

Game Theoretical Analysis of Cooperation and Cheating Among Lipase Producing *Candida rugosa* Sub-Cultures

Özgür Yüksel¹ and Emrah Nikerel¹

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. 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. In this study a methodology was developed that enables the analysis of evolutionary dynamics of lipase producing *Candida rugosa* from kinetic models of fermentation. 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. Although Cheaters benefits higher initial densities due to increase in lipase excretion rates, at highest densities Cooperators halt enzyme production to regain their dominance. Optimal productivity has achieved around 0.06 g/L initial total density where final extracellular lipolytic activity reaches 4.68 U/mL in the Evolutionary stable state where Cooperators occupy 64% of the population. This result indicate that complete elimination of Cheaters is not necessary for the optimization of exoenzyme production. The methodology of finding the Evolutionarily 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.

Keywords: Evolutionary Game Theory, Cooperation, Cheating, Lipase, *Candida rugosa*

Özgür Yüksel
ozgur.yuksel@std.yeditepe.edu.tr
Emrah Nikerel
emrah.nikerel@yeditepe.edu.tr

¹ Department of Biotechnology Yeditepe University, Atasehir, Istanbul, Turkey 34755

1 Introduction

1.1 Game Theory and Evolutionary Dynamics

Game theory is a mathematical tool for understanding and solving conflicts of interest between agents. 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 (Vincent and Brown 2005). 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 (Flux 1896; Von Neumann and Morgenstern 1944; Nash 1951; Von Stackelberg et al. 1952).

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 (Smith 1982a; Hofbauer and Sigmund 1998a). Aspects of living organisms such as evolution of cooperation (Hauert and Doebeli 2004; Perc and Szolnoki 2010; Tomassini et al. 2010), sex ratios (Smith 1982a), selection of biochemical pathways (Pfeiffer and Schuster 2005) and biofilms (Kreft 2004) were analyzed by EGT and furthermore, it can be used for the classification in data analysis (Cohen et al. 2007).

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 would correspond to switches between strategies (Schuster et al. 2010). 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 (Schuster et al. 2010). Although increase in exoenzyme production is desired, exoenzyme production is often decreased by the non-producing subpopulation of microorganisms (Allison 2005; Modak et al. 2007).

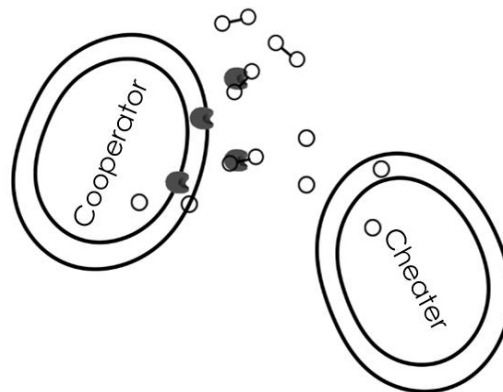


Fig. 1 Interplay between microbial cells for the exoenzyme secretion. Complex substrates are indicated by linked circles. Cooperating cells secrete exoenzyme (black sliced circles) to release growth substrate (single circles). Cheater cells do not produce exoenzymes yet benefit from the growth substrate.

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 (Fig 1).

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 (Naumov et al. 1996). Moreover, there are species that doesn't carry any *SUC* genes like *S. italicus* (Schaefer and Cooney 1982). 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 (Greig and Travisano 2004).

1.3 Extracellular Lipase from *Candida rugosa*

Lipases, otherwise known as triacylglycerol acylhydrolases (EC 3.1.1.3) constitute a wide family of enzymes. They hydrolyze ester bonds in triglycerides to produce diglycerides, monoglycerides, glycerol and fatty acids. Their stability in organic solvents, substrate specificity and high enantioselectivity provides biotechnological advantages (Ferrer et al. 2001). Due to its substrate specificity and high activity, lipases from yeast *Candida rugosa* is one of the most often used lipases in the industry. Encoded by the LIP gene family, several isoenzymes are secreted by the *C. rugosa*. (Vanleeuw et al. 2019). In the presence of olive oil and its fatty acid oleic acid *C. rugosa* produces isoenzymes Lip2 and Lip3 (Domínguez de María et al. 2005).

Since the *C. rugosa* extracellular lipase is biocatalyst for biotechnological processes, food, pharmaceutical and environmental industries are interested in large scale production of the enzyme. Large scale production processes require development and validation of mathematical models for optimization and process control. The aim of our study is to construct a game theoretical model to analyze the evolutionary dynamics and the lipase productivity of the *C. rugosa* sub-population using structured mathematical models. In particular, necessary mathematical tools will be developed in order to determine the frequencies of the lipase producer and the non-producer (Cooperator and Cheater) sub-cultures. Ultimately, significance of evolutionary pressures in the fermentation environment and possible improvements in terms of lipase productivity will be analyzed.

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

S. cerevisiae exhibits producer, non-producer sub-cultures and experimental studies has shown that increase in initial cell density has benefitted Cheater sub-cultures (Gore et al. 2009). There were no records for producer and non-producer sub-cultures for *C. rugosa* in the literature. However, like extracellular invertase produced by *S. cerevisiae*, the extracellular lipase produced by *C. rugosa* is “Public good”. Therefore, the following hypothesis is proposed. In an artificial fermentation environment where Cooperators and Cheaters co-exist, increase in 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” sub-cultures of *C. Rugosa* respectively. The produced lipase is assumed to be in the 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.

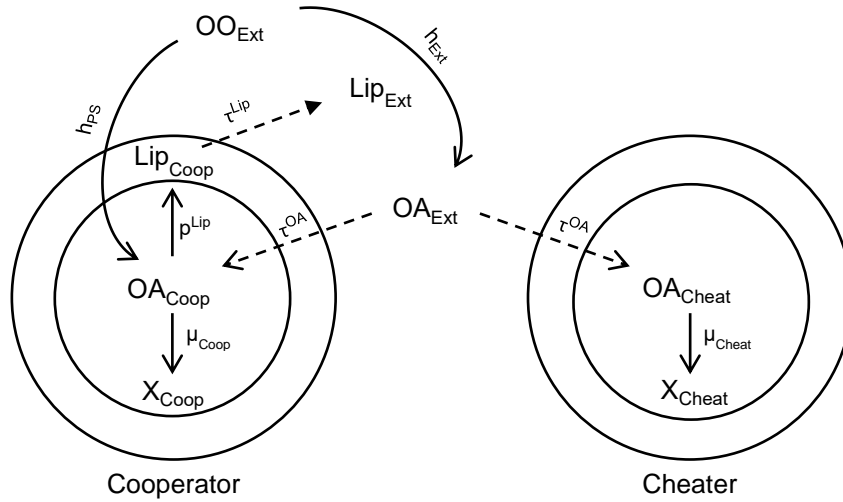


Fig 2 Model representation of Multi-player fermentation game. Olive Oil OO_{Ext} is being hydrolyzed both at the extracellular and the periplasmic space by the extracellular lipase Lip_{Ext} and the periplasmic lipase Lip_{Coop} , resulting in intracellular oleic acid OA_{Coop} and extracellular oleic acid OA_{Ext} 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 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 (**Fig 2**). Previous mathematical model of *C. Rugosa* (Montesinos et al. 1997) 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. Additionally, an extracellular olive oil state variable and it’s hydrolysis via lipase is introduced to generate a public goods scenario. The following system of equations represents the mass balances for the Multi-player fermentation game.

Table 1. System of mass balance equations for the Multi-player fermentation game

$$\frac{dX_{Coop}}{dt} = \mu_{Coop} \cdot X_{Coop} \quad (1)$$

$$\frac{dX_{Cheat}}{dt} = \mu_{Cheat} \cdot X_{Cheat} \quad (2)$$

$$\frac{dOA_{Coop}}{dt} = \tau^{OA} + h_{PS} - c \cdot OA_{Coop} \cdot p^{Lip} - Y_{SX} \cdot \mu_{Coop} - \mu_{Coop} \cdot OA_{Coop} \quad (3)$$

$$\frac{dOA_{Cheat}}{dt} = \tau^{OA} - Y_{SX} \cdot \mu_{Cheat} - \mu_{Cheat} \cdot OA_{Cheat} \quad (4)$$

$$\frac{dOA_{Ext}}{dt} = h_{Ext} - \tau^{OA} \cdot (X_{Coop} + X_{Cheat}) \quad (5)$$

$$\frac{dOO_{Ext}}{dt} = feed - h_{Ext} - h_{PS} \cdot X_{Coop} \quad (6)$$

$$\frac{dLip_{Coop}}{dt} = p^{Lip} - \tau^{Lip} - \mu_{Coop} \cdot Lip_{Coop} \quad (7)$$

$$\frac{dLip_{Ext}}{dt} = \tau^{Lip} \cdot X_{Coop} \quad (8)$$

The cellular growth is regulated by intracellular concentration of oleic acid and the Monod's equations are separately expressed for the Cooperator and the Cheater sub-cultures as,

$$\mu_{Coop} = \frac{\mu_{max} \cdot OA_{Coop}}{k_{ss} + OA_{Coop}} \quad (9)$$

and

$$\mu_{Cheat} = \frac{\mu_{max} \cdot OA_{Cheat}}{k_{ss} + OA_{Cheat}} \quad (10)$$

respectively. Extracellular oleic acid is transported towards each sub-culture equally and it is expressed in terms of active transportation with saturation

$$\tau^{OA} = \frac{k_{so} \cdot OA_{Ext}}{k_{so1} + OA_{Ext}} \quad (11)$$

Lipase production is assumed to be induced by extracellular oleic acid and regulated by the substrate to biomass ratio. The production described only for the lipase producer "Cooperator" as,

$$p^{Lip} = \frac{k_{1m} \cdot \frac{OA_{Ext}}{X_{Total}}}{k_{1s} + \frac{OA_{Ext}}{X_{Total}} + k_i \cdot \left(\frac{OA_{Ext}}{X_{Total}}\right)^2} \quad (12)$$

Cooperator also pays cost for the produced lipase and it is introduced as a reduction in intracellular substrate (Eq. 3). Here c is an arbitrary value, multiplication with intracellular oleic acid approximates to a yield coefficient of substrate to enzyme. The excretion of lipase is described as active transport with saturation

$$\tau^{Lip} = \frac{k_m \cdot Lip_{Coop}}{k_s + Lip_{Coop}} \cdot \frac{1}{k_\mu + \mu_{Coop}} \cdot \delta S \quad (13)$$

The rate is limited as the specific growth rate increases. A necessary amount of internal substrate is being consumed and its accounting function is introduced as

$$\delta S = \frac{OA_{Coop}}{k_{es} + OA_{Coop}} \quad (14)$$

Cooperator can perform cell-bound hydrolysis

$$h_{PS} = Y_{SS} \cdot k_{oh} \cdot OO_{Ext} \cdot Lip_{Coop} \quad (15)$$

at periplasmic space and excreted lipase hydrolyses olive oil at extracellular space which is expressed with,

$$h_{Ext} = Y_{SS} \cdot k_{oh} \cdot OO_{Ext} \cdot Lip_{Ext}. \quad (16)$$

The main compound of olive oil is oleic acid (65 to 85 percent) (Ramirez-Tortosa et al. 2006). Here we assumed that the oil hydrolysis yields 70 percent oleic acid and represented as yield coefficient Y_{SS} and k_{oh} is the olive oil hydrolysis parameter and its value has been estimated as 0.5 (Serra et al. 1992).

Fermentation simulations were started with equal initial densities of Cooperator and Cheater sub-cultures with the duration of 40 hours throughout the study. Olive oil was introduced at the beginning for the batch fermentation and at 0th 20th and 30th hours for the fed-batch fermentation (Eq. 6). Initial values of other state variables were set to 0, except for the extracellular oleic acid OA_{Ext} , which is necessary for the initial lipase production and is set to minimum of 10^{-4} g/L throughout the study.

Table 2. Multi-player fermentation game parameters adopted from *C. rugosa* model (Montesinos et al. 1997).

$k_{ss} = 0.01 \text{ g g}^{-1}$	$k_{so} = 0.153 \text{ g g}^{-1} \text{ h}^{-1}$	$k_{so1} = 0.135 \text{ g L}^{-1}$	$k_{1m} = 2.5 \text{ U mg}^{-1} \text{ h}^{-1}$
$k_{1s} = 0.08 \text{ g g}^{-1}$	$k_i = 12 \text{ g g}^{-1}$	$k_s = 0.25 \text{ U mg}^{-1}$	$k_m = 0.06 \text{ U mg}^{-1} \text{ h}^{-1}$
$k_{\mu} = 0.075 \text{ h}^{-1}$	$k_{es} = 10^{-7} \text{ g g}^{-1}$	$\mu_{max} = 0.253 \text{ h}^{-1}$	$k_{oh} = 0.5$
	$Y_{SS} = 0.70$	$Y_{XS} = 0.86 \text{ g g}^{-1}$	

2.2 Stable State Finder Algorithm

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. Also, such solution would further abstract the fermentation environment. Here we are interested in practical improvements in terms of lipase productivity and therefore preserved the concrete elements of the fermentation such as duration and substrate feed. Therefore, the Evolutionary stability analysis is performed with successive forward simulations.

In order to find the equilibrium frequencies of the player types we need to find the attractors of the system which corresponds to the Evolutionarily 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. Each arrangement of initial frequencies is used as initial condition and simulated for specified time. If the end frequencies have differed from the initial frequencies than the end frequencies are used as initial conditions for the next simulation. If the end frequencies are the same as the input frequencies than it is concluded that the system has reached to the steady state.

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 Evolutionarily stable state when their fitnesses are equal. In order to find the Evolutionarily 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} .

3 Results

3.1 Multi-player Fermentation Model

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 0.2 g/L each. The batch fermentation (**Fig. 3a-b**) starts with 3 g/L olive oil while the fed-batch fermentation (**Fig. 3c-d**) starts with 1 g/L olive oil and additional 2 pulses of 1 g/L olive oil added at 20th and 30th hours.

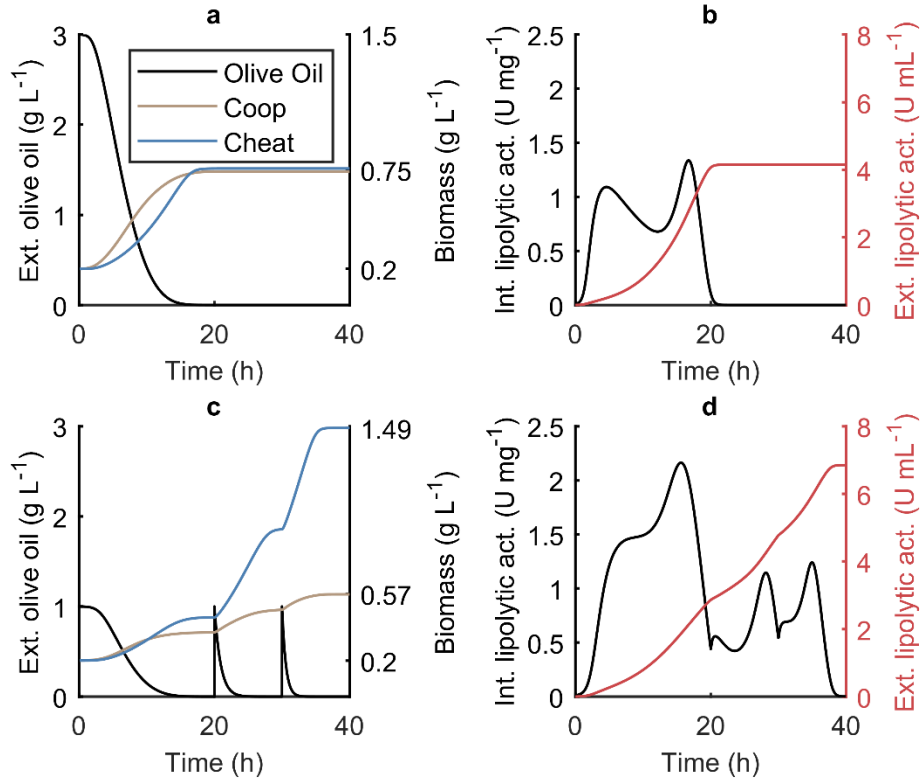


Fig 3. Multi-player fermentation game employing Cooperator and Cheater sub-populations for batch (**a-b**) and fed-batch (**c-d**) modes. **a** Batch fermentation starts with 0.3 g/L olive oil. Starting from initial densities of 0.2 g/L each, Cooperator and Cheater reach to 0.74 g/L and 0.76 g/L respectively. **b** Cooperator produces lipase and excretes to the extracellular space. Extracellular lipolytic activity reaches to 4.1 U/mL at the end of the fermentation. **c** Fed-batch fermentation starts with 1 g/L olive oil and 2 pulses of additional 1 g/L olive oil is added at 20th and 30th hours. Starting from initial densities of 0.2 g/L each, Cooperator and Cheater reach to 0.57 g/L and 1.49 g/L respectively. **d** Extracellular lipolytic activity reaches to 6.8 U/mL at the end of the fermentation.

3.2 Lipase Production Cost and Game Regimes

The lipase production cost is expressed as parameter c . 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.

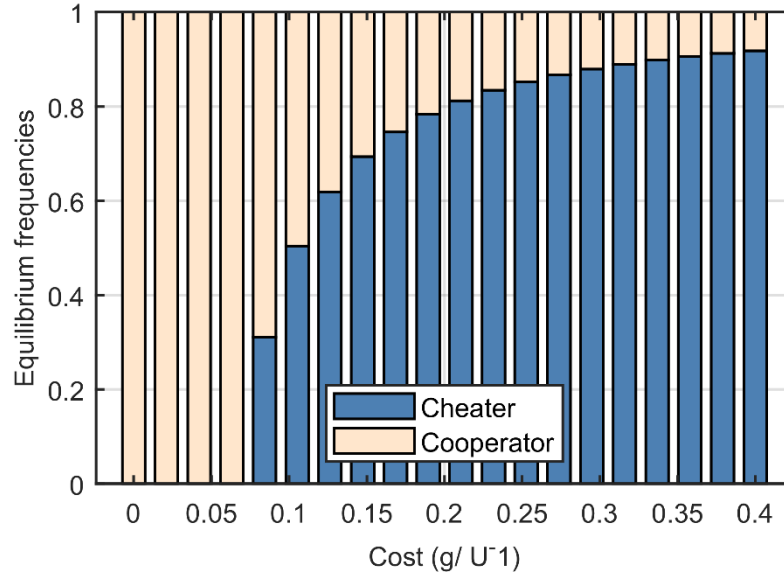


Fig 4. Effects of the lipase production cost parameter c on the equilibrium frequency of the Cooperator in batch fermentation settings. Cooperator and Cheater frequencies are shown as stacked bars with cream and blue colors respectively. Below the cost parameter value of 0.075 the game regime is “No conflict” where Cooperation is the dominant strategy. Above the cost parameter value of 0.075 the game regime is “Snowdrift” where Cooperators and Cheaters coexist.

In stable state finder algorithm, each consecutive batch simulation starts with 3 g/L olive oil and 1 g/L initial total density and runs for 40 hours. The final frequencies are presented in **Fig 4**. Equilibrium frequency of the Cooperator is decreased with the increase in the lipase production cost. More specifically, below the cost parameter value 0.075, the produced lipase fully compensates its own production cost with periplasmic hydrolysis before finally transported to the extracellular space, resulting in “No conflict” scenario where Cooperation is the dominant strategy. Above the cost parameter value 0.075, the produced lipase partly compensates its own production cost before being released to the extracellular space resulting in “*Snowdrift*” scenario where Cooperators and Cheaters coexist. The game doesn’t result in Prisoner’s Dilemma even in extreme production costs. Furthermore, “Coordination” regime is not observed since the equilibrium frequencies were not affected by initial composition of the populations (not shown). Therefore, Later simulations were done for equal initial Cooperator and Cheater compositions and the cost parameter value is assumed as 0.12.

3.3 Equilibrium Frequencies and Lipolytic Activities

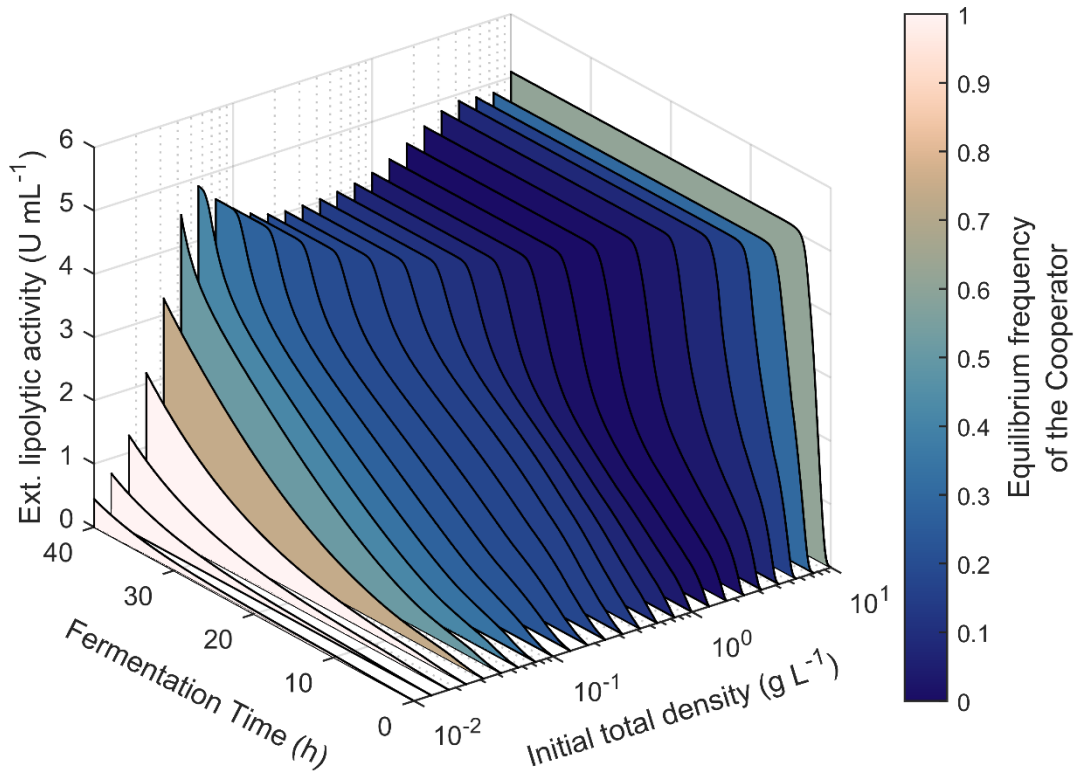


Fig 5. Effects of Initial total density on the Equilibrium frequencies and Extracellular lipolytic activities during fermentation. Extracellular lipolytic activities were plotted during batch fermentations on 25 different initial total densities where Cheater and Cooperator sub-populations are at equilibrium. Face color of each plot represents the Equilibrium frequency of the Cooperator at corresponding Initial total density. Higher Initial total densities cause earlier excretion of the lipase, and hence most of the Olive oil is being hydrolyzed by extracellular lipase resulting in increase in Cheater frequency.

Effects of the initial total density, initial olive oil and 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.

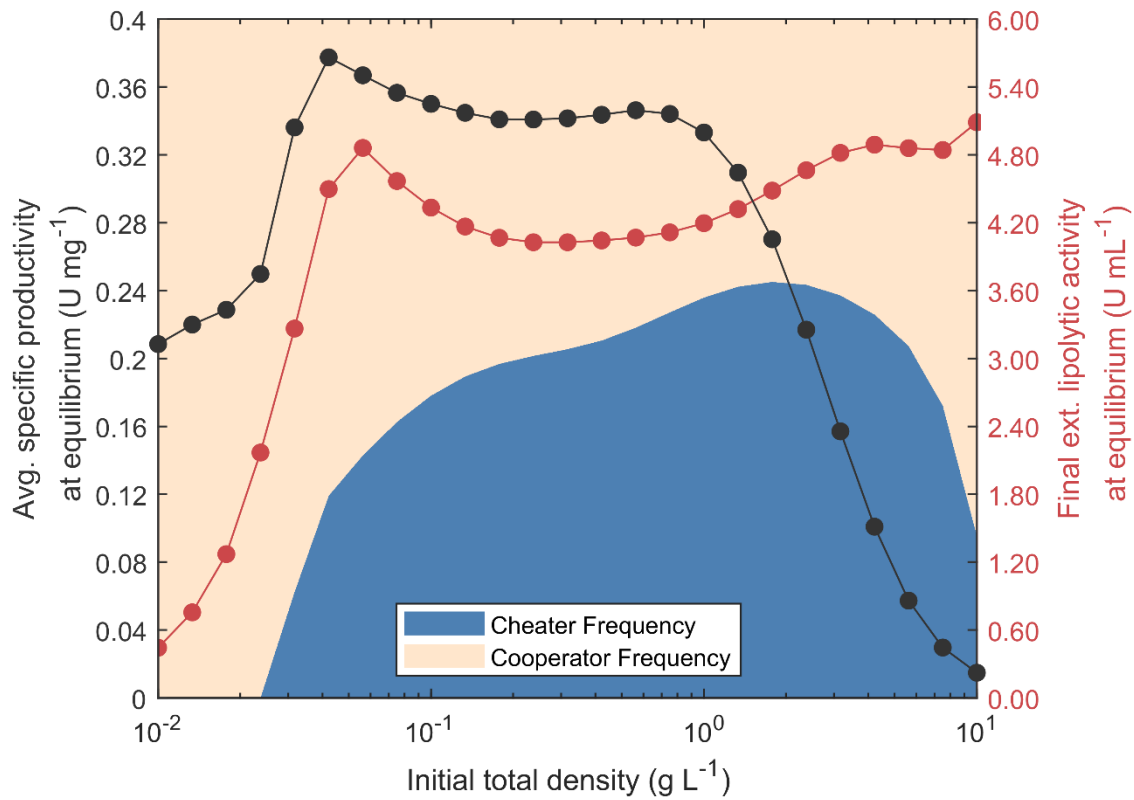


Fig 6 Effects of Initial total density on the Equilibrium frequencies and the Average specific productivity during a batch fermentation at equilibrium. Cooperator and Cheater frequencies are represented as cream and blue stacked areas respectively. Black dotted line represents the Average specific productivity (left y axis) at equilibrium. Red dotted line (right y axis) represents the Final extracellular lipolytic activity at equilibrium.

For initial total density experiments, each consecutive batch simulation starts with 3 g/L olive oil and runs for 40 hours. At low densities (between 0.01 g/L and 0.03g/L) Cooperators dominate the population. Up until 0.03g/L increase in initial total density increases specific productivity. At medium densities (between 0.03g/L and 1g/L) Cheaters begins to populate the population. At highest densities (between 1g/L and 10g/L) Cooperators halt their enzyme production. Spared from paying the production cost, Cooperators able to maintain their dominancy.

4 Discussion

The multi-player fermentation game was constructed from the mathematical model of the lipase producing *C. Rugosa* by deriving an additional “non-producer” Cheater mutant. The two types only differ in their lipase production mechanisms where the Cheater do not participate in the production of the lipase. In the constructed multi-player fermentation game, the same parameter values as the previous mathematical model were used. Throughout this study it is assumed that model parameters are applicable to multi-player fermentation settings where non-producers and producers coexist.

In order to study the social dilemma among sub-populations, extracellular olive oil is added to the *C. Rugosa* model because in the preceding model extracellular lipase had no utility and therefore wasn't a public good. Accordingly, olive oil hydrolysis is described as a first order kinetics and oleic acid content is assumed as 70 percent. The 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 Cooperators at early stages (0th to 10th hour) of fermentations (**Fig 3a**). However, at later stages of the fermentations (10th to 20th hour), lipase is excreted and thus Cheaters can benefit from products of extracellular lipase hydrolysis. 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 Cooperators to produce more lipase. This increase in lipase production is due to suitable extracellular oleic acid to biomass ratios as described in **Eq 12** that fed-batch environment provides.

The stable state finder algorithm generates initial frequencies by distributing between the minimum frequency and the maximum possible frequencies. 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 simulation ran for fixed duration. The end densities of the solution were converted back into the frequencies and then introduced into the next simulation and the process is repeated until the frequencies within the final simulation 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 then 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 then 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 Cooperators and Cheaters 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 **SI Table 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 (**SI Fig 1**). The **SI Fig 1** 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 Evolutionarily stable states of the Multi-player fermentation game.

Higher costs result in decrease in the Equilibrium frequency of the Cooperators and the “*Snowdrift*” dynamics can be observed (**Fig 4**). 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 equilibrium frequencies are not affected by alternating initial frequencies, therefore for the later experiments Cooperator and Cheater sub-populations started with equal initial frequencies.

Initial total cell density results (**Fig 5,6**) indicate that higher initial total densities not always benefitted to Cheaters. In the original *C. Rugosa* model (Montesinos et al. 1997) the lipase induction is considered to be related to the extracellular oleic acid to biomass ratio. 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, there were no records of polymorphism in LIP gene family indicating that *C. rugosa* may have more intricate lipase induction and transportation mechanisms to prevent tragedy of commons in nature where as *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. Since cooperators can adjust their lipase production, for practical and biotechnological purposes its necessary to examine extracellular lipolytic activities at the Evolutionarily stable states.

At low initial densities (between 0.01 g/L and 0.03g/L) Cooperators dominate the population as most of the olive oil undergoes periplasmic hydrolysis. Up until 0.03g/L increase in initial total density increases specific productivity which is modulated by substrate to biomass ratios (**Eq. 12**). This increase in specific productivity combined with the increase in initial total density results in drastic increase in extracellular lipolytic activities (**Fig 6**). At medium initial densities (between 0.03g/L and 1g/L) increase in initial total density increases excretion rate of the lipase (**Fig 5**), causing olive oil to undergo extracellular hydrolysis and ultimately resulting in increase in Cheater frequency. Specific productivity appears stable at medium initial densities indicating slight decrease in final extracellular lipolytic activity is due to increase in Cheater frequency. At highest densities (between 1g/L and 10g/L) Cooperators halt their enzyme production due to unsuitable substrate to biomass ratios (**Fig 6**). Spared from paying the production cost, Cooperators able to maintain their dominancy. Even though specific productivity completely halts, final extracellular lipolytic activity slightly increases due to decrease in Cheater frequency.

For biotechnological purposes 0.06 g/L initial total density is a sweet spot where final extracellular lipolytic activity reaches 4.68 U/mL in the Evolutionary stable state where Cooperators occupy 64% of the population (**Fig 6**). It is an important result showing that complete elimination of Cheaters is not necessary for the optimization of exoenzyme production.

Other extrinsic elements that could be modified in a batch fermentation are initial Olive oil and Oleic acid concentrations. Stable state finder results indicated that the increase in initial olive oil has resulted in increase in equilibrium frequency of Cooperator and Extracellular lipolytic activities (**SI Fig 2**). Whereas, higher amounts of initial oleic acid have negative impact on the equilibrium frequency of the Cooperators and extracellular lipolytic activities since, while Cooperators are investing in lipase production, Cheaters can use the available oleic acid.

In multi-player fermentation game, the oleic acid is the sole inducer of the lipase production. Along with free fatty acids, LIP gene can be induced via extracellular triacylglycerols, a potential game changer which was not taken into the consideration in this paper.

The multi-player fermentation model allows only for parent to offspring (vertical) gene transfer, therefore phenotypic frequency change depends on differences in growth/death rates. Even without including the between organisms (horizontal) gene transfer, a fermentation model can reach to an Evolutionarily 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 (Gore et al. 2009). 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 the desired outcome.

5 Conclusion

In order to analyze the Evolutionarily stable states of multi-player fermentation model, Stable state finder algorithm was developed and then validated by comparing the results against Nash equilibria of 2-player social dilemma games. The algorithm was used to analyze the effects of intrinsic cost of lipase production and the extrinsic factors such as initial cell density, initial olive oil and oleic acid concentrations.

The multi-player fermentation game regime is “No Conflict” for cost parameter values under 0.075 and “Snowdrift” for cost parameter values over 0.075 and “Prisoner’s Dilemma” and “Coordination game” regimes were not observed. Increase in olive oil and oleic acid initial concentrations had positive and negative effects on Cooperator frequencies respectively due to utilization of produced lipase enzyme or lack thereof. Finally, the increase in initial densities has not always benefitted Cheater sub-cultures and lipase production can be halted even in completely Cooperating populations.

As a conclusion, a game theoretical model was constructed to analyze the evolutionary dynamics and lipase productivity in terms of structured kinetic models. Evolutionary pressures in biotechnological settings effecting metabolic strategies and enzymatic activities were analyzed in order to understand and further improve the productivity. Optimal productivity has achieved around 0.06 g/L initial total density where final extracellular lipolytic activity reaches 4.68 U/mL in the Evolutionary stable state where Cooperators occupy 64% of the population (**Fig 6**). This result indicate that complete elimination of Cheaters is not necessary for the optimization of exoenzyme production.

In this study a social dilemma in a microbial population is examined, where same species acts out different phenotypes. The methodology of finding the Evolutionarily 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.

List of Symbols/Abbreviations

k_{ss}	Saturation constant of the growth	μ_{Coop}	Specific growth rate of the cooperator
k_{so}	Substrate transport rate coefficient	μ_{max}	Maximum specific growth rate
k_{so1}	Transport saturation constant for substrate	c	Lipase production cost parameter
k_{1m}	Lipase synthesis maximum rate coefficient	δS	Accounts for substrate consumption
k_{1s}	Lipase synthesis saturation constant	Lip_{Ext}	Extracellular lipase
k_i	Lipase synthesis inhibition coefficient	Lip_{Coop}	Intracellular lipase of the Cooperator
k_m	Lipase transport rate coefficient	OA_{Cheat}	Intracellular oleic acid of the cheater
k_s	Lipase transport saturation constant	OA_{Coop}	Intracellular oleic acid of the cooperator
k_μ	Growth related transport saturation constant	OA_{Ext}	Extracellular oleic acid
k_{es}	Lipase excretion saturation constant	OO_{Ext}	Extracellular olive oil
k_{oh}	Olive oil hydrolysis parameter	X_{Cheat}	Biomass of the Cheater
Y_{ss}	Oleic acid/olive oil yield coefficient	X_{Coop}	Biomass of the Cooperator
Y_{sx}	Substrate/biomass yield coefficient	X_{Total}	Total Biomass
μ_{Cheat}	Specific growth rate of the cheater		

6 References

- Aledo JC, Pérez-Claros JA, del Valle AE (2007) Switching between cooperation and competition in the use of extracellular glucose. *J Mol Evol* 65:328–339. <https://doi.org/10.1007/s00239-007-9014-z>
- Allison SD (2005) Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments. *Ecol Lett* 8:626–635. <https://doi.org/10.1111/j.1461-0248.2005.00756.x>
- Archetti M, Scheuring I (2012) Review: Game theory of public goods in one-shot social dilemmas without assortment. *J Theor Biol* 299:9–20. <https://doi.org/10.1016/j.jtbi.2011.06.018>
- Cohen S, Dror G, Ruppin E (2007) Feature selection via coalitional game theory. *Neural Comput* 19:1939–1961. <https://doi.org/10.1162/neco.2007.19.7.1939>
- Desfougères T, Haddouche R, Fudalej F, et al (2010) SOA genes encode proteins controlling lipase expression in response to triacylglycerol utilization in the yeast *Yarrowia lipolytica*: RESEARCH ARTICLE. *FEMS Yeast Res* 10:93–103. <https://doi.org/10.1111/j.1567-1364.2009.00590.x>
- Domínguez de María P, Sánchez-Montero JM, Alcántara AR, et al (2005) Rational strategy for the production of new crude lipases from *Candida rugosa*. *Biotechnol Lett* 27:499–503. <https://doi.org/10.1007/s10529-005-2540-0>
- Ferrer P, Montesinos L, Valero F, Solà C (2001) Production of Lipases of *C. rugosa* 221 Production of Native and Recombinant Lipases by *Candida rugosa* A Review
- Flux AW (1896) Vilfredo Pareto. *Cours d'Economie Politique*. Tome Premier. *The Economic Journal* 6:249–253. <https://doi.org/10.2307/2956507>
- Gore J, Youk H, van Oudenaarden A (2009) Snowdrift game dynamics and facultative cheating in yeast. *Nature* 459:253–256. <https://doi.org/10.1038/nature07921>
- Greig D, Travisano M (2004) The Prisoner's Dilemma and polymorphism in yeast SUC genes. *Proceedings of the Royal Society B: Biological Sciences* 271:25–26. <https://doi.org/10.1098/rsbl.2003.0083>
- Hauert C (2006) Spatial effects in social dilemmas. *J Theor Biol* 240:627–636. <https://doi.org/10.1016/j.jtbi.2005.10.024>
- Hauert C, Doebeli M (2004) Spatial structure often inhibits the evolution of cooperation in the snowdrift game. *Nat Mater* 428:643–646. <https://doi.org/10.1038/nature02360>
- Hofbauer J, Sigmund K (1998a) *Evolutionary Games and Population Dynamics*
- Hofbauer J, Sigmund K (1998b) *Evolutionary Games and Population Dynamics*. Cambridge University Press
- Kreft J-U (2004) Conflicts of interest in biofilms. *Biofilms* 1:265–276. <https://doi.org/10.1017/s1479050504001486>
- Modak T, Pradhan S, Watve M (2007) Sociobiology of biodegradation and the role of predatory protozoa in biodegrading communities. *J Biosci* 32:775–780. <https://doi.org/10.1007/s12038-007-0078-0>
- Montesinos JL, Gordillo MA, Valero F, et al (1997) Improvement of lipase productivity in bioprocesses using a structured mathematical model. *J Biotechnol* 52:207–218. [https://doi.org/10.1016/S0168-1656\(96\)01646-X](https://doi.org/10.1016/S0168-1656(96)01646-X)
- Nash J (1951) Non-Cooperative Games. *Ann Math* 54:286–295. <https://doi.org/10.2307/1969529>

- Naumov GI, Naumova ES, Sancho ED, Korhola MP (1996) Polymeric SUC genes in natural populations of *Saccharomyces cerevisiae*. FEMS Microbiol Lett 135:31–35. [https://doi.org/10.1016/0378-1097\(95\)00424-6](https://doi.org/10.1016/0378-1097(95)00424-6)
- Perc M, Szolnoki A (2010) Coevolutionary games-A mini review. BioSystems 99:109–125. <https://doi.org/10.1016/j.biosystems.2009.10.003>
- Pfeiffer T, Schuster S (2005) Game-theoretical approaches to studying the evolution of biochemical systems. Trends Biochem Sci 30:20–25. <https://doi.org/10.1016/j.tibs.2004.11.006>
- Ramirez-Tortosa MC, Granados S, Quiles JL, others (2006) Chemical composition, types and characteristics of olive oil. Olive Oil Health 45–61
- Schaefer EJ, Cooney CL (1982) Production of maltase by wild type and a constitutive mutant of *Saccharomyces italicus*. Appl Environ Microbiol 43:75–80. <https://doi.org/10.1128/aem.43.1.75-80.1982>
- Schuster S, Kreft JU, Brenner N, et al (2010) Cooperation and cheating in microbial exoenzyme production - Theoretical analysis for biotechnological applications. Biotechnol J 5:751–758. <https://doi.org/10.1002/biot.200900303>
- Schuster S, Kreft JU, Schroeter A, Pfeiffer T (2008) Use of game-theoretical methods in biochemistry and biophysics. J Biol Phys 34:1–17. <https://doi.org/10.1007/s10867-008-9101-4>
- Serra P, del Río JL, Robusté J, et al (1992) A model for lipase production by *Candida rugosa*. Bioprocess Engineering 8:145–150. <https://doi.org/10.1007/BF01254230>
- Smith JM (1982a) Evolution and the Theory of Games. Cambridge University Press
- Smith JM (1982b) Evolution and the Theory of Games. Cambridge University Press
- Tomassini M, Pestelacci E, Luthi L (2010) Mutual trust and cooperation in the evolutionary hawks-doves game. BioSystems 99:50–59. <https://doi.org/10.1016/j.biosystems.2009.08.008>
- Vanleeuw E, Winderickx S, Thevissen K, et al (2019) Substrate-Specificity of *Candida rugosa* Lipase and Its Industrial Application. ACS Sustain Chem Eng 7:15828–15844. <https://doi.org/10.1021/acssuschemeng.9b03257>
- Vincent TL, Brown JS (2005) Evolutionary Game Theory, Natural Selection, and Darwinian Dynamics. Cambridge University Press, Cambridge
- von Neumann J, Morgenstern O (1944) Theory of games and economic behavior. Princeton University Press, Princeton, NJ, US
- von Stackelberg H, Von SH, Peacock AT (1952) The Theory of the Market Economy. Oxford University Press

7 SUPPLEMENTARY INFO

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 (Archetti and Scheuring 2012). 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 in **SI Table 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 (Aledo et al. 2007).

SI Table 1 Pairwise representation of social dilemmas. Letters represent the pay-offs for the focal player

		Opponent	
		Coop	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 (Hofbauer and Sigmund 1998a; Hauert and Doebeli 2004; Hauert 2006).

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 (Smith 1982b; Hofbauer and Sigmund 1998b; Tomassini et al. 2010).

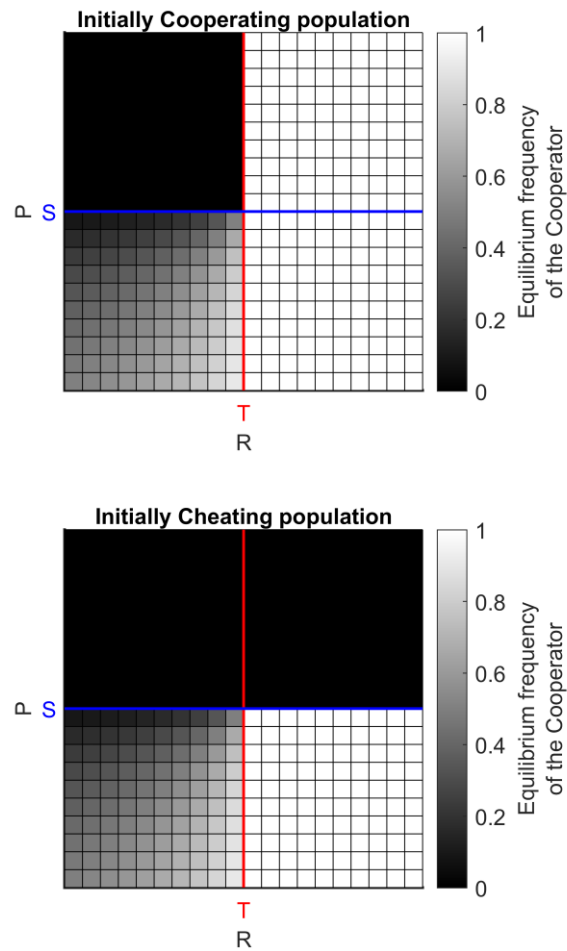
Stag Hunt (Coordination): If $R > P > T$, S , “mutual Cooperation” is better than “mutual Cheating”, and both “mutual Cheating” and “mutual Cooperation” give better results than lack of coordination. This is a coordination game, with two symmetric equilibria with pure strategies. It has received little 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”.

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.

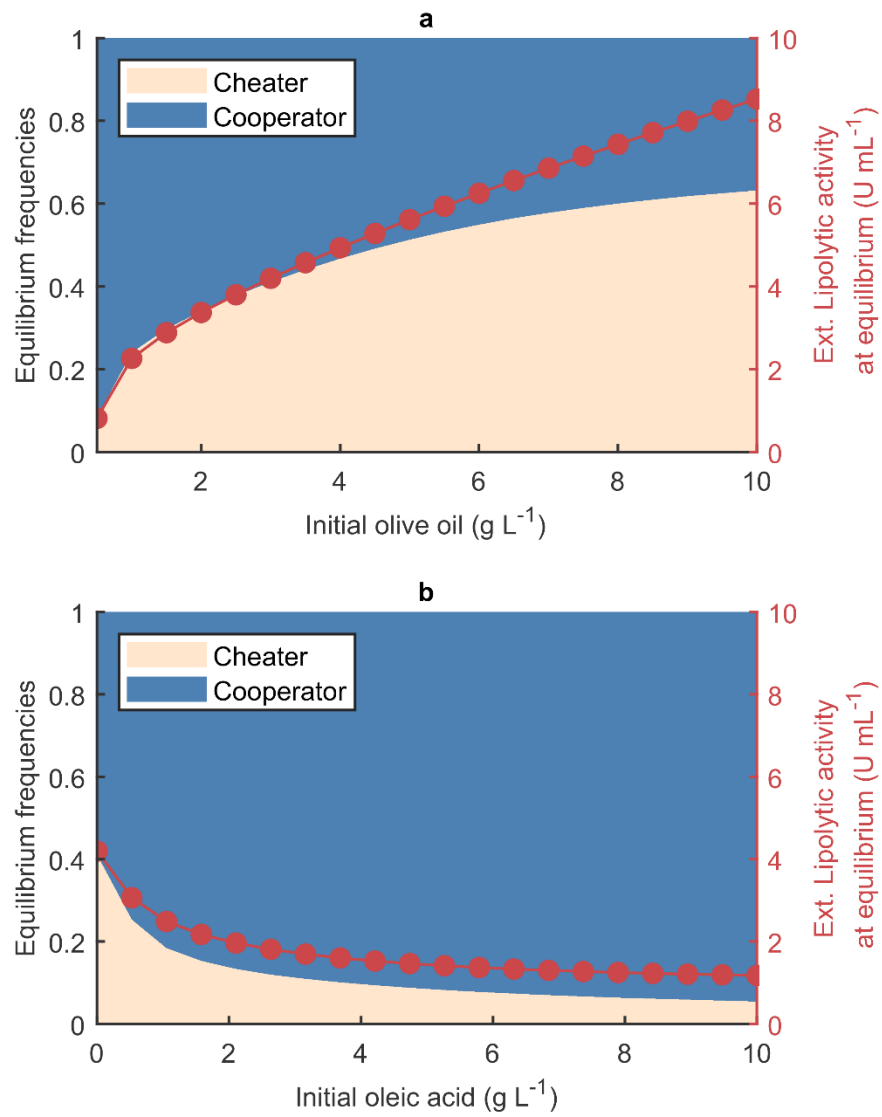
The population dynamics of the social dilemma games (*Prisoner’s dilemma*, *Snowdrift*, *Coordination*, *No-conflict*) were simulated with the numeric (R, T, S, P) pay-off values (**SI Table 1**) and the replicator dynamics. With a fixed S and T and varying R and P values Equilibrium frequencies for 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 (**SI Fig 1 Top**) and under 1 percent Equilibrium frequency of the Cooperator in Initially Cheating population (**SI Fig 1 Bottom**).



SI Fig 1 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*: $R > P > T, S$. S and T values are fixed whereas axes represent varying R and P values.



SI Fig 2. Effects of Initial olive oil a and Initial oleic acid b on Equilibrium frequencies and Extracellular lipolytic activities.