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

Özgür Yüksel 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 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 long-term evolutionary dynamics of lipase-producing *Candida rugosa* that make use of structured mathematical models at the ecological scale. Key results show that fermentation dynamics are influenced by substrate availability and initial population densities. Batch fermentations favor Cooperators due to localized hydrolysis, while fed-batch conditions benefit Cheaters by increasing extracellular hydrolysis. The Stable State Finder algorithm highlights that high lipase production costs and suboptimal substrate concentrations can lead to Cheater dominance and reduced enzymatic output. For biotechnological applications, optimizing initial total density and substrate concentrations is essential to maximizing enzymatic activity while preserving cooperative stability. Overall, our study provides a comprehensive understanding of environmental currency in evolutionary games and offers a novel and robust framework for analyzing evolutionary stable states in microbial populations.

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

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1 Introduction

1.1 Game Theory and Evolutionary Dynamics

Game theory is a mathematical framework for understanding and resolving conflicts of interest among agents, conceptualized as players who possess a set of strategies and aim to maximize their individual payoffs. Unlike standard optimization problems, the optimal strategy in game theory depends on the strategies chosen by other players (Vincent and Brown 2005). Key solution concepts in this field include the min-max principle, 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) extends classical game theory to evolving biological populations. EGT examines the evolutionary stability of strategies and explores population dynamics where an organism's fitness depends on both its own strategy and those of others (Smith 1982a; Hofbauer and Sigmund 1998a). This approach has been used to analyze various aspects of living systems, including the evolution of cooperation (Hauert and Doebeli 2004; Perc and Szolnoki 2010; Tomassini et al. 2010), sex ratios (Smith 1982a), the selection of biochemical pathways (Pfeiffer and Schuster 2005) and biofilm formation (Kreft 2004). Additionally, EGT has applications beyond biology, such as classification tasks 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 viewed as strategies within an evolutionary game, where the growth rates of cells represent the fitness associated with each strategy. In this context, epigenetic and genetic states correspond to different strategies, while gene silencing and mutations act as switches between these strategies (Schuster et al. 2010). Evolutionary game theory can be applied to optimize and analyze biotechnological setups, where population productivity is influenced by the interplay between exoenzyme-producing and non-producing subcultures (Schuster et al. 2010). Although increased exoenzyme production is generally desirable, it is often reduced due to competition from the non-producing subpopulation of microorganisms (Allison 2005; Modak et al. 2007).

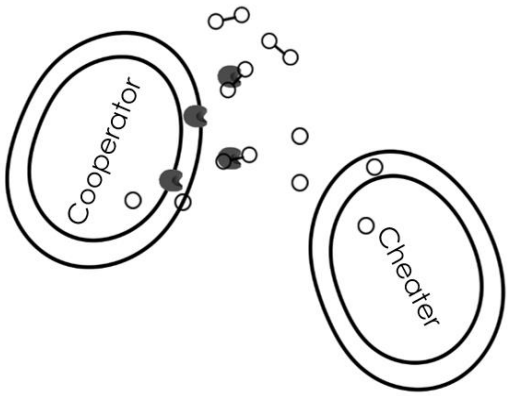

Fig. 1 Interplay between microbial cells for the exoenzyme secretion. Complex substrates are indicated by linked circles. Cooperating cells secrete exoenzyme (black sliced circles) to release growth substrate (single circles). Cheater cells do not produce exoenzymes yet benefit from the growth substrate.

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.

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).

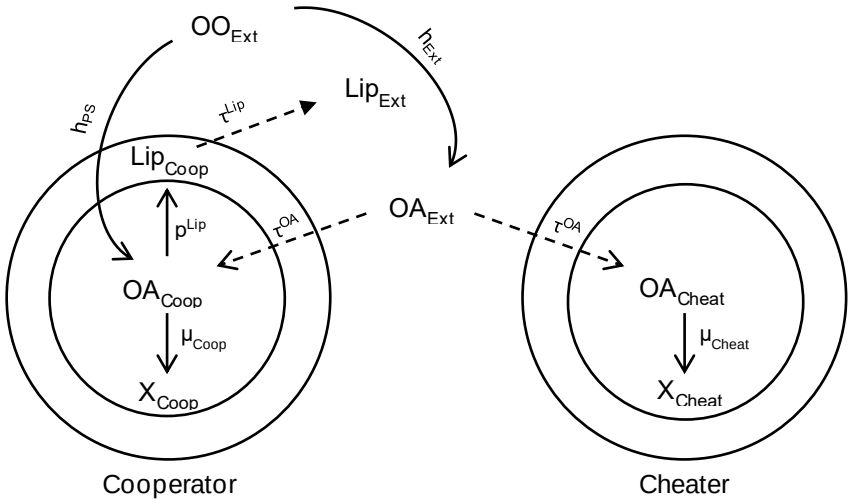

Fig 2 Model representation of Fermentation game. Inner circles represent the border between intracellular space and the periplasmic space. Outer circles represent the border between periplasmic space and the extracellular space. Solid arrows are production and growth processes, dashed arrows indicate 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

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

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

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

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

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

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

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

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

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). The transport process is assumed to be active and subject to saturation,

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

In Cooperator cells, oleic acid is used for the lipase production. The reduction in oleic acid is regulated by both the cost of production c and the rate of lipase production p^{Lip}. Here c is an arbitrary value, it approximates to a yield coefficient of substrate to enzyme. Lipase production p^{Lip} 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 concentration in the vicinity of the cell. Accordingly, the lipase production of Cooperator described as,

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

This version of the Michaelis-Menten equation incorporates an inhibition term. In many enzymatic systems, the reaction rate increases with substrate concentration. However, at very high substrate concentrations, the substrate can inhibit the enzyme, reducing its efficiency. This inhibitory effect is represented by the quadratic term in the denominator: as the substrate-to-biomass ratio increases significantly, the inhibition becomes more pronounced. The last term δS modulates the lipase production according to Cooperator's available substrate to make sure it only produces when it has necessary substrate,

$$\delta S = \frac{OA_{Coop}}{k_{es} + OA_{Coop}}. \quad (13)$$

When OA_{Coop} is low, the lipase production operates in low-efficiency/resource-limited regime. Y_{SX} is the yield coefficient (amount of substrate consumed per unit of biomass produced), $Y_{SX} \cdot \mu$ represents oleic acid consumption associated with growth. Additional substrate is available to Cooperator with cell-bound/periplasmic hydrolysis h_{PS}, this is the privileged share of the Cooperator that justifies the production of the common good enzyme. We first describe the extracellular hydrolysis process h_{Ext} which is simpler due to the assumption of a well-mixed environment,

$$h_{Ext} = Y_{SS} \cdot k_{oh} \cdot OO_{Ext} \cdot Lip_{Ext}. \quad (14)$$

The main compound of olive oil is oleic acid (65 to 85 percent) (Ramirez-Tortosa et al. 2006). We assume that oil hydrolysis yields 70 percent oleic acid, represented by the yield coefficient Y_{SS} and the olive oil hydrolysis parameter k_{oh} is estimated to be 0.5 (Serra et al. 1992). The reaction rate depends on both the availability of olive oil and the enzyme concentration. In contrast, the availability of olive oil for periplasmic hydrolysis h_{PS} performed by the cooperator, depends on the concentration of olive oil around the cell,

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

If the olive oil concentration is too high relative to cell density, the equation approximates the extracellular hydrolysis. Conversely, if the cell concentration is too high compared to the olive oil concentration, periplasmic hydrolysis ceases.

The excretion of lipase is modeled as an active transport process with saturation. There is an inverse relationship with the cooperator growth rate, as faster growth may reduce the relative efficiency of lipase export due to resource competition.

$$\tau^{Lip} = \frac{k_m \cdot Lip_{Coop}}{k_s + Lip_{Coop}} \cdot \frac{1}{k_\mu + \mu_{Coop}}. \quad (16)$$

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^{-3} g/L throughout the study.

Table 2. Fermentation game parameters, adopted from *C. rugosa* model (Montesinos et al. 1997).

$k_{ss} = 0.01\ g\ g^{-1}$	$k_{so} = 0.153\ g\ g^{-1}\ h^{-1}$	$k_{so1} = 0.135\ g\ L^{-1}$	$k_{1m} = 2.5\ U\ mg^{-1}\ h^{-1}$
$k_{1s} = 0.08\ g\ g^{-1}$	$k_i = 12\ g\ g^{-1}$	$k_s = 0.25\ U\ mg^{-1}$	$k_m = 0.06\ U\ mg^{-1}h^{-1}$

$k_\mu = 0.075\ h^{-1}$	$k_{es} = 10^{-7} g\ g^{-1}$	$\mu_{max} = 0.253\ h^{-1}$	$k_{oh} = 0.5$
$k_o = 25$	$Y_{SS} = 0.70\ g\ g^{-1}$	$Y_{XS} = 0.86\ g\ g^{-1}$	

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_{Cheat}	Specific growth rate of the cheater
k_{so1}	Transport saturation constant for substrate	μ_{max}	Maximum specific growth rate
k_{1m}	Lipase synthesis maximum rate coefficient	c	Lipase production cost parameter
k_{1s}	Lipase synthesis saturation constant	δS	Accounts for substrate consumption
k_i	Lipase synthesis inhibition coefficient	Lip_{Ext}	Extracellular lipase
k_m	Lipase transport rate coefficient	Lip_{Coop}	Intracellular lipase of the Cooperator
k_s	Lipase transport saturation constant	OA_{Cheat}	Intracellular oleic acid of the cheater
k_μ	Growth related transport saturation constant	OA_{Coop}	Intracellular oleic acid of the cooperator
k_{es}	Lipase excretion saturation constant	OA_{Ext}	Extracellular oleic acid
k_{oh}	Olive oil hydrolysis parameter	OO_{Ext}	Extracellular olive oil
k_o	Olive oil saturation constant	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

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

Fermentation game 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 0.15 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.

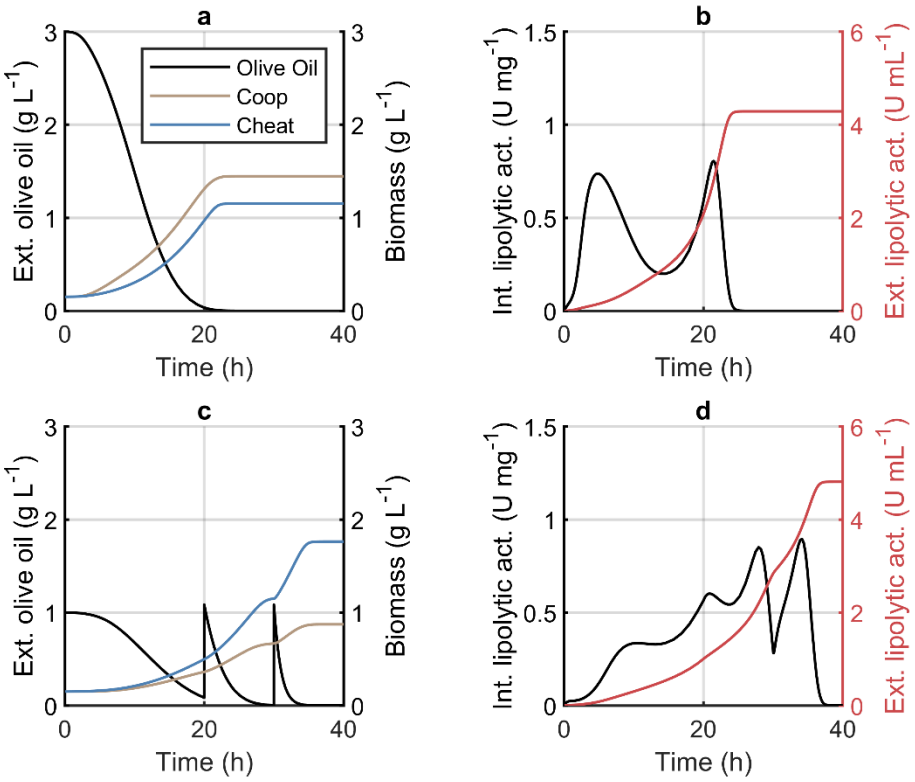

Fig 3. Fermentation game employing Cooperator and Cheater sub-populations for batch **(a-b)** and fed-batch **(c-d)** modes. **a** Batch fermentation starts with 3 g/L olive oil. Starting from initial densities of 0.15 g/L each, Cooperator and Cheater reach to 1.44 g/L and 1.15 g/L respectively. **b** Cooperator produces lipase and excretes to the extracellular space. Extracellular lipolytic activity reaches to 4.28 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 20^{th} and 30^{th} hours. Starting from initial densities of 0.15 g/L each, Cooperator and Cheater reach to 0.87 g/L and 1.76 g/L respectively. **d** Extracellular lipolytic activity reaches to 4.81 U/mL at the end of the fermentation.

The batch simulation (**Fig. 3a-b**) yielded higher Cooperator densities, attributed to their access to abundant olive oil and their ability to hydrolyze it into oleic acid within the periplasmic space. In contrast, the fed-batch simulation (**Fig. 3c-d**) resulted in higher Cheater densities due to a decrease in olive oil concentration and accessibility. The impact of olive oil concentration on evolutionary outcomes is analyzed in the subsequent sections. Although the frequency of non-producer "Cheaters" increased during fed-batch fermentation, extracellular lipolytic activity showed a slight improvement compared to batch fermentation. This improvement is attributed to the optimal substrate feeding concentration, which avoids triggering adverse effects on enzyme production, as outlined in Eq. 12.

3.2 Lipase Production Cost and Game Regimes

In this study, the cost of lipase production c was incorporated to enhance biological realism and better align with the game-theoretical framework. It is reasonable to assume that enzyme production competes with cellular growth, as both processes draw from the shared currency of intracellular substrate. Thus, we introduced this cost parameter to represent the expenditure of intracellular resource required for producing unit of enzyme. The exact value of the cost associated with lipase production remains challenging to estimate. To address this uncertainty, we examined a range of cost parameters and analyzed their impacts at larger scales, including population dynamics, stable population states, and the prevailing game-theoretical regime.

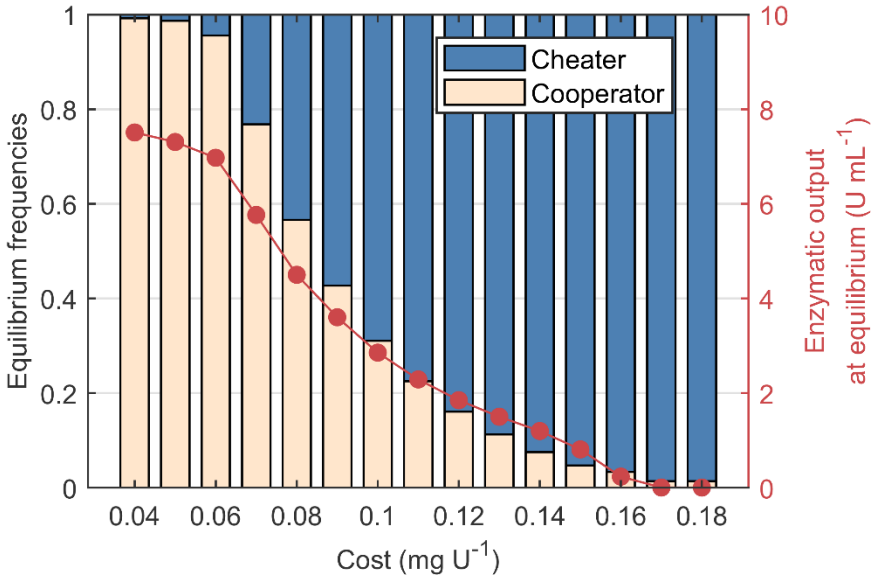

Fig 4. Effects of the lipase production cost parameter **c** on the equilibrium frequencies in batch fermentation settings. Cooperator and Cheater frequencies are shown as stacked bars with cream and blue colors respectively. The red dotted line represents the lipase activity level measured at the end of simulations performed under the corresponding equilibrium frequencies.

In the stable-state finder algorithm, each consecutive batch simulation begins with 3 g/L olive oil and an initial total cell density of 0.4 g/L, running for 40 hours. The final frequencies are shown in **Fig. 4.** The equilibrium frequency of cooperators decreases as the lipase production cost increases. Produced lipase partially offsets its production cost before being released into the extracellular space, leading to a "Snowdrift" scenario where Cooperators and Cheaters coexist. At high production costs (>0.16), the game shifts to a "Prisoner's Dilemma" scenario, where both enzymatic output and cell growth diminish (not shown). Additionally, the "Coordination" game behavior is absent in the fermentation game, as the equilibrium frequencies remained unaffected by the initial population composition (not shown). Based on these findings, subsequent simulations were performed using equal initial cooperator and cheater compositions, using a cost parameter set to a reasonable yet arbitrary value of 0.08.

3.3 Equilibrium Frequencies and Enzymatic Outputs

Effects of the initial total density, initial olive oil and the initial oleic acid on the "Equilibrium frequencies" and the "Enzymatic output" in batch fermentation settings were analyzed with the Stable state finder algorithm.

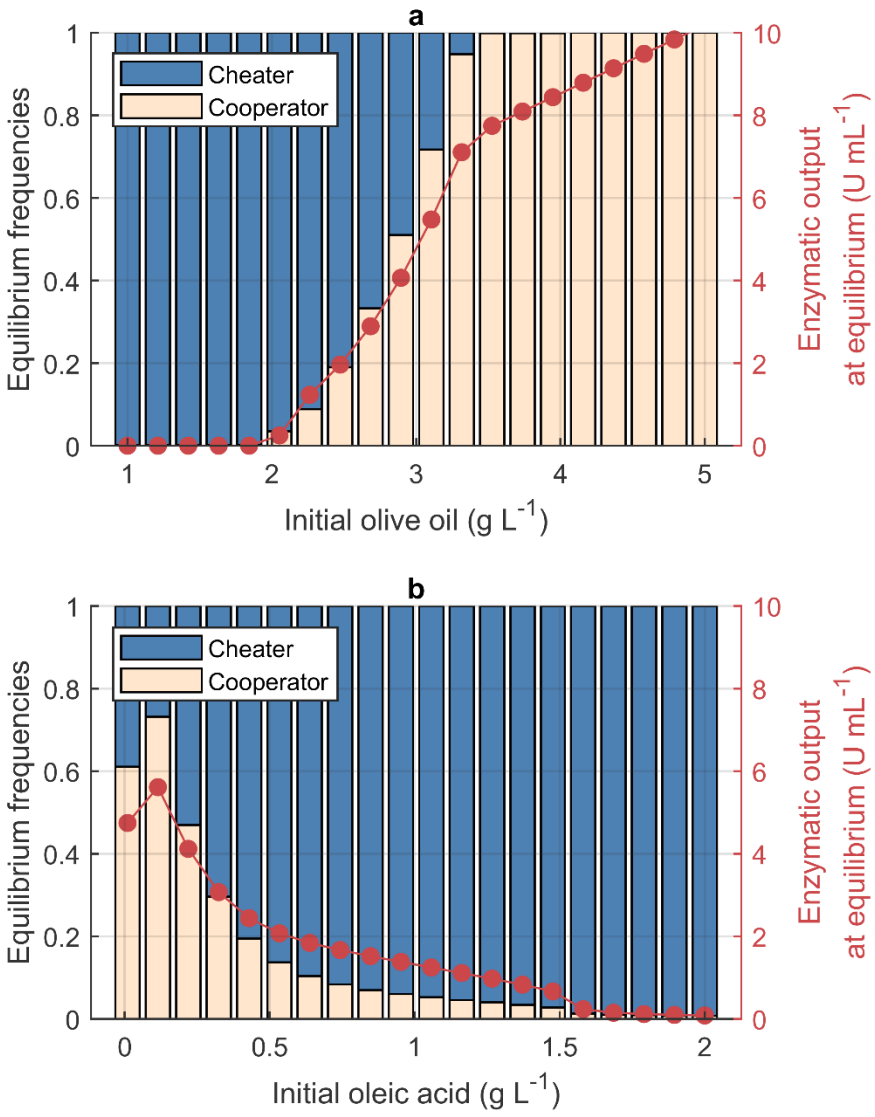

Fig 5 Effects of initial olive oil concentration (**a**) and initial oleic acid concentration (**b**) on equilibrium frequencies and enzymatic outputs. Cooperator and cheater frequencies are shown as cream and blue stacked areas, respectively. The red dotted line (corresponding to the right y-axis) represents the enzymatic output, measured as the final extracellular lipolytic activity at equilibrium.

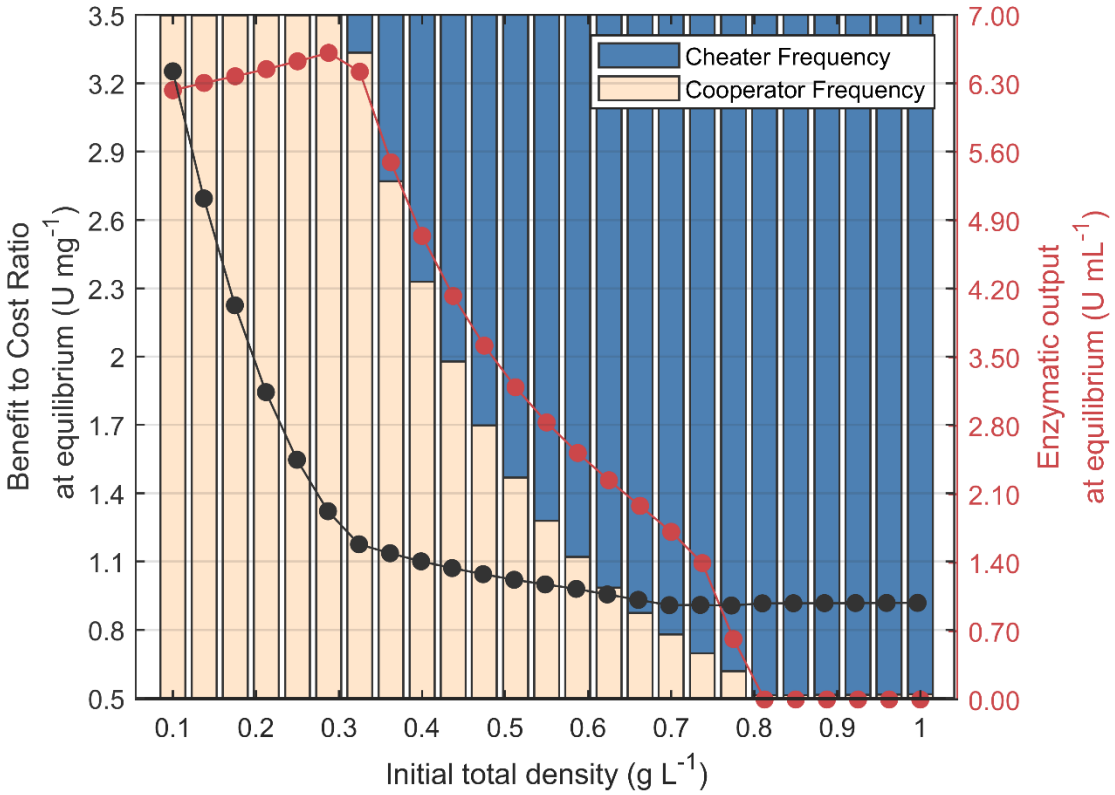

Fig 6 Effects of initial total density on equilibrium frequencies (stacked bars), enzymatic outputs (red dotted line), and the benefit-to-cost ratio of lipase production (black dotted line) during batch fermentation at equilibrium.

In initial total density experiments, each consecutive batch simulation begins with 3 g/L olive oil and runs for 40 hours. At low densities (0.01–0.03 g/L), the production of costly lipase is advantageous, providing returns that exceed its production cost, leading to cooperator dominance in the population. The enzymatic output peaks at approximately 0.3 g/L of initial total density. At medium densities (0.03–1 g/L), cheaters begin to emerge as the benefits of lipase production barely offset its costs, resulting in a decline in enzymatic output. At the highest densities (0.8–1 g/L), producing the enzyme becomes entirely unprofitable, leading to the cessation of cooperation and, consequently, a complete halt in enzyme production.

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 explore the social dynamics between these 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.

A critical aspect of public goods games is that cooperation entails costs. 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 this refinement, Fermentation simulations were conducted for batch and fed-batch operation modes.

Fermentation game involve sub-cultures starting at a concentration of 0.15 g/L each, with a total of 3 g/L olive oil supplied at the beginning of batch fermentation and in three pulses during fed-batch fermentation. Batch fermentations, where the entire substrate is provided upfront, tend to favor the Cooperator sub-culture (**Fig. 3a**). In contrast, fed-batch fermentation significantly benefits Cheaters (**Fig. 3c**). This outcome arises for two main reasons: first, lipase produced by Cooperators is initially localized within their periplasmic space (**Fig. 3c,3d black lines**), where it facilitates the hydrolysis of adjacent olive oil. At later stages, however, lipase is secreted into the extracellular medium (**Fig. 3c,3d red lines**), allowing Cheaters to capitalize on its activity. When olive oil is introduced during these later stages (**Fig. 3c**), extracellular hydrolysis becomes more dominant than periplasmic hydrolysis, providing an advantage to free riders. Second, the fed-batch environment promotes an increase in lipase production due to favorable extracellular oleic acid-to-biomass ratios, as detailed in **Eq. 12**. This dynamic results in a higher overall yield, even in conditions that favor the Cheater sub-culture. These findings challenge the assumption that Cooperators inherently serve as proxies for the common good, emphasizing the importance of explicitly considering the production and transport mechanisms of lipase.

The Stable State Finder algorithm was utilized to explore long term dynamics comprehensively. By initializing frequencies across the full range of possible values, the algorithm ensured coverage of all attractors, providing insights into scenarios where one strategy dominates, both coexist, or coordination dynamics emerge. Applied to the Fermentation game, the algorithm identified evolutionary stable states, linking ecological growth dynamics with evolutionary game theory. This integration offers a nuanced understanding of how ecological factors influence the stability and evolution of strategies, with implications for biotechnological optimization.

For instance, the algorithm revealed that higher lipase production costs reduce the equilibrium frequency of Cooperators, shifting the system toward "*Snowdrift*" dynamics **(Fig 4)**. As the lipase production cost increases, the Fermentation game does transition into a "*Prisoner's dilemma*" as production of highly costly lipase hardly returns the investment. Since estimating the exact cost of production provides difficulty, we set cost to a reasonable yet arbitrary value of 0.08. Furthermore, the system exhibited equilibrium dynamics that were insensitive to initial conditions, as both Cooperator and Cheater sub-populations converged to the same equilibrium regardless of their initial frequencies. Consequently, for subsequent experiments, Cooperator and Cheater sub-populations were initiated with equal initial frequencies.

It is important to estimate optimal feeds and density of inoculum in a biotechnological setup for economic purposes. Here the fermentation game coupled with stable state finder provides important insights. As the olive oil density increases, which serves as the substrate for lipase hydrolysis, Cooperators gain an advantage, and the enzymatic output increases (Fig. 5a). When the olive oil density exceeds 3.4 g/L, the Cooperator subtype

dominates the population. This is because the concentration of olive oil near the cell becomes sufficiently high, making periplasmic lipase hydrolysis highly profitable, as indicated by **Eq. 15**. Inversely, low olive oil concentration (< 2 g/L) are inappropriate for the production of the public good. The product of hydrolysis, oleic acid, is necessary to introduce in small amounts to initiate cell growth and lipase production. However, when the optimal amount is exceeded, it becomes highly detrimental (**Fig. 5b**). As described by **Eq. 12**, lipase production is modulated by the concentration of oleic acid near the cell. The oleic acid-to-biomass ratio must be optimized, as excessive oleic acid has inhibitory effects on lipase production. The beneficial initial concentration is estimated to be around 0.1 g/L, as shown in **Fig. 5b**. Beyond this threshold, Cheater sub-types increasingly benefit, as they no longer depend on Cooperators and their enzymatic products.

The initial total density and its effects on cooperative dynamics are well-established in the literature. While previous studies often rely on readers' intuition to grasp the adverse impact of high density on cooperation, our work offers a mechanistic understanding of how cell density influences cooperative behavior. By delving into the interplay between enzymatic production, resource availability, and sub-population dynamics, we elucidate the precise conditions under which cooperation thrives or deteriorates, bridging gaps in prior research and providing actionable insights for both theoretical and practical applications. At low initial total densities (0.1–0.3 g/L), Cooperators dominate the population as the benefit of producing the enzyme significantly outweighs its production cost, primarily due to the high relative availability of olive oil (**Eq. 15**). Enzymatic output peaks around 0.3 g/L, where Cooperators still maintain dominance, though the relative benefit of enzyme production diminishes compared to lower densities. At medium initial densities (0.3–0.8 g/L), the benefit of lipase production barely exceeds its cost, allowing a Cheater sub-culture to emerge and causing a corresponding decline in enzymatic output. At high densities (above 0.8 g/L), enzyme production results in a net loss for Cooperators, driving a population shift toward Cheater dominance. For biotechnological purposes, an initial total density of 0.3 g/L emerges as an optimal point. At this density, the final extracellular lipolytic activity reaches 6.48 U/mL.

Finally, several assumptions underpin this study. Oleic acid was assumed to be the sole inducer of lipase production, although extracellular triacylglycerols can also induce the LIP gene, a factor not incorporated here. Additionally, the fermentation model assumes only parent-to-offspring (vertical) gene transfer, meaning that changes in phenotypic frequencies are driven solely by differences in growth and death rates. Despite excluding horizontal gene transfer between organisms, the model demonstrates that a fermentation setup can still reach an evolutionarily stable state based on initial conditions and the costs of lipase production within typical fermentation time frames. This finding is consistent with experimental results observed in well-mixed *S. cerevisiae* cultures (Gore et al. 2009), highlighting the critical role of evolutionary pressures in shaping metabolic strategies and enzymatic activities. Such considerations are essential for optimizing biotechnological processes to achieve desired outcomes.

5 Conclusion

This study analyzed the evolutionary dynamics and enzymatic productivity of microbial populations using the Fermentation game, a game-theoretical model incorporating structured kinetic principles. By integrating ecological growth factors and evolutionary game theory, we explored the interplay between lipase-producing Cooperators and non-producing Cheaters in batch and fed-batch fermentation setups.

Key findings underscore that fermentation dynamics are sensitive to substrate availability and initial population densities. Batch fermentations favor Cooperators due to localized periplasmic hydrolysis, while fed-batch fermentation benefits Cheaters by increasing extracellular hydrolysis opportunities. The Stable State Finder algorithm further reveals that high lipase production costs and suboptimal olive oil concentrations shift the system towards Cheater dominance or sub-optimal enzymatic output.

For biotechnological applications, optimizing inoculum density and substrate concentrations is critical. An initial density of 0.3 g/L, olive oil concentration of 3.4g/L and an oleic acid concentration of 0.1 g/L maximize enzymatic activity while maintaining cooperative stability. Additionally, while oleic acid was assumed to be the sole inducer of lipase production, the potential role of extracellular triacylglycerols warrants further exploration to refine predictions.

Our findings bridge theoretical and practical considerations, offering actionable insights into the evolutionary pressures that shape metabolic strategies. This approach enables the optimization of cooperative processes in biotechnological systems, paving the way for more efficient and sustainable applications.

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.

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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).

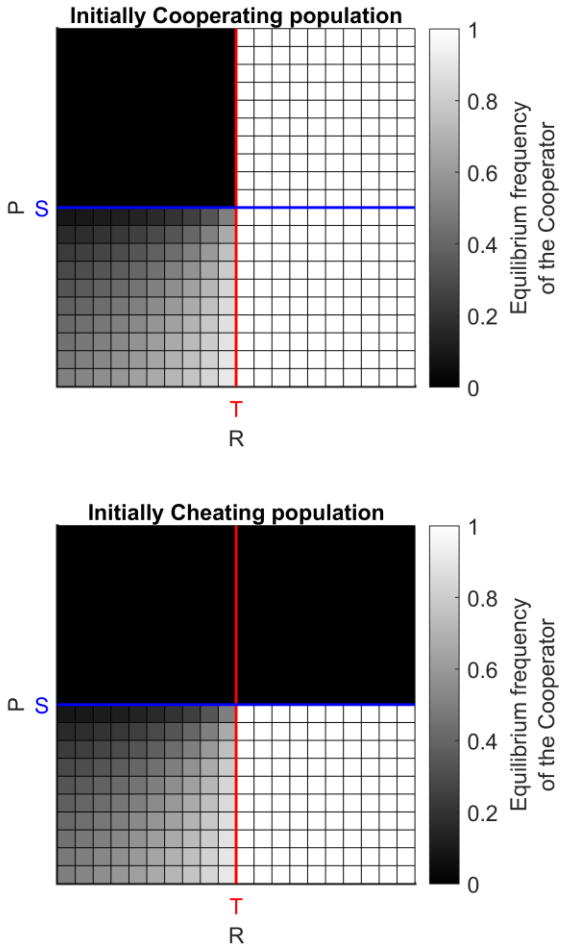
Snowdrift: If T > R > S > P, "mutual Cooperation" is better than "Cooperating while the other player Cheats", but "Cheating while the other player cooperates" is better than "mutual Cooperation", and "mutual Cheating" is the worst possible outcome. This is an anti-coordination game, with two asymmetric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

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.