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

Özgür Yüksel[1] and Emrah Nikerel[1]

Game theory provides a mathematical framework for understanding social dilemmas and conflicts of interest among agents striving to maximize personal gain. In microbial populations, different phenotypes, such as complete or partial failure in exoenzyme production, can be seen as strategies in a game where cell growth rates indicate the fitness of each strategy. This research focuses on the interplay between wildtype "Cooperator" cells which exhibit plastic behavior by producing exoenzymes based on substrate concentrations, and "Cheater" cells that do not produce, with population productivity depending on the dynamics of exoenzyme production, transportation, and hydrolysis. We present a novel successive simulation methodology to analyze the evolutionary dynamics of lipase-producing *Candida rugosa* using kinetic fermentation models. Two main game regimes emerge: wildtype Cooperators dominate when the cost of lipase production is low, while at intermediate and higher costs, wildtype Cooperators coexist with Cheaters. Despite Cheaters benefiting from higher initial densities due to increased lipase excretion rates, wildtype Cooperators can cease enzyme production at utmost densities to regain dominance. Optimal productivity is attained at an initial total density of 0.06 g/L, with a final extracellular lipolytic activity of 4.68 U/mL, and Cooperators occupying 64% of the population in the evolutionary stable state. This suggests that complete elimination of Cheaters is not necessary to optimize exoenzyme production. Overall, our study provides a novel and comprehensive understanding of environmental currency and offers an innovative and robust framework for analyzing evolutionary stable states in microbial populations.

Keywords: Evolutionary Game Theory, Cooperation, Cheating, Social Dilemma, Exoenzyme, 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 resolving conflicts of interest among agents, who are treated as players with a set of strategies and the goal of maximizing personal interests (Payoffs). The game problem is distinguished from the standard optimization problem because the best strategy choice depends on the strategies chosen by other players (Vincent and Brown 2005). The optimal strategies and the outcomes of games are studied using solution concepts such as min-max, Nash equilibrium, Stackelberg equilibrium and Pareto optimality (Flux 1896; Von Neumann and Morgenstern 1944; Nash 1951; Von Stackelberg et al. 1952).

Evolutionary game theory (EGT) applies classical game theory to evolving populations in biology. EGT analyzes the evolutionary stability of strategies and specifies population dynamics 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 the growth rates of cells represent the fitness of each strategy. Epigenetic and genetic states correspond to strategies, while gene silencing and mutations would correspond to switches between strategies (Schuster et al. 2010). The optimization and analysis of biotechnological setups can be performed with 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 an increase in exoenzyme production is desired, exoenzyme production is often decreased by the non-producing subpopulation of microorganisms (Allison 2005; Modak et al. 2007).

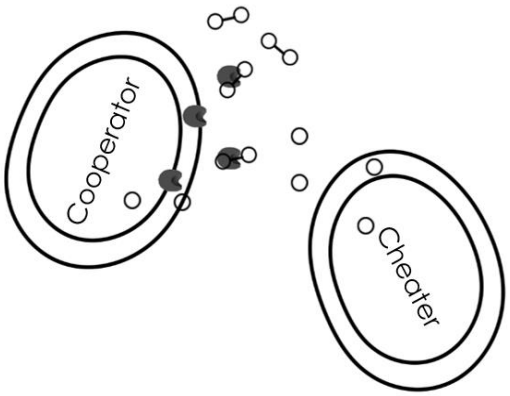


Fig. 1 Interplay between microbial cells for the exoenzyme secretion. Complex substrates are indicated by linked circles. Cooperating cells secrete exoenzyme (black sliced circles) to release growth substrate (single circles). Cheater cells do not produce exoenzymes yet benefit from the growth substrate.

Throughout the study, we refer to exoenzyme producers as the Cooperators and 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 a non-functional *SUC2* gene as the only *SUC* gene (Naumov et al. 1996). Moreover, there are species that do not 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 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 that 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 Fermentation Game

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. Environmental dimensions are typically abstracted away such that the amount of public good is simply defined by a proxy such as the number of cooperators in the neighborhood of recipients (Estrela et al. 2019). In this study we explicitly consider the environmental currency and its dynamics (**Fig 2**). 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.

Traditionally, an evolutionary game is presented with the consequences of interaction (payoffs) provided in matrix form. Then, if one is interested in population dynamics, the replicator equation can be used to derive fitness values from these payoffs. In this study, we initiate our analysis at the fitness equation level, represented by Monod growth equations. The reason for this choice is that our players do not directly influence each other's fate; instead, their strategies collectively affect the population, which can be described as 'playing the field'. Even without a direct impact on each other, the frequency of Cheaters has a detrimental effect on the fitness of the Cooperators. This frequency-dependent fitness, where one's fitness depends on both its own and other players' strategies, serves as an indicator of the evolutionary game (Sigmund and Nowak 1999).

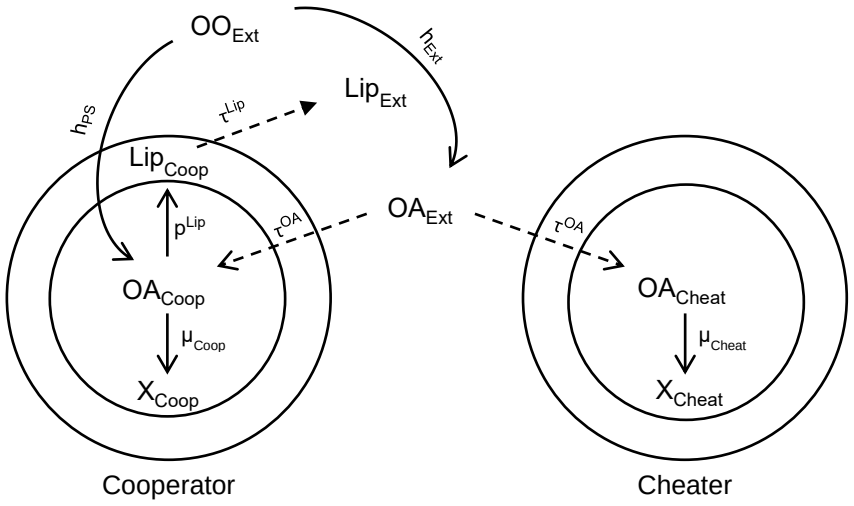


Fig 2 Model representation of Fermentation game. Inner circles represent the border between intracellular space and the periplasmic space. Outer circles represent the border between periplasmic space and the extracellular space. Solid arrows are production and growth processes, dashed arrows indicate transportation and curved arrows are hydrolysis reactions. Olive Oil OO_{Ext} is being hydrolyzed both at the extracellular (h_{Ext}) and the periplasmic space (h_{PS}) by the extracellular lipase Lip_{Ext} and the periplasmic lipase Lip_{Coop}, resulting extracellular oleic acid OA_{Ext} and intracellular oleic acid OA_{Coop} respectively. Then extracellular oleic acid is equally shared and transported (τ^{OA}) towards both the Cooperator and the Cheater. While both types of cells use intracellular oleic acid for the cell growth (μ), only the Cooperator produce lipase (p^{Lip}) which then is transported into the extracellular space (τ^{Lip})

The Fermentation game consist of common (i.e., extracellular substrate, extracellular enzyme) and sub-culture specific (i.e., 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 its hydrolysis via lipase is introduced to generate a public goods scenario. The following system of equations represents the mass balances for the Fermentation game.

Table 1. System of mass balance equations for the Fermentation game

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

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

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

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

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

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

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

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

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 equally transported towards each sub-culture where it is stored, consumed and transformed (Eqs. 3 and 4). 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. Where the expression of lipase genes would be controlled at the transcription level by oleic acid. This assumption entails that genetically determined "Cooperator" phenotype behaves plastically depending on the environmental conditions. The lipase production described only for the "Cooperator" (Eq. 7) 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).

When the microorganism grows, both intracellular oleic acid and intracellular lipase are affected by a dilution factor, $\mu_{Coop} \cdot OA_{Coop}$ and $\mu_{Coop} \cdot Lip_{Coop}$, derived from mass balancing (Eqs. 3,4 and 7).

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. Fermentation game parameters adopted from *C. rugosa* model (Montesinos et al. 1997).

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

Table 3. 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		

2.2 Stable State Finder Algorithm

Analyzing the Evolutionary stability of the 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 may depend 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 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 finesses 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 Fermentation simulations

Constructed 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.

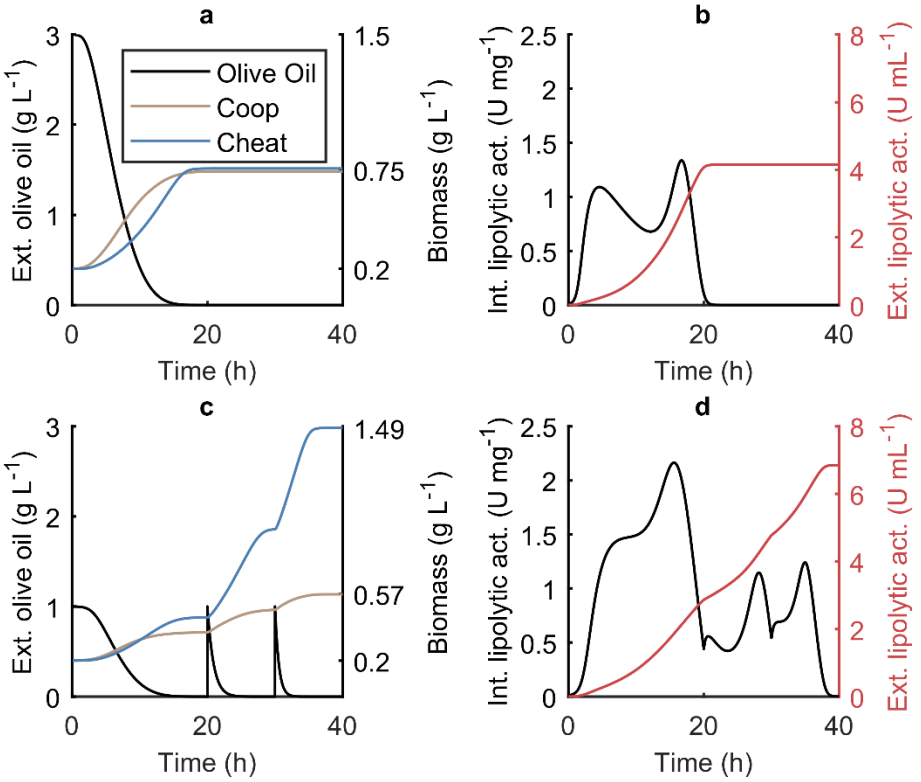


Fig 3. Fermentation game employing Cooperator and Cheater sub-populations for batch **(a-b)** and fed-batch **(c-d)** modes. **a** Batch fermentation starts with 0.3 g/L olive oil. Starting from initial densities of 0.2 g/L each, Cooperator and Cheater reach to 0.74 g/L and 0.76 g/L respectively. **b** 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.

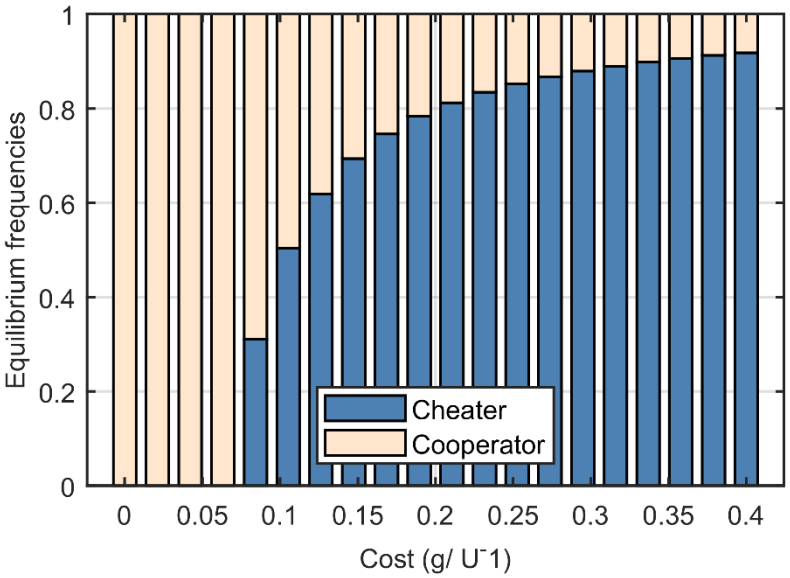


Fig 4. Effects of the lipase production cost parameter **c** on the equilibrium frequency of the Cooperator in batch fermentation settings. Cooperator and Cheater frequencies are shown as stacked bars with cream and blue colors respectively. Below the cost parameter value of 0.075 the game regime is "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

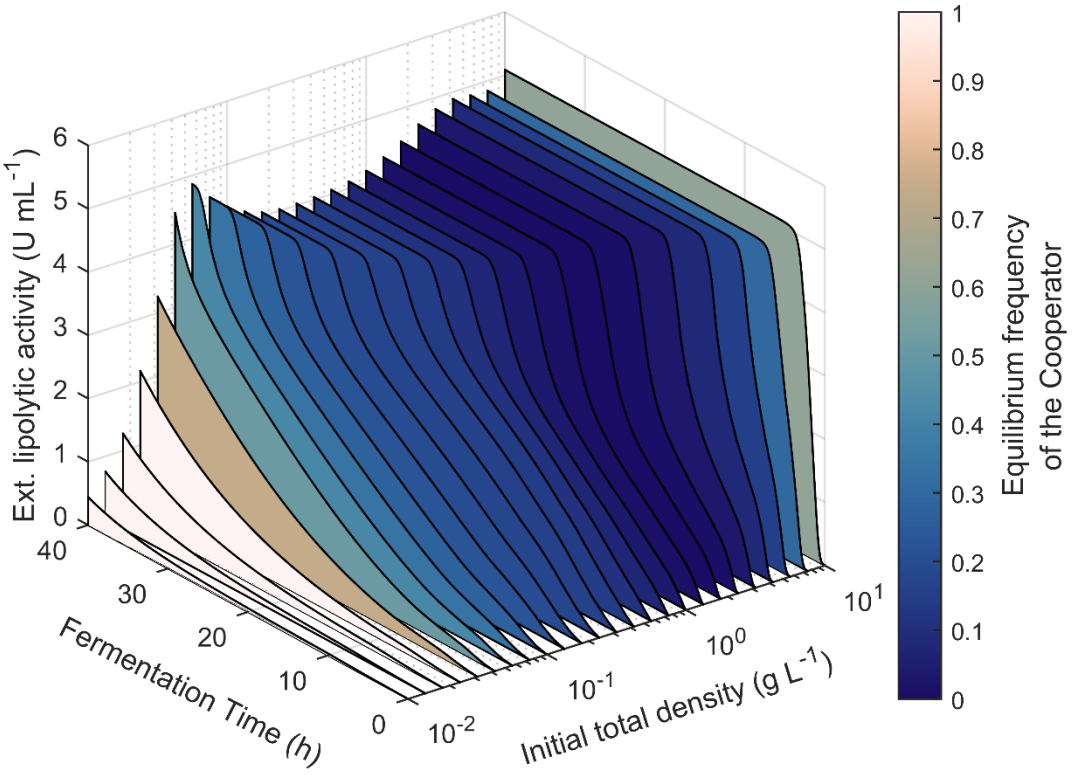


Fig 5. Effects of Initial total density on the Equilibrium frequencies and Extracellular lipolytic activities during fermentation. Extracellular lipolytic activities were plotted during batch fermentations on 25 different initial total densities where Cheater and Cooperator sub-populations are at equilibrium. 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.

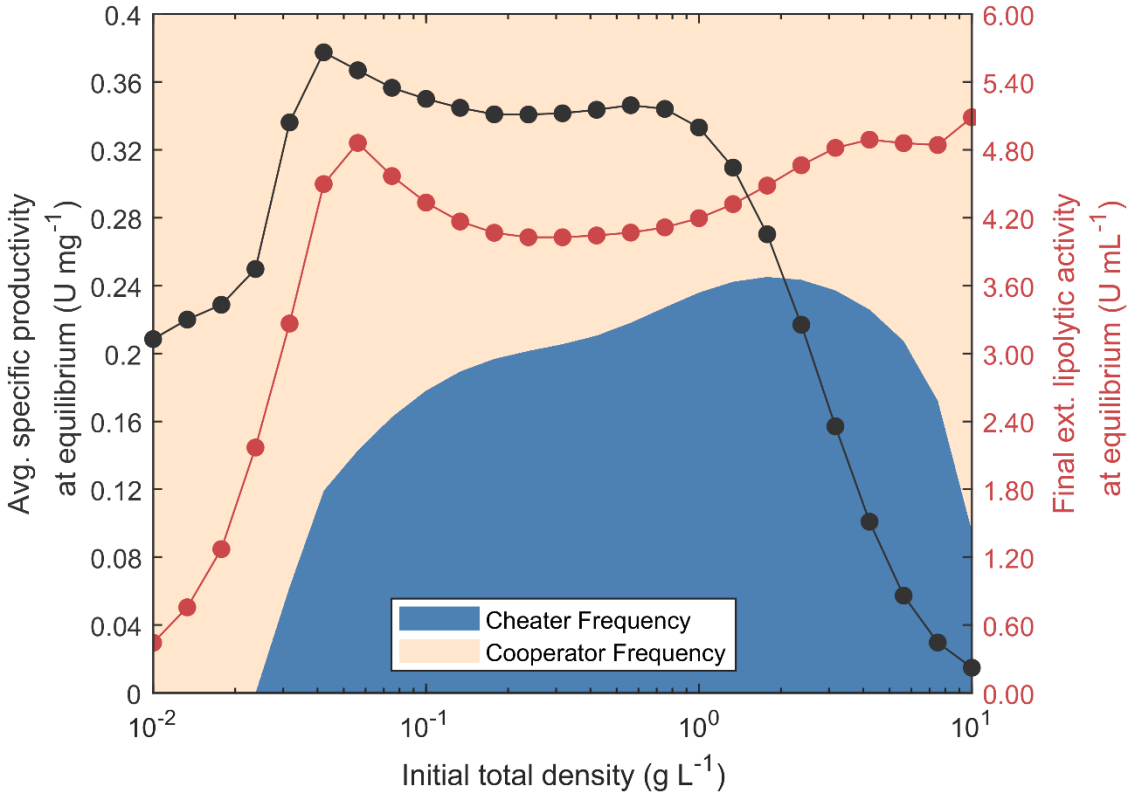


Fig 6 Effects of Initial total density on the Equilibrium frequencies and the Average specific productivity during a batch fermentation at equilibrium. Cooperator and Cheater frequencies are represented as cream and blue stacked areas respectively. Black dotted line represents the Average specific productivity (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 Fermentation game was developed based on the mathematical model of lipase-producing *C. Rugosa*, incorporating an additional "non-producer" Cheater mutant. These two types differ solely in their lipase production mechanisms, with Cheaters abstaining from contributing to lipase production. In this constructed Fermentation game, we employed the same parameter values as in the previous mathematical model. Throughout this study, it is assumed that model parameters are applicable to multi-player fermentation settings where non-producers and producers coexist.

To investigate the social dilemma among sub-populations, we introduced extracellular olive oil to the *C. Rugosa* model, as the previous model did not consider the utility of extracellular lipase, making it a non-public good. Consequently, we describe olive oil hydrolysis as following first-order kinetics, with an assumed oleic acid content of 70 percent. Hydrolysis occurs at two distinct locations: the periplasmic space and the extracellular space. It is assumed that intracellularly produced lipase directly catalyzes periplasmic hydrolysis, capturing the resulting oleic acid, while extracellular lipase generates extracellular oleic acid that must be transported.

Another crucial aspect of public good games is that cooperation comes at a cost. The previous mathematical model overlooked the consumption of essential intracellular substrate. Consequently, in this study, we introduced the lipase production cost, denoted as "*c*", which represents the reduction in intracellular substrate per unit of lipase produced. The cost needed to be linked to intracellular substrate levels, leading to the assumption that the lipase production cost is directly proportional to the intracellular substrate quantity. Following the construction of the Fermentation game, simulations were conducted for both batch and fed-batch operation modes.

Cell-bound hydrolysis of olive oil confers an advantage to Cooperators during the early stages (0^{th} to 10^{th} hour) of fermentations (**Fig 3a**). However, at later stages of the fermentations (10^{th} to 20^{th} hour), lipase is excreted, and thus Cheaters can benefit from products of extracellular lipase hydrolysis. Despite the Cheater sub-culture benefiting more from fed-batch fermentation, extracellular lipolytic activity has increased in comparison to batch fermentation. This outcome suggests that, in contrast to batch fermentation, fed-batch fermentation has induced Cooperators to produce more lipase. This increase in lipase production is attributed to the favorable extracellular oleic acid to biomass ratios, as outlined in **Eq 12**, which the fed-batch environment offers.

The stable state finder algorithm generates initial frequencies by distributing them between the minimum and maximum possible frequencies. This approach encompasses all attractors of the system, as demonstrated in subsequent validation experiments. The initial frequencies were transformed into initial densities by multiplying them by the total initial density, and the simulation was run for a fixed duration. The final densities of the solution were converted back into frequencies, which were then used in the subsequent simulation. This process iterated until the frequencies in the final simulation remained unchanged, indicating that the system had reached a stable state. In two-player Cooperation and Cheating games, the algorithm generates two potential starting scenarios: one with 99 percent Cooperators and 1 percent Cheaters, and the other with 1 percent Cooperators and 99 percent Cheaters. We assumed that if, in both scenarios, the Cooperator frequency exceeds 99 percent, the game regime is classified as "*No-conflict*", signifying that Cooperation is the optimal strategy for all members of the population. If both scenarios result in the same frequencies, and Cooperation and Cheating coexist, with their frequencies ranging between 99 percent and 1 percent, the game regime is termed "*Snowdrift*." This indicates that Cooperators and Cheaters have equal fitnesses in equilibrium and a slight deviation towards one side will make the opposite strategy stronger. Lastly, the game regime is classified as a "*Coordination game*" if Cooperators prevail when their initial frequency is 99 percent but are eliminated when their initial frequency is 1 percent. The efficacy of the Stable State Finder algorithm in identifying all attractors within a given dynamical system was assessed through a series of steps. First, two-player population dynamics were generated using replicator equations, incorporating the payoffs from the four aforementioned social dilemma games (as detailed in **SI Table 1**). Subsequently, the algorithm was employed to determine the equilibrium frequency of Cooperators. Finally, the results obtained were compared to the Nash solutions of these two-player games (**SI Fig 1**). Following the successful testing of the Stable State Finder algorithm, it was then applied to analyze the evolutionarily stable states of the Fermentation game.

Higher costs result in a decrease in the equilibrium frequency of the Cooperators, and the "*Snowdrift*" dynamics can be observed **(Fig 4)**. As the lipase production cost increases, the Fermentation game does not

transition into a "*Prisoner's dilemma*" because the equilibrium frequency of the Cooperators never falls below the assumed threshold of 1 percent at any cost. Furthermore, the game does not qualify as a "*Coordination game*" because the equilibrium frequencies remain unaffected by alternating initial frequencies. Consequently, for subsequent experiments, Cooperator and Cheater sub-populations were initiated with equal initial frequencies as opposed to the two scenarios where initial frequencies were set at 99% to 1% and 1% to 99%.

Results from the initial total cell density (**Fig 5,6**) suggest that a higher initial total density does not always favor Cheaters. In the original *C. Rugosa* model (Montesinos et al. 1997) the lipase production 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 game model, the wildtype Cooperator can behave plastically and reduce the lipase production and therefore pay less cost and maintain its advantage over the Cheater type. These results demonstrate that plastic responses to the environment can be the starting point of evolutionary change. In fact, there is no documented polymorphism in the LIP gene family for *C. rugosa*, suggesting that it may possess more complex lipase production and transportation mechanisms to prevent the tragedy of the commons in nature. In contrast, *S. cerevisiae* exhibits high polymorphism in SUC2 genes and regulates its invertase production at the gene level to maintain fitness in diverse environments. Because Cooperators can regulate their lipase production, it is essential for practical and biotechnological purposes to investigate extracellular lipolytic activities at the evolutionarily stable states.

At low initial densities (ranging from 0.01 g/L to 0.03 g/L), Cooperators dominate the population, primarily because most of the olive oil undergoes periplasmic hydrolysis. This dominance persists until an increase in initial total density up to 0.03 g/L, which elevates specific productivity due to modulation by substrate to biomass ratios (**Eq 12**). This increase in specific productivity, coupled with the rise in initial total density, leads to a significant surge in extracellular lipolytic activities (**Fig 6**). At medium initial densities (between 0.03g/L and 1g/L) the increase in initial total density result in an increased lipase excretion rate, leading to elevated levels of extracellular hydrolysis **(Fig 5)**. Simultaneously, higher initial densities enhance the collective effort of periplasmic hydrolysis. Unlike extracellular hydrolysis, the product of periplasmic hydrolysis is selfishly retained by individual cooperators rather than shared among community members. Consequently, an increase in the collective effort of periplasmic hydrolysis does not directly impact the fitness of an individual cooperator, as more cooperators invest equally in this intracellular process. In contrast, the product of extracellular hydrolysis is shared among all community members. Therefore, higher initial densities primarily benefit cheaters, who exploit communal resources without proportionately investing. While specific productivity remains relatively stable at medium initial densities, there is a slight decrease in final extracellular lipolytic activity attributed to the rise in Cheater frequency. At the highest densities (ranging from 1 g/L to 10 g/L), Cooperators cease their enzyme production due to unfavorable substrate to biomass ratios (**Fig 6**). Freed from the burden of production costs, Cooperators maintain their dominance. Although specific productivity comes to a complete halt, the final extracellular lipolytic activity experiences a slight increase due to a decrease in Cheater frequency.

For biotechnological purposes, an initial total density of 0.06 g/L emerges as an optimal point. At this density, the final extracellular lipolytic activity reaches 4.68 U/mL in the evolutionarily stable state, with Cooperators comprising 64% of the population (**Fig 6**). This result highlights that complete elimination of Cheaters is not necessary for optimizing exoenzyme production.

Other extrinsic elements that can be modified in a batch fermentation include the initial concentrations of olive oil and oleic acid. Stable state finder results suggest that an increase in initial olive oil concentration results in an increase in the equilibrium frequency of Cooperators and extracellular lipolytic activities (**SI Fig 2**). Conversely, higher initial oleic acid concentrations negatively impact the equilibrium frequency of Cooperators and extracellular lipolytic activities. This occurs because, while Cooperators invest in lipase production, Cheaters can utilize the available oleic acid.

In the Fermentation game, oleic acid serves as the sole inducer of lipase production. In addition to free fatty acids, the LIP gene can be induced via extracellular triacylglycerols, a potential game-changing factor that was not considered in this paper.

The fermentation model only allows for parent-to-offspring (vertical) gene transfer, which means that phenotypic frequency changes depend on differences in growth and death rates. Even without including between-organisms (horizontal) gene transfer, a fermentation model can reach an evolutionarily stable state depending on

the initial conditions and the costs associated with lipase production within the time frames of fermentation setups. This result has been experimentally proven in well-mixed *S. cerevisiae* cultures (Gore et al. 2009). It underscores the significant impact of evolutionary pressures in biotechnological settings on metabolic strategies and enzymatic activities, necessitating consideration for achieving desired outcomes.

5 Conclusion

To analyze the evolutionarily stable states of the fermentation game, we developed the Stable State Finder algorithm and tested it by comparing the results against Nash equilibria of 2-player social dilemma games. The algorithm was employed to investigate the effects of the intrinsic cost of lipase production and extrinsic factors such as initial cell density, initial olive oil, and oleic acid concentrations.

The fermentation game regime is characterized as "*No Conflict*" for cost parameter values under 0.075 and as "*Snowdrift*" for cost parameter values over 0.075. We did not observe "*Prisoner's Dilemma*" and "*Coordination Game*" regimes. Increases in olive oil and oleic acid initial concentrations had positive and negative effects on Cooperator frequencies, respectively, due to the utilization of the produced lipase enzyme or its absence. The increase in initial densities did not always benefit Cheater sub-cultures. Wildtype Cooperators can cease enzyme production at utmost densities to regain dominance. These results demonstrate that plastic behaviors can have significant leading role in the evolution of genetically determined phenotypes.

In summary, this study constructed a game theoretical model to analyze evolutionary dynamics and lipase productivity using structured kinetic models. We examined the evolutionary pressures in biotechnological settings affecting metabolic strategies and enzymatic activities to enhance productivity. Optimal productivity was achieved at an initial total density of approximately 0.06 g/L, where the final extracellular lipolytic activity reached 4.68 U/mL in the evolutionarily stable state, with Cooperators occupying 64% of the population (**Fig 6**). This result emphasizes that complete elimination of Cheaters is not necessary for optimizing exoenzyme production.

In this study, we examined a social dilemma in a microbial population where the same species exhibit different phenotypes. The methodology for finding the evolutionarily stable state can also be applied to scenarios involving different species as players. In the growing biotechnological literature, co-cultures are becoming prominent examples of such scenarios.

Declarations

Ethical Approval
Not applicable.

Competing interests
The authors have no competing interests to declare that are relevant to the content of this article.

Authors' contributions
All authors contributed to the study conception and design. Model construction and analysis were performed by Özgür Yüksel. The first draft of the manuscript was written by Özgür Yüksel and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding
The authors did not receive support from any organization for the submitted work.

Availability of data and materials
Not applicable.

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

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

Estrela S, Libby E, Van Cleve J, et al (2019) Environmentally Mediated Social Dilemmas. Trends Ecol Evol 34:6–18

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

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

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

Sigmund K, Nowak MA (1999) Evolutionary game theory. Current Biology 9:R503–R505. https://doi.org/https://doi.org/10.1016/S0960-9822(99)80321-2

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

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

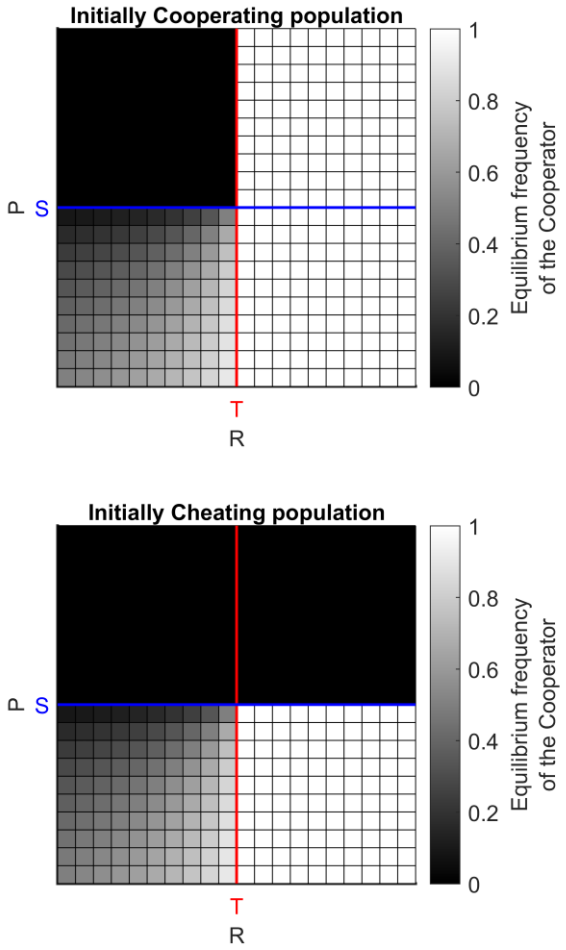
Snowdrift: If T > R > S > P, "mutual Cooperation" is better than "Cooperating while the other player Cheats", but "Cheating while the other player cooperates" is better than "mutual Cooperation", and "mutual Cheating" is the worst possible outcome. This is an anti-coordination game, with two asymmetric 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.

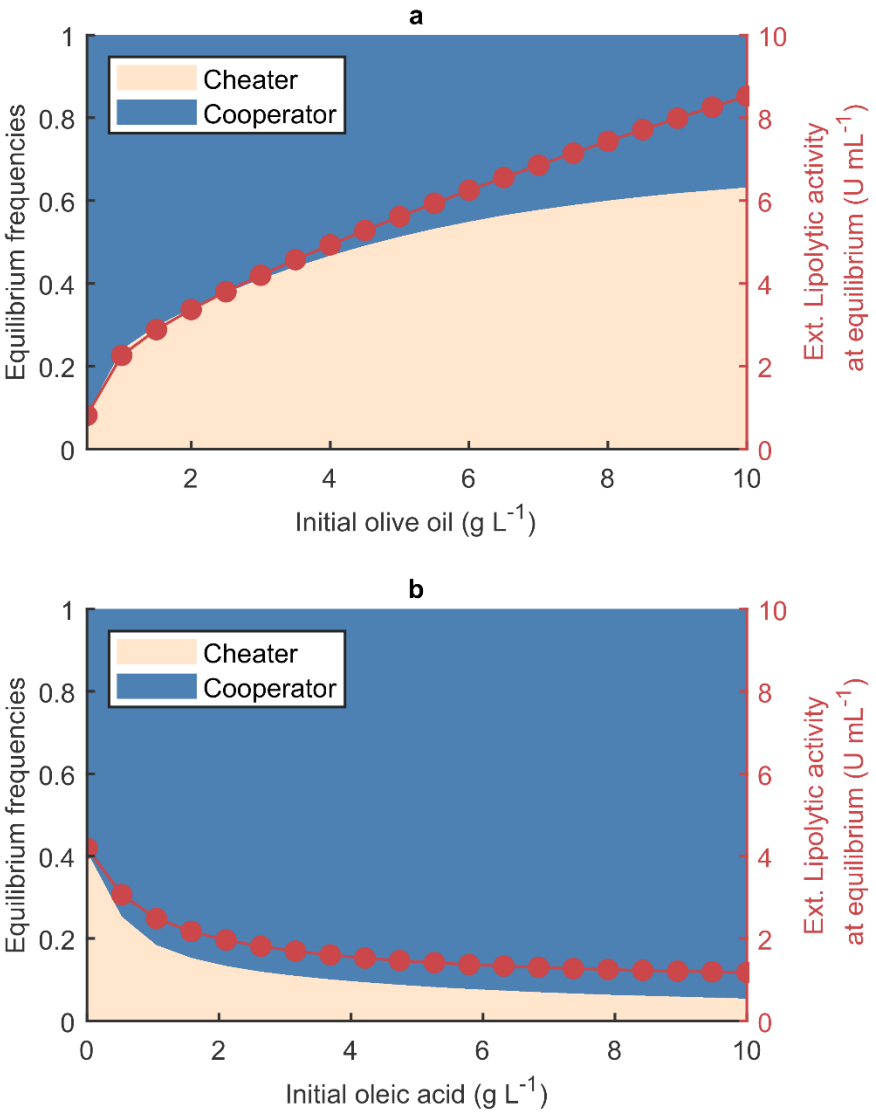
The population dynamics of the social dilemma games (*Prisoner's dilemma*, *Snowdrift*, *Coordination*, *No-conflict*) were simulated with the numeric (R, T, S, P) pay-off values **(SI Table 1**) and the replicator dynamics. With a fixed S and T and varying R and P values Equilibrium frequencies for 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, we assume in this domain the game regime is Snowdrift. The values set T > R > P > S, results under 1 percent Equilibrium frequency of the Cooperator, we assume in this domain the game regime is Prisoner's Dilemma. The values set R > T, S > P results over 99 percent Equilibrium frequency of the Cooperator, we assume in this domain 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)**. We assume this behavior indicates the Coordination game.



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 axises represent varying R and P values.

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.



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