamics. Evolutionary stable states were found by using the stable state finder algorithm. Finally, the determined stable states were compared with the Nash equilibria of the 2-player games (Table 1.1.).

First, fitnesses of the Cooperator f_{Coop} and the Cheater f_{Cheat} are found by multiplying the frequencies of strategies (x_{Coop} and x_{Cheat}) with numeric payoff values (*R, T, S, P*) as following equations of

$$f_{Coop} = \left(x_{Coop} \cdot R\right) + \left(x_{Cheat} \cdot S\right) \quad (2.17)$$

and

$$f_{Cheat} = \left(x_{Coop} \cdot T\right) + \left(x_{Cheat} \cdot P\right). \quad (2.18)$$

Then, the weighted average fitness of the population $\emptyset$ is calculated as,

$$\emptyset = x_{Coop} \cdot f_{Coop} + x_{Cheat} \cdot f_{Cheat}. \quad (2.19)$$

The frequency changes of the players are found by multiplying current frequency with the fitness differences between the player and the population average and described as,

$$\frac{x_{Coop}}{dt} = x_{Coop} \cdot [f_{Coop} - \emptyset] \quad (2.20)$$

and

$$\frac{x_{Cheat}}{dt} = x_{Coop} \cdot \left[f_{Coop} - \emptyset\right]. \quad (2.21)$$

The attractors of replicator equations were determined with the stable state finder algorithm and the results were compared with the Nash equilibria of 2-player games.

2.2.2. Stable States of the Multi-Player Fermentation Game

In the Multi-Player Fermentation Game, growth rates (fitness) of phenotypes are affected by their metabolic mechanisms, such as uptake and utilization of the substrate, and production and transportation of the enzyme. The frequencies of the phenotypes will reach to an Evolutionary stable state when their finesses are equal. In order to find the Evolutionary stable state of the 2-player batch fermentation, the stable state finder algorithm was used. The algorithm first, generates set of possible initial frequencies, that is x_{Coop} = 0.99 with x_{Cheat} = 0.01 ,and x_{Coop} = 0.01 with x_{Cheat} = 0.99 (minimum allocated frequency by a player is x_{min} = 0.01) and multiplies with the total initial density td_i (sum of the initial densities of Cooperator and Cheater sub-cultures) to convert frequencies into densities. Then, for each set of initial densities, ODE is initialized and iterated by passing the end frequencies of the current ODE to the next ODE until the frequency stabilizes.

Throughout the study, the frequency is assumed to be stable if its change is smaller than 10^{-6}.

First, the Evolutionary stable state and the game regime is then interpreted over range of cost parameters. Next, the effects of initial total cell density to the equilibrium frequency of the Cooperator sub-culture were observed by utilizing the stable state finder algorithm by applying different initial total cell densities between 0.5 g/L to 5 g/L. The final extracellular lipolytic activity at every stable state was recorded. In the same manner, the effects of the initial olive oil concentration and the initial oleic acid concentration was observed and for every stable state the final extracellular lipolytic activities were recorded.

3. RESULTS

3.1. Multi-Player Fermentation Model

Multi-player fermentation model (Equations 2.1-2.15) was derived from the mathematical model of lipase producing *Y. lipolytica* (Equations 1.11-1.18) [40]. The second player, lipase non-producing Cheater is derived from the wildtype Cooperator by removing the lipase induction rate equations. Along with simple substrate extracellular oleic acid, complex substrate extracellular olive oil was added as state variable to be hydrolyzed into extracellular oleic acid by the extracellular lipase and into intracellular oleic acid by the cell-bound (intracellular) lipase. Constructed Multi-player fermentation model was simulated in batch and fed-batch modes. For both batch and fed-batch fermentation simulations, initial density of Cooperator and Cheater sub-cultures were set to 0.5 g/L each. The batch fermentation starts with 3 g/L olive oil while the fed-batch fermentation starts with 1 g/L olive oil and 1 g/L olive oil added at 20^{th} and 30^{th} hours. Initial values of other state variables were set to 0, except the intracellular lipase of the Cooperator sub-cultures (Lip_{Coop}) which is necessary for the oil hydrolysis and is set to 10^{-4} U/mg throughout the study. The simulation ran for 40 hours duration and the results were generated. The results of the batch fermentation simulation are shown in Figure 3.1. The results of the batch fermentation simulation are shown in Figure 3.2.

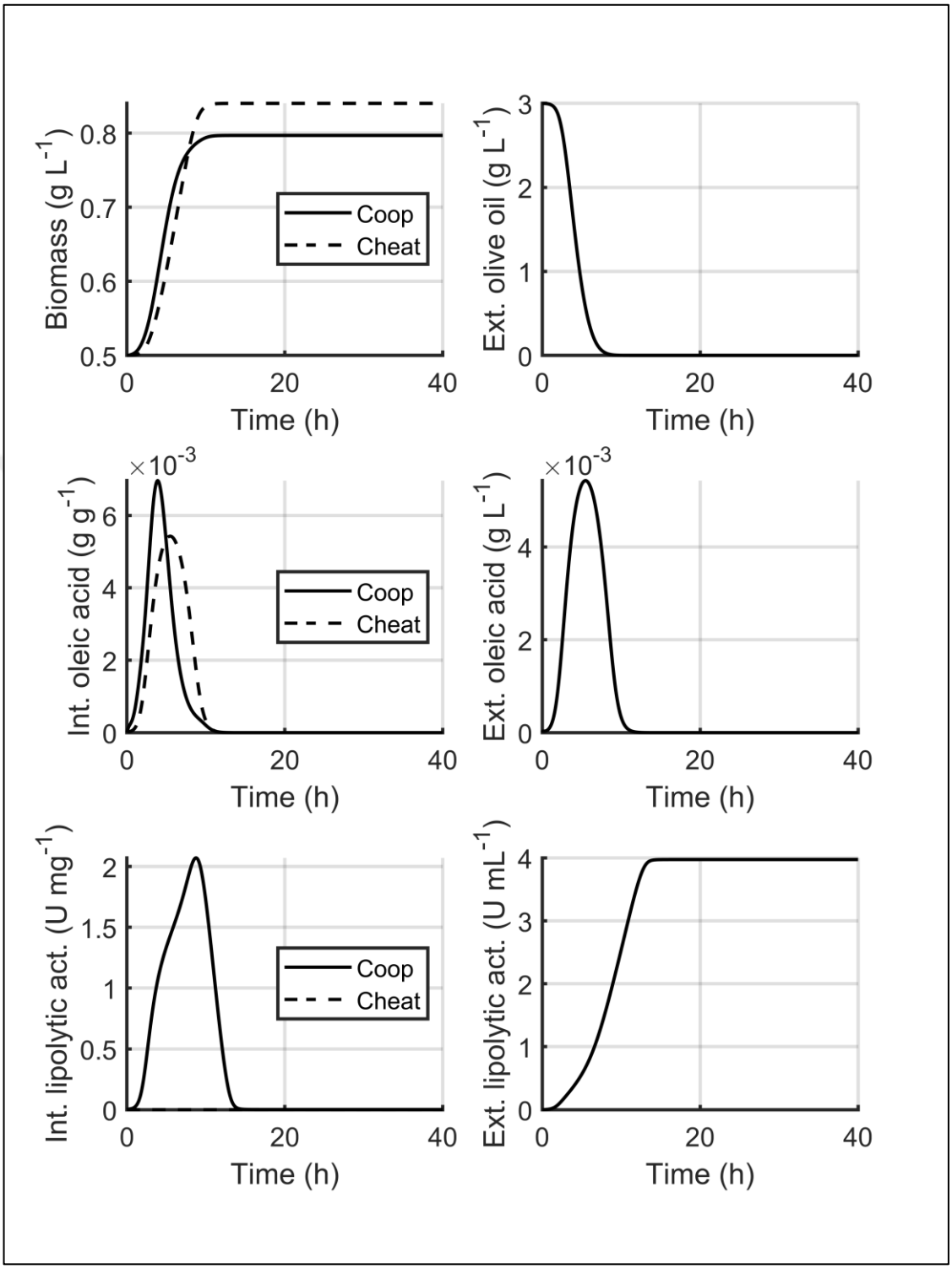


Figure 3.1. Multi-player batch fermentation model between Cooperator and Cheater sub-populations. Total duration is 40 hours. Cooperator and the Cheater starts with equal initial density of 0.5 g/L and reaches to 0.80 g/L and 0.84 g/L respectively. The fermentation starts with 3 g/L olive oil and gets depleted until the end of the fermentation. Extracellular lipolytic activity reaches to 3.97 U/mL at the end of the fermentation

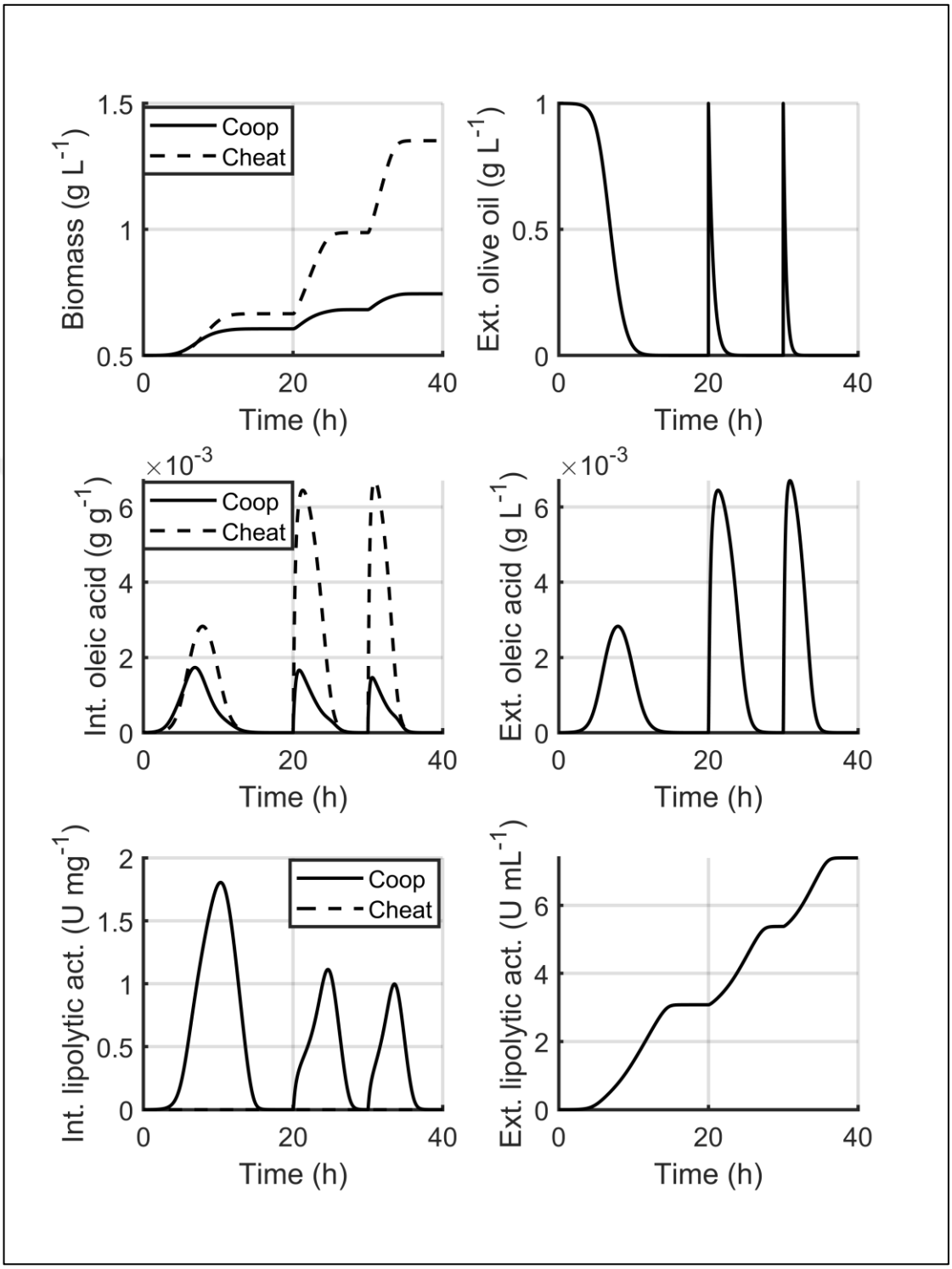


Figure 3.2. Multi-player fed-batch fermentation model Cooperator and Cheater subpopulations. Total duration is 40 hours. Cooperator and the Cheater starts with equal initial density of 0.5 g/L and reaches to 0.74 g/L and 1.35 g/L respectively. The fermentation starts with 1 g/L olive oil and 1 g/L olive oil added at 20th and 30th hours. Extracellular lipolytic activity reaches to 7.39 U/mL at the end of the fermentation

3.2. Validation of Stable State Finder

In order to evaluate the stability of a dynamical system with set of various ordinary differential equations a Stable state finder algorithm was developed. In order to validate the algorithm, the population dynamics of the social dilemma games (*Prisoner's dilemma*, *Snowdrift*, *Coordination*, *No-conflict*) were simulated with the numeric (R, T, S, P) pay-off values (Table 1.1.) and the replicator dynamics (Equations 2.17-2.21). With a fixed S and T and varying R and P values Equilibrium frequencies for the Cooperator were found with Stable state finder algorithm and surf plotted as in Figure 3.3.

The values set T > R > S > P, results between 1 percent and 99 percent Equilibrium frequency of the Cooperator, indicating the game regime is *Snowdrift*. The values set T > R > P > S, results under 1 percent Equilibrium frequency of the Cooperator, indicating the game regime is *Prisoner's Dilemma*. The values set R > T, S > P results over 99 percent Equilibrium frequency of the Cooperator, indicating the game regime is *No Conflict*. Finally, the values set R > P > T, S results over 99 percent Equilibrium frequency of the Cooperator in Initially Cooperating population (Figure 3.3.top.) and under 1 percent Equilibrium frequency of the Cooperator in Initially Cheating population (Figure 3.3.bottom.).

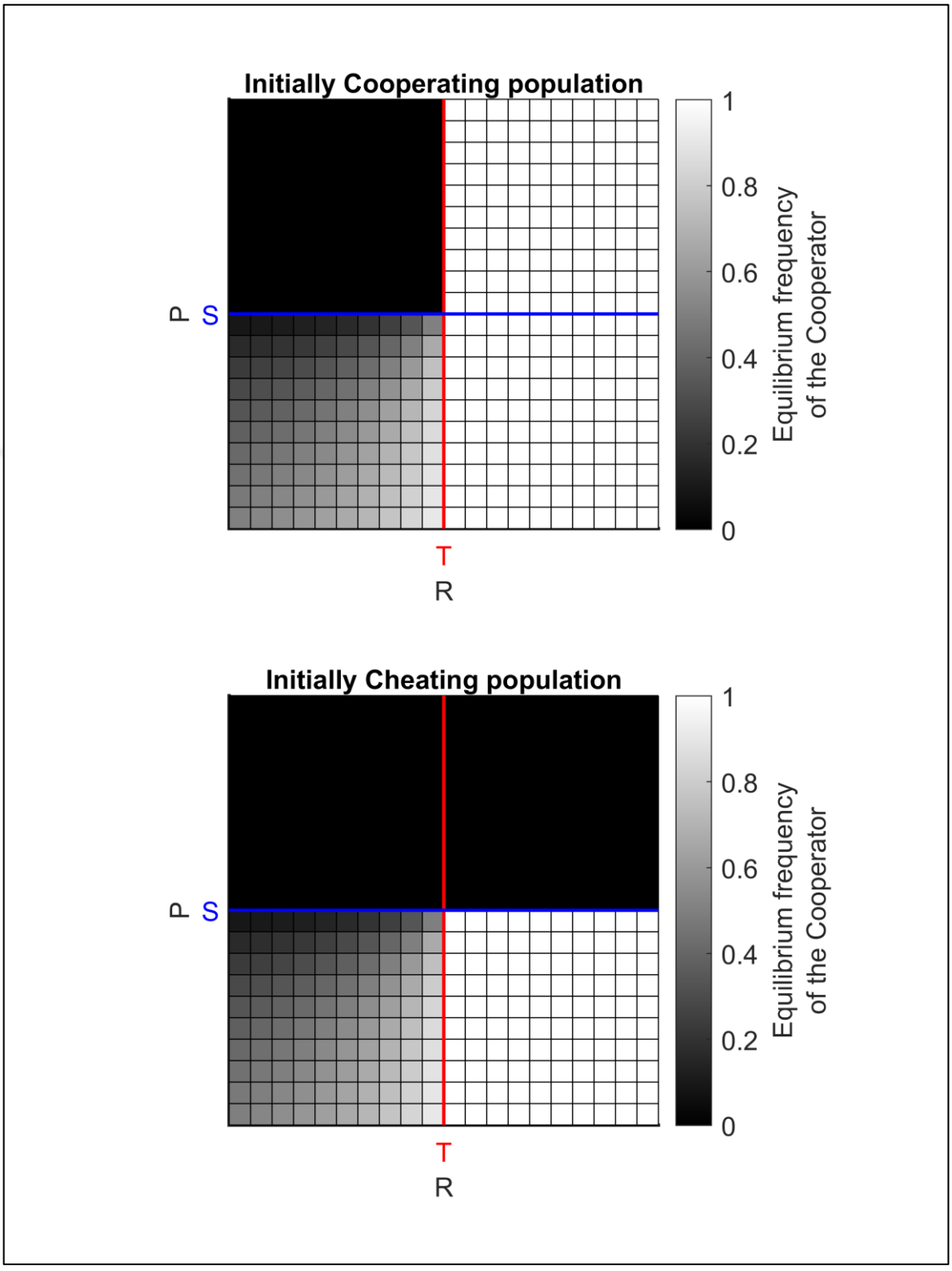


Figure 3.3. Comparison between the stable state finder algorithm results (tiles) and the pay-off values of the social dilemma games: *Snowdrift*: T > R > S > P, *Prisoner's dilemma*: T > R > P > S, *No Conflict:* R > T, S > P, *Coordination game*: R > P > T, S

3.3. Lipase Production Cost and Game Regimes

The lipase production cost "*c*" expressed in Equation 2.15 is a necessary component of the Multi-player fermentation game. However, the exact cost value of the lipase production to its producer is hard to estimate. In this study we analyzed varying cost parameters and its effects on the larger scales, i.e., population dynamics, stable states of the population and the game regime.

In stable state finder algorithm, each iteration of ODE solver started with 3 g/L olive oil and 1 g/L initial total density for 40 hours duration. ODE solver iterations finalized when the frequencies are stabilized and the final frequencies are recorded (Figure 3.4.). In both initially Cooperating population (Figure 3.4.top.) and initially Cheating population (Figure 3.4.bottom.) equilibrium frequency of the Cooperator is decreased with the increase in the lipase production cost. More specifically, below the cost value 60 the game regime is "No conflict" where Cooperation is the dominant strategy. Above the cost value 60 the game regime is "*Snowdrift*" where Cooperators and Cheaters coexist. Later simulations were done for only initially Cooperating populations and the cost value is assumed as 120.

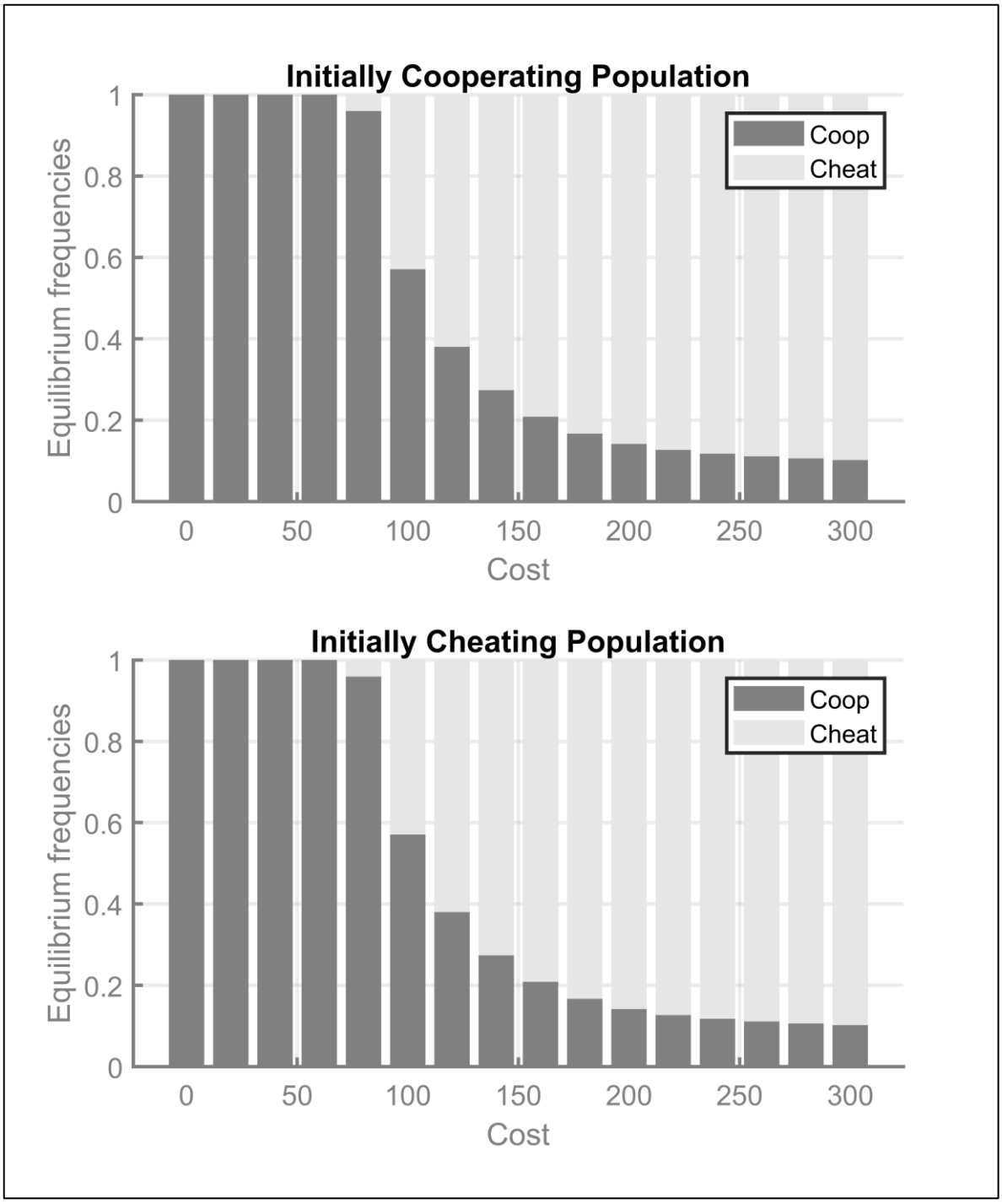


Figure 3.4. Effects of the lipase production cost c on the equilibrium frequency of the Cooperator in batch fermentation settings. In both populations (top and bottom figure), below the cost value 60 the game regime is "No conflict" where Cooperation is the dominant strategy. Above the cost value 60 the game regime is "*Snowdrift*" where Cooperators and Cheaters coexist

3.4. Equlibrium Frequencies and Lipolytic Activities

Effects of the initial total density on the “Equilibrium frequency of the Cooperator” and the “Extracellular lipolytic activity at equilibrium” in batch fermentation settings were analyzed with the Stable state finder algorithm (Figure 3.5.). Each iteration of ODE solver started with 3 g/L olive oil for 40 hours duration. ODE solver iterations finalized when the frequencies are stabilized and the final frequencies are recorded. Equilibrium frequency of the Cooperator is decreased along with initial total density and.

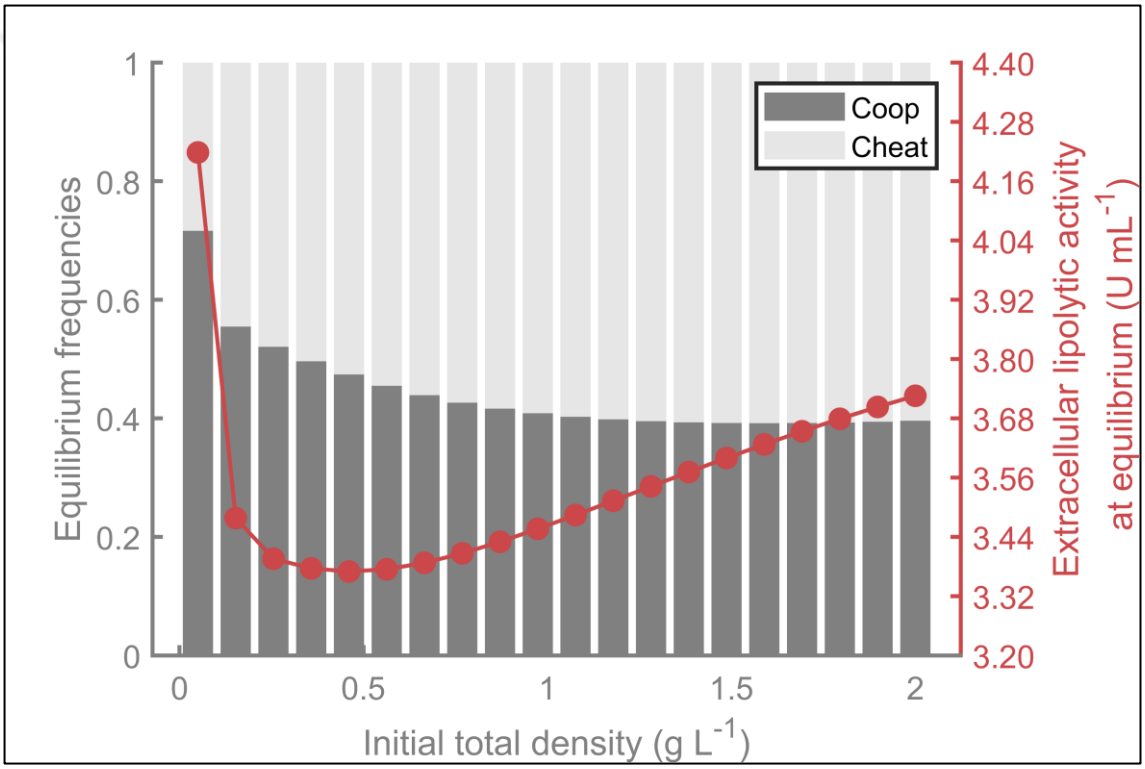


Figure 3.5. Effects of the “Initial total density” on the “Equilibrium frequency of the Cooperator” and the “Extracellular lipolytic activity at equilibrium” in batch fermentation settings. Equilibrium frequency of the Cooperator is decreased along with initial total density

Next, effects of the initial olive oil on the “Equilibrium frequency of the Cooperator” and the “Extracellular lipolytic activity at equilibrium” in batch fermentation settings were analyzed with the Stable state finder algorithm. Each iteration of ODE solver started with 1 g/L initial total density for 40 hours duration. ODE solver iterations finalized when the frequencies are stabilized and the final frequencies are recorded (Figure 3.6.). Equilibrium frequency of the Cooperator is increased along with initial olive oil. Extracellular lipolytic activity at equilibrium is increased along with initial olive oil.

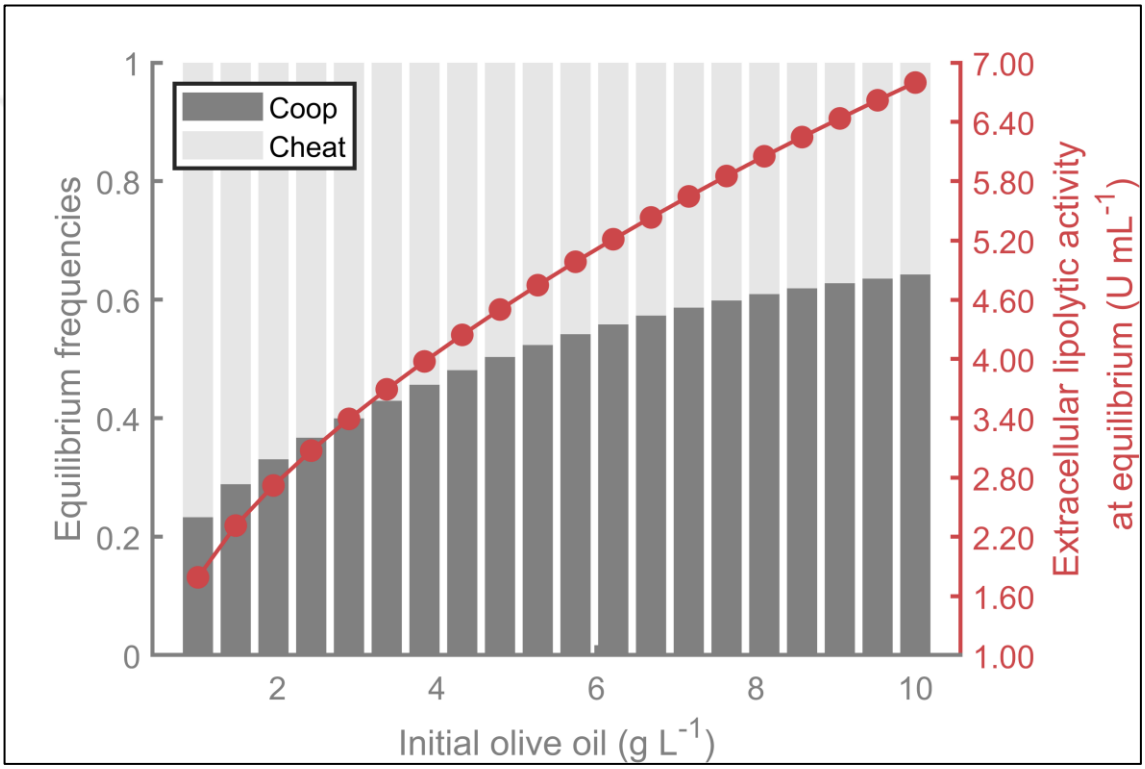


Figure 3.6. Effects of the “Initial olive oil” on the “Equilibrium frequency of the Cooperator” and the “Extracellular lipolytic activity at equilibrium” in batch fermentation settings. Equilibrium frequency of the Cooperator is increased along with initial olive oil and over 6 g/L the population is dominated by the Cooperator strategy. Extracellular lipolytic activity at equilibrium is increased along with initial olive oil but with the slower rates over 6g/L initial olive oil

Finally, effects of the “Initial oleic acid” on the “Equilibrium frequency of the Cooperator” and the “Extracellular lipolytic activity at equilibrium” in batch fermentation settings were analyzed with the Stable state finder algorithm. Each iteration of ODE solver started with 1 g/L initial total density and 3 g/L initial olive oil for 40 hours duration (Figure 3.7.). ODE solver iterations finalized when the frequencies are stabilized and the final frequencies are recorded. Equilibrium frequency of the Cooperator is decreased with increasing initial oleic acid.

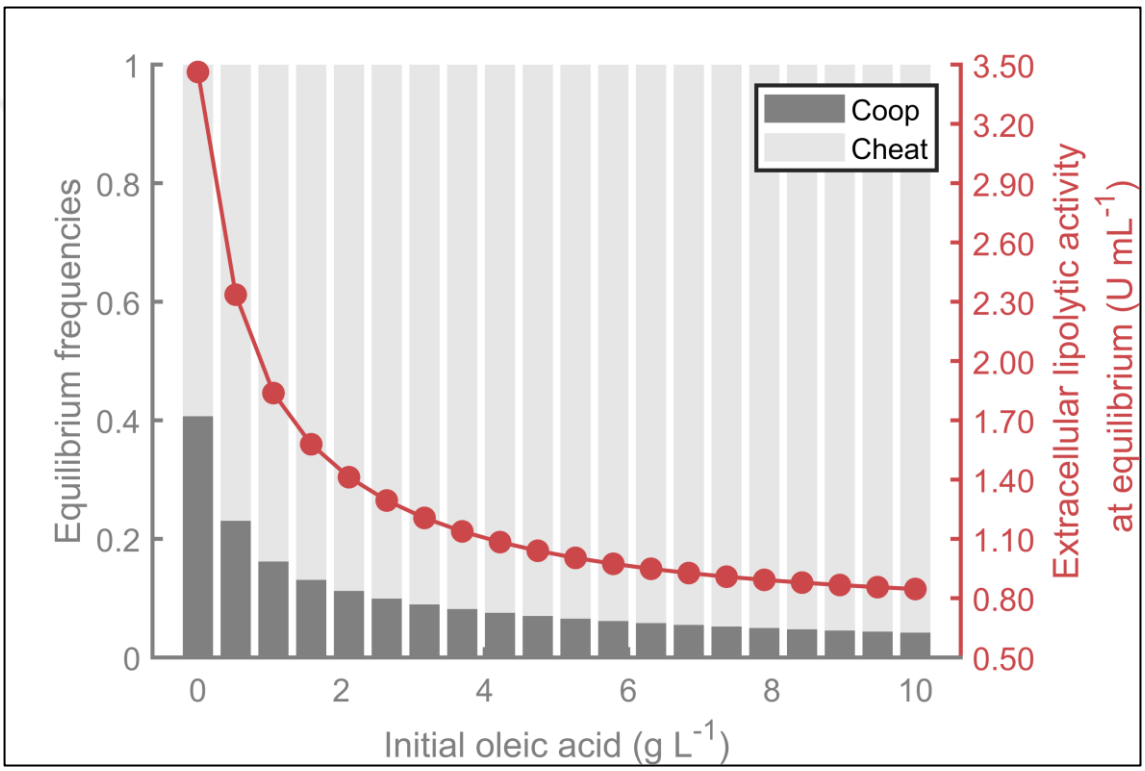


Figure 3.7. Effects of the “Initial oleic acid” on the “Equilibrium frequency of the Cooperator” and the “Extracellular lipolytic activity at equilibrium” in batch fermentation settings. Equilibrium frequency of the Cooperator is decreased with increasing initial oleic acid

4. DISCUSSION

The multi-player fermentation game was constructed from the mathematical model of the lipase producing *Y. lipolytica* by deriving an additional "non-producer" Cheater mutant. The two types only differ in their lipase production mechanisms where the Cheater do not induce the production of the lipase. In the constructed multi-player fermentation game, the same parameter values as the previous mathematical model were used. Since these parameters are calibrated for the wildtype *Y. lipolytica*, addition of a non-producing type can affect these values. Throughout this study it is assumed that model parameters are applicable to multi-player fermentation settings.

In order to generate a social dilemma, extracellular olive oil is added to the *Y. lipolytica* model since in the previous mathematical model extracellular lipase had no utility therefore wasn't a public good. Olive oil hydrolysis is described as a first order kinetics and oleic acid content is assumed as 70 percent. The olive oil hydrolysis is performed at two different locations, one, at periplasmic space and second, at extracellular space. It is assumed that the produced intracellular lipase can directly perform periplasmic hydrolysis and the resulting oleic acid is directly captured by the cell whereas extracellular lipase yields extracellular oleic acid which then has to be transported.

Another important aspect of the public good games is the Cooperation is a costly behavior. The previous mathematical model has neglected the consumption of necessary intracellular substrate. Therefore, in this study, the lipase production cost c is implemented as reduction in intracellular substrate per unit lipase produced. The cost had to be related to the intracellular substrate, therefore it is assumed that the lipase production cost is proportional to the amount of intracellular substrate. After constructing the multi-player fermentation game, the simulations ran for batch and fed-batch operation modes and the results were generated.

The cell-bound hydrolysis of olive oil provides advantage to the wildtype Cooperators at early stages (0^{th} to 15^{th} hour) of fermentations as can be seen in Figure 3.1. and Figure 3.2. However, at later stages of the fermentations (15^{th} to 40^{th} hour), lipase is excreted and thus Cheaters can benefit from products of extracellular lipase hydrolysis.

With an equal starting density of 0.5 g/L, in batch fermentation, Cooperator and Cheater reached to cell densities of 0.80 g/L and 0.84 g/L respectively and extracellular lipolytic activity has reached to 3.97 U/mL. In fed batch fermentation Cooperator and Cheater reached to cell densities of 0.74 g/L and 1.35 g/L respectively and extracellular lipolytic activity has reached to 7.39 U/mL. Even though, cheater sub-culture has benefited more from the fed-batch fermentation the extracellular lipolytic activity has increased compared to batch fermentation. This result indicates that compared to batch fermentation, fed-batch fermentation has led Cooperator sub-cultures to produce more lipase by providing more suitable substrate to biomass ratio since the induction of lipase production is related to substrate to biomass ratio as described in Equation 2.8.

Analyzing the Evolutionary stability of the multi-player fermentation game with analytical solutions is a difficult task since it's a dynamical system with multiple state variables. Therefore, the Evolutionary stability analysis is performed with stock Matlab ODE45 solver. The stable state finder algorithm generates initial frequencies by distributing between the minimum possible frequency (1 percent throughout the study) and the maximum possible frequency (Equation 2.16). This approach captures all attractors of the system as shown in later validation experiments. The initial frequencies were converted to the initial densities by multiplying with the total initial density and the ODE set was solved for a fixed duration. The end densities of the solution were converted back into the frequencies and then implemented into the next ODE set and the process is repeated until the frequencies within the final ODE solution is remained unchanged. Which is regarded as a cue that the system has reached a stable state. For two-player, Cooperation and Cheating games, the algorithm generates two possible starting scenarios, 99 percent Cooperator, 1 percent Cheater and 1 percent Cooperator, 99 percent Cheater. If in both scenarios the Cooperator frequency has reached a value over 99 percent than the game regime is "*No-conflict*", indicating that the Cooperation is the best strategy for all members of the population. If in both scenarios the Cheater frequency has reached a value over 99 percent than the game regime is "*Prisoner's dilemma*", indicating that the Cheating is the best strategy for all members of the population. If in both scenarios Cooperation and Cheating coexists, meaning that their frequencies are equal and between 99 percent and 1 percent, then the game regime is *"Snowdrift"* indicating that Cooperation and Cheating have equal fitnesses in equilibrium and a slight deviation towards one side will make opposite strategy

stronger. Finally, the game regime is "*Coordination game*" if Cooperators dominate when their initial frequency is 99 percent but eliminated when their initial frequency is 1 percent. These claims were validated by first, generating 2-player population dynamics using replicator equations with the payoff Table 1.1. and then the equilibrium frequency of the Cooperator was found with the stable state finder algorithm. Finally, the results were compared with the Nash Equilibria of the 2-player games (Figure 3.3.). The Figure 3.3. shows that initially Cooperator population (99 percent Cooperator, 1 percent Cheater) at the top figure and initially Cheater population (1 percent Cooperator, 99 percent Cheater) at the bottom figure has the same results for, "*Snowdrift*" (T > R > S > P), "*Prisoner's dilemma*" (T > R > P > S) and "No Conflict" (R > T , S > P) areas and contrasting results for "*Coordination game*" (R > P > T ,S) which is the only game regime that depends on the initial frequency. After validating the stable state finder algorithm, it is used for the analysis of the Evolutionary stable states of the Multi-player fermentation game.

First, the Equilibrium frequencies of the Cooperators on different values of lipase production cost were evaluated. When the cost is low (c < 60) the game regime is "*No-conflict*" as the Equilibrium frequency of the Cooperators reach to 100 percent (Figure 3.4.).

Higher costs result in decrease in the Equilibrium frequency of the Cooperators and the "*Snowdrift*" dynamics can be observed. The Multi-player fermentation game in increasing lipase production cost doesn't become a "*Prisoner's dilemma"* since equilibrium frequency of the Cooperators does not reach below 1 percent in any cost. Additionally, the game is not a "*Coordination game*" since the top and bottom figure reaches to same Evolutionary stable states, therefore for the next experiments the initial starting frequencies reduced to one scenario only. Initial total cell density results (Figure 3.5.) indicate that the Cheaters are at advantage at higher initial total densities. In the original *Y. lipolytica* model [40] the lipase induction is considered to be related to the oleic acid to biomass ratio **(**Equation 1.15). If this ratio is too high or too low the lipase production is reduced. Therefore, in the proposed two player model here, the Cooperator can adjust and reduce the lipase production and therefore pay less cost and maintain its advantage over Cheater type. Indeed, S. cerevisiae is highly polymorphic in *SUC2* genes and regulates its capacity to produce invertase at gene level to maintain its fitness in varying environments where as

Y. lipolytica has intricate lipase induction and transportation mechanisms to prevent tragedy of commons in nature.

As mentioned before, rather than having a fixed amount of lipase production, Cooperators adjust their lipase production depending on the oleic acid to biomass ratio. Therefore, for practical and biotechnological purposes its necessary to examine the extracellular lipolytic activity at the Evolutionary stable (equilibrium) state.

Figure 3.5. shows that higher initial total densities benefitted Cheaters. The decrease in lipolytic activity between 0.1g/L and 0.5g/L is due to decrease in the frequency of the lipase producing Cooperators and the increase in the lipolytic activity between 0.5 g/L and 2 g/L is due to stabilization of Cooperator frequency and the increase in initial total density. The increase in initial olive oil has resulted in increase in both the equilibrium frequency of the Cooperators and extracellular lipolytic activity at the equilibrium (Figure 3.6.). Unlike initial total density, increase in olive oil does not reduce the specific productivity hence the lipolytic activity continues to increase. Furthermore, Figure 3.7. shows that higher amounts of initial oleic acid have negative impact on the equilibrium frequency of the Cooperators since, while Cooperators are investing in lipase production, Cheaters can use the available oleic acid.

Multi-player fermentation game is derived from the mathematical model of lipase producing *Y. lipolytica* where the oleic acid is the sole inducer of the lipase production. Along with free fatty acids, *LIP2* gene can be induced via extracellular triacylglycerols [43], a potential "game changer" which was not taken into the consideration in this thesis.

The multi-player fermentation model allows only for the vertical gene transfer, therefore phenotypic frequency change depends on differences in growth/death rates. Even without including the horizontal gene transfer, a fermentation model can reach to an Evolutionary stable state depending on the initial conditions and the lipase production costs within the time frames of a fermentation setups, a result that is experimentally proven in the well-mixed *S. cerevisiae* culture (Figure 1.3.) [29]. The evolutionary pressures in biotechnological settings have significant effect on the metabolic strategies and enzymatic activities and therefore must be taken into the consideration for desired outcomes.

5. CONCLUSION

The aim of this study was to construct a game-theoretical model to analyze *Y. lipolytica* population structure and lipase productivity in terms of biotechnologically relevant parameters with the emphasis on, cooperation and cheating tendencies within the population, regulations of the *Y. lipolytica* community and possible improvements in terms of lipase production. The game theoretical model was built upon lipase production fermentation model by deriving new player, a lipase non-producer Cheater. Batch and fed-batch fermentations were modeled. Model results showed that, fed-batch mode promoted Cheating.

In order to analyze the Evolutionary stable states of multi-player fermentation model, Stable State Finder Algorithm was developed and then validated by comparing the results against the 2-player games. The algorithm was used to analyze the effects of the extrinsic factors such as initial cell density, initial olive oil and oleic acid concentrations and intrinsic factors such as cost of lipase production. The multi-player fermentation game regime is "*No Conflict"* for cost parameter values under 60 and "*Snowdrift"* for cost parameter values over 60 and *"Prisoner's Dilemma"* and *"Coordination game"* regimes were not observed.

As proposed, the higher initial population densities benefitted Cheater sub-cultures and consequently inhibit the lipase production. As a conclusion, a game theoretical model was constructed to analyze the evolutionary dynamics and lipase productivity in terms of structured kinetic models. A stable state finder algorithm was developed for games in biotechnological settings. Finally, evolutionary pressures in biotechnological settings that affects metabolic strategies and enzymatic activities were analyzed.

6. FUTURE DIRECTIONS

Throughout this study oleic acid is considered to be the sole inducer of the lipase production. However, *LIP2* gene can be also induced by extracellular triacylglycerols. Therefore, the mathematical model of lipase producing *Y. lipolytica* can be further improved with the addition of *LIP2* gene induction via extracellular triacylglycerols.

In this study a social dilemma in a microbial population, where the same species acts out different phenotypes, is analyzed. The methodology of finding the Evolutionary stable state can also be applied to scenarios where different species are considered as players. In growing biotechnological literature, co-cultures are becoming prominent examples of such scenarios.

APPENDIX A: STABLE STATE FINDER ALGORITHM

Algorithm. A.1. Stable state finder algorithm. The algorithm is computed with the MATLAB programming language version R2021a

```
function stablestatefinder

td      =   1;
n       =   2;
xmin    =   0.01;

for m           = 1:n-1
    xmax(m)     = (1 - xmin*m)/(n-m);
    v1          = xmin*ones(1,m);
    v2          = xmax(m)*ones(1,n-m);
    v3          = [v1 v2];
    fi1(:,:,m)  = perms(v3);
end

fi2     = permute(fi1,[2 1 3]);
fi3     = reshape(fi2,n,[]).';

for r = 1:size(fi3,1)

    while   1
            Xi      = td*fi3(r,:);
            tspan   = [0 40];
            [t, x]  = ode45(@fermODE_2p, tspan, Xi);

            for i       = 1:n
                gi(r,i) =  x(end,i)/(sum(x(end,1:n)));
            end

            if  all(abs(gi(r,:)-fi3(r,:)) <= 1e-6*ones(1,n))
                fprintf('Run %d, Coop. eq. freq. = %4.2f\n',r,gi(r,1))
            break
            else
                fi3(r,:) = gi(r,:);
            end
     end
end
```

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