A Unique Bacterial Pelletized Cultivation Platform in *Rhodococcus opacus* PD630 Enhanced Lipid Productivity and Simplified Harvest for Lignin Bioconversion

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ABSTRACT

Pelletized liquid cultivation has been widely explored due to its advantages in biomanufacturing such as easier biomass harvesting, higher product yield, lower medium viscosity and energy consumption. In this study, we discovered that a non-filamentous bacterium *R. opacus* PD630 could form pellets during the fermentation of alkaline pretreatment liquor (APL) containing lignin as carbon source. This discovery advanced our understanding of the bacterium pelletization, considering that only filamentous fungi and filamentous bacteria were reported to form the pellets without addition of external agents such as flocculants or polymers in the previous research. Several factors were investigated to understand how they affect the process of pelletization. Notably, the lipid content in the pellets was much higher than in the scattered bacteria at low nitrogen concentration (<0.5g/L), under which condition (high carbon to nitrogen ratio) the industrial microbial production for lipids was carried out. Moreover, the highest pellet percentage (~60% of the total biomass) was observed at 30 g/L soluble solid content (SSC), 180 rpm, 1.4 g/L $(NH_4)_2SO_4$, 10 initial OD_{600} (optical density) and 6000 rpm of centrifugation speed. The study also open new avenues to decrease harvesting and cultivation cost as well as energy consumption for microbial fermentation.

KEYWORDS

Lignin bioconversion, lipid production, pellet formation, biomass harvesting, *R. opacus*

INTRODUCTION

Microbial growth in the form of pellets has long been sought after in industrial biotechnology [1-6]. The first works on pellet formation were reported in the late of the 19th and beginning of the 20th centuries, when authors found the formation of spherical colonies or mycelial globules in different species of the filamentous fungi *Aspergillus sp.*[1]. Modern pelletized cultivation was advanced as a major platform for bioproduction and biomanufacturing, where the pellet size was controlled to be at less than 5 micrometer in scale. Such pelletized liquid cultivation possesses several advantages over single-cell suspended growth such as higher biomass density, simplified cell harvesting, lower medium viscosity, reduced energy consumption, improved mass transfer, and higher yield of products such as fatty acids, citric acid and enzymes [2-4, 6-7]. Pelletized fungus have also been exploited to harvest single cell oil (SCO) algae from liquid cultivation, which synergized higher lipid yield[8]. Despite the progress, most of the current success in pelletized cultivation was achieved in filamentous fungus. Recent works have evaluated the formation of pellets from non-filamentous microorganisms by adding external agents like aluminum sulfate and alginates to enhance cell harvest [6]. Nevertheless, the pelletized cultivation without flocculants remains highly challenging. Even though granule cultivation has been achieved, these granules are generally much larger and less uniform, which is significantly different in their morphology and impact on cultivation. Overall, the advancement of novel pelletized cultivation strategy for bacteria could open new avenues to reduce the energy consumption, improve product yield, simplify harvest, and enhance both cost-effectiveness and sustainability of bioconversion. We hereby report establishing a new pelletized cultivation platform for bacterial conversion of lignin.

Rhodococcus opacus is a non-filamentous bacterium that can accumulate up to 70% of triacylglycerol (TAG) lipids of its dry weight under conditions of carbon excess and nitrogen depletion [9-10]. Importantly, this microorganism has evolved efficient catabolic pathways, such as the β-ketoadipate pathway, that enable the degradation and bioconversion of lignin into lipids [11-12]. Such characteristics make *R. opacus* a great candidate for producing lipid, and ultimately, biodiesel, from lignin. Lignin-based fuel represents an environment-friendly alternative for petroleum-based fuels, considering that lignin is the second most abundant biopolymer on earth after cellulose and constitutes 15-35% of the dry lignocellulosic biomass. Moreover, the conversion of lignin into fungible fuels and chemicals will increase the carbon efficiency and overall sustainability of lignocellulosic biorefinery. Recently, we reported the enhancement of lipid production using *R. opacus* PD630 and lignin from corn stover as substrate through systems biology-guided design of more efficient laccase secretion, fatty acid and triglyceride biosynthesis[13]. Further improvement was achieved by novel combinatorial pretreatment to improve lignin fractionation and fermentation optimization along with carbohydrate conversion into biofuels [14]. Despite the potential of *R. opacus* PD630 to produce lipids as biofuels precursors, the economic viability of the process depends significantly on lipid yield, energy consumption, and harvest cost. Pelletized cultivation has been widely used in fungal bioproduction to improve the yield and efficiency [2-5, 7, 15-18]. Pelletization was also achieved in some filamentous bacteria [19-21]. However, to the best of our knowledge there is no evidence on the current literature of pellet formation from non-filamentous bacteria without the addition of flocculants, polymers or by co-cultivation with filamentous microorganisms [16-17]. If achievable, pelletized cultivation will open new avenues for bacterial bioproduction to improve yield and conversion efficiency.

In this study, we demonstrate a novel system to achieve pelletized cultivation without the addition of external coagulant agents by using the unique characteristics of *R. opacus* PD630 and lignin as substrate. Pellet formation and lipid production were evaluated at different culture conditions by using lignin from corn stover as substrate. The factors studied were the soluble solid content (SSC), agitation, nitrogen content, inoculum size and centrifugation speed to harvest the initial inoculum (Table 1). The pellet structure was analyzed by scanning electron microscopy (SEM) and the possible mechanisms of the pellet formation were discussed with Nuclear Magnetic Resonance (NMR) analyses.

Table 1. Evaluated conditions of lipid fermentation for pellet formation.

Evaluated effect	**Culture conditions**					
	Substrate	SSC (g/L)	Shaking speed (rpm)	Nitrogen concentration, NH_4NO_3 (g/L)	Initial OD_{600}	Centrifugation speed (rpm)
Substrate	APL, glucose	30	180	1.4	5	4000
SSC	APL	10, 30, 50	180	1.4	5	4000
Shaking speed	APL	30	180, 250	1.4	5	4000
Nitrogen concentration	APL	30	180	0, 0.2, 0.5, 1, 1.4, 2	5	4000
Initial OD_{600}	APL	30	180	1.4	5, 10, 15, 20	4000
Centrifugation speed	APL	30	180	1.4	10	4000, 6000, 8000

APL: Alkaline pretreatment liquor

RESULTS AND DISSCUSION

Lignin induces pellet formation

The major constituents of the corn stover used in this study were glucan (33.1%), lignin (22%) and xylan (17.5%)[22]. Although lignin is the second most abundant polymer after glucan, its valorization is typically hindered by the high molecular weight and chemical composition consisting of phenyl propanol units[23-24]. As reported in our previous work, after alkaline treatment with 1% sodium hydroxide, corn stover lignin is mainly composed of guaiacyl and syringyl units, and fractionated to approximately one-third of the original molecular weight in untreated corn stover [22]. This lignin stream has demonstrated to be a potential carbon source for lipid production in *R. opacus* PD630 aiming biofuel production [14]. One of the interesting phenomena we observed during lipid fermentation is that the *R. opacus* PD630 cells can form pellets as filamentous microorganisms[8].

Fig. 1 shows that *R. opacus* PD630 grew exclusively as suspended biomass when glucose was used as the only carbon source, while with other components of the hydrolysis of corn stover such as xylose, *R. opacus* PD630 did not grow. However, when alkaline pretreated liquor (APL) was used as substrate, 30% of the biomass grew in the form of pellets. To the best of our knowledge, this is the first work reporting the occurrence of pellet formation in non-filamentous microorganisms without the addition of external agents (see photographic evidence in Fig. S1). We demonstrate that besides inducing pellet formation, lignin in APL is consumed by *R. opacus* PD630 and used as carbon source. In average, the lignin removal during the present study was 58%, which is similar to the values reported elsewhere [14].

Recently, Liu et al (2019) demonstrated that lignin consumption, biomass generation and product yield can be improved by cooperative valorization of lignin and limited sugar concentrations [25]. In such study, the production of polyhydroxyalkanoates (PHA) from *P. putida* was increased by integrating lignin and residual sugars from corn stover under a biorefinery scheme. Therefore, during the present study we evaluated the effect of glucose addition in APL on biomass generation, lipid production and pellet formation (Fig. S2). The results were similar to those reported by Liu et al (2019) [25]. Nonetheless, pellet formation only increased when glucose was added at 2 g/L, while no significant differences were found at higher glucose concentrations. Furthermore, at glucose concentrations higher than 2 g/L, the size distribution of pellets was more variable and the morphology changed from compact to loose pellets (Fig. S2). Therefore, for the following experiments we decided to use APL as the only carbon source.

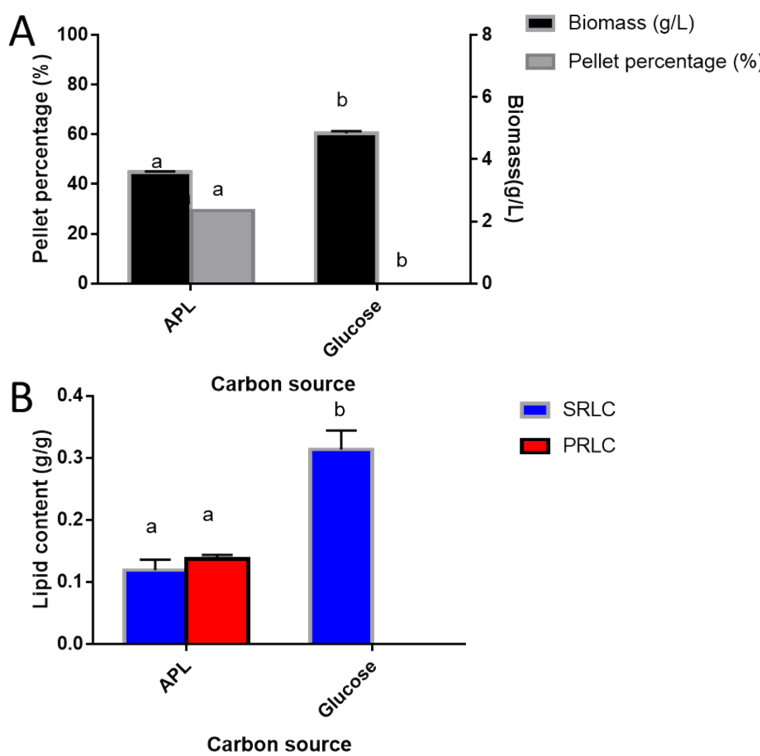

Fig. 1 Effect of the type of substrate on lipid fermentation by *R. opacus* PD630. (A) Effect on biomass growth and pellet formation. (B) Effect on lipid content in suspended biomass and pellets. SRLC: Suspended *R. opacus* PD630 lipid content, PRLC: Pellet *R. opacus* PD630 lipid content.

High Soluble solid content (SSC) increase biomass production and pellet formation

Lignin concentration is directly correlated to SSC in APL. Lignin is the main component of APL (34%) followed by sugars (8% glucose and 7% xylose) and other components such as extractives, ash, salts, proteins, etc. The SSC concentration in APL was around 60 g/L, but it varies from batch to batch. Therefore, 50 g/L SSC was evaluated as the highest concentration during lipid fermentation. Theoretically, high SSC should promote high metabolic fluxes and increase biomass production and desired product. However, the concentration of degradation products of lignocellulosic pretreatment such as weak acids, furans, and other derivatives also increase at high SSC and might have inhibitory effects on *R. opacus* [14, 25]. Therefore, low (10 g/L), moderate (30 g/L) and high (50 g/L) SSC were evaluated in this study. Fig. 2 shows that biomass increased from 2.9 g/L at 10 g/L SSC to 4.9 g/L at 50 g/L SSC. Interestingly, pellets were attained at the end of the fermentation for all of the tested conditions. Pictures and size distributions can be found in Fig. S3. The pellet percentage in the recovered biomass was also significant for all of the SSC tested ($P < 0.05$). Pellet percentages increased from 24% at 10g/L SSC to 42% at 50g SSC/L, suggesting that pellet formation is correlated to lignin concentration. In this context, Guiot *et al* (1992) reported that higher concentrations of lignin from pulping spent liquor promoted microbial aggregation in the form of large size granules (not in pelletized cultivation) during wastewater treatment[26]. The authors found that lignin did not affect the cell surface properties, so they suggested that granules were formed by entrapping and forming clusters due to the branched structure of lignin [26]. Nonetheless, it is also widely reported that microbial cells have negative charges as a consequence of the carboxylic (–COOH) group. Therefore, strong positive-charged polymers are suggested for chemical polymeric coagulation [27]. Considering that APL is constituted of many organic polymers such as lignin, sugars, proteins, etc. [14, 28], as well as other charged ions and molecules such as Na^{+} and CH_3COO^{-} [14], pellet formation could be the result of both electrostatic force and entrapping by the branched structure and high polymerization degree of lignin.

It is widely reported that lipid accumulation in oleaginous microorganisms depends of high C/N ratios[29-31]. Therefore, higher lipid contents in biomass were expected when higher SSC was applied. Fig. 2 shows that in the present study no significant difference was found in the lipid content of the pellets and suspended biomass at different SSC. The probable reason for this behavior is that at higher SSC not only the lignin content increases but also the concentration of lignocellulosic degradation products that can affect the microbial metabolism. These results are in agreement with our previous study in which the lipid content did not increase at SSC>15 g/L [14].

Thus, even though the highest SSC of 50 g/L attained the highest biomass production and pellet percentage in biomass (Fig. 2), 30 g SSC/L was selected for the following experiments to maintain moderate concentrations of lignocellulosic degradation products that might affect the microbial metabolism and lipid production (Table 1).

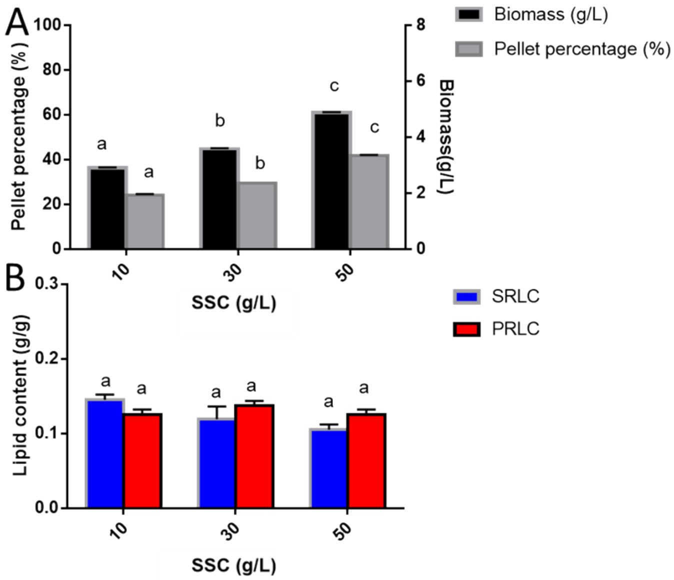

Fig. 2 Effect of SSC on lipid fermentation by using APL as substrate. (A) Effect on biomass growth and pellet formation. (B) Effect on lipid content in suspended biomass and pellets. SRLC: Suspended *R. opacus* PD630 lipid content, PRLC: Pellet *R. opacus* PD630 lipid content.

Effect of agitation speed on pellet formation

Mass transfer and homogeneity of the culture mainly depend on the agitation speed. At high agitation speeds both parameters are enhanced. However, the share forces and particle collision that affect pellet formation/morphology can be also increased at high agitation speeds[4]. In the present study, the increase of the shaking speed in fermentation was evaluated from 180 to 250 rpm. Fig. S4 and Fig. 3 show that at 250 rpm no pellet formation was attained although biomass and lipids were produced. Similarly, Roa *et al* (2011) found that the pellet size of *R. orzyae* decreased from 2.5 mm to 0.9 mm when the shaking speed was increased from 150 to 225 rpm[4]. They also suggested by using flow regimes studies to maintain the shaking speed above 150 rpm in order to have good shaking conditions that allow high mass transfer and homogeneity of the culture [4]. Therefore, for the next experiments the shaking speed of 180 rpm was selected, since it allows good mass transfer, homogeneous conditions and pellet formation.

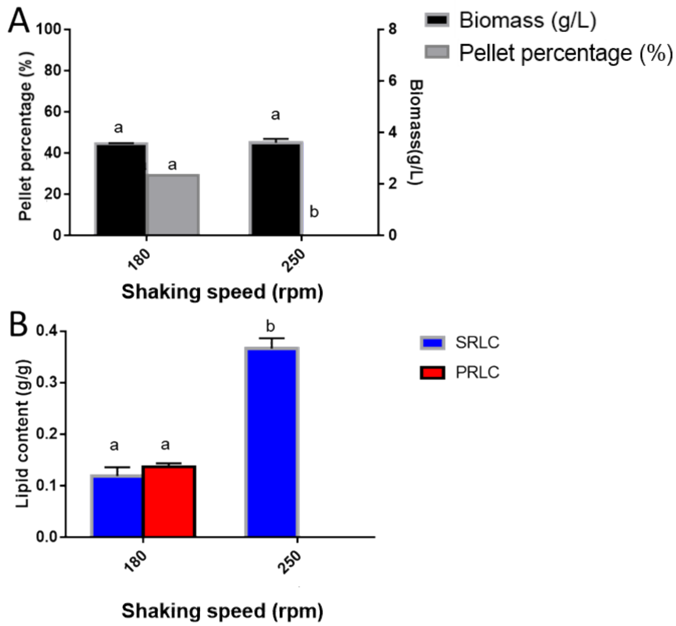

Fig. 3 Effect of speed agitation on lipid fermentation by using APL as substrate. (A) Effect on biomass growth and pellet formation. (B) Effect on lipid content in suspended biomass and pellets. SRLC: Suspended *R. opacus* PD630 lipid content, PRLC: Pellet *R. opacus* PD630 lipid content.

Pelletized cultivation improves lipid yield at low nitrogen condition

Previous research have reported that the oleaginous potential of microorganisms is influenced by the nitrogen concentration [32-33]. It is widely accepted that nutrient deficiency (low nitrogen or phosphate conditions) can induce lipid accumulation[34-35]. Therefore, it is critical to compare the lipid production for pelletized and un-pelletized cells under low nitrogen condition to evaluate the real potential for palletization to promote lipid accumulation. Our previous work with *R. opacus* PD630 on APL substrate at different culture conditions (15 g SSC/L, OD_{600}=1.0, and 200 rpm) demonstrated that nitrogen concentration plays a key role in the cell growth and lipid accumulation

[14]. According to Kosa and Ragauskas (2012), this strain and other *R. opacus* strains can effectively convert lignin model compounds into lipids at C/N molar ratios of 5:1 to 10:1 [36]. In the present study by using APL as substrate, biomass production and pellet percentage in biomass presented no significant difference ($P > 0.05$) at different concentrations of nitrogen source (Fig. 4). Fig. S5 shows that pellets with very uniform size distribution were formed at all the evaluated conditions. In contrast, as shown in Fig. 4, lipid production in both pellets and suspended biomass presented significant differences at evaluated conditions ($P < 0.05$). Lipid content in pellets increased from 0.196 g/g biomass to 0.254g/g biomass when the nitrogen source was increased from 0 to 0.5 g/L but then decreased to 0.13 g/g biomass for higher concentrations of nitrogen source. Similarly, Liu et al (2018) reported lower lipid content can be obtained at high nitrogen concentrations [14]. The latter results suggested that low nitrogen concentrations can enhance the lipid content in *R. opacus* PD630, which is helpful for improving the lipid extraction efficiency. Our observations are in agreement to previous studies that reported that lipid accumulation in oleaginous microorganisms depends of high C/N molar ratios [32-33]. More importantly, lipid production for pelletized cells are significantly higher than those of un-pelletized cells at 0, 0.2, and 0.5g/L nitrogen (Fig. 4). In particular, at 0.5g/L nitrogen concentration, the lipid production in pelletized cells was about 1.6-fold higher than those of the un-pelletized cells. The magnitude of this increase is even bigger than previously established algae-fungus co-cultivation system[8]. It is intriguing to observe that the increase in lipid production by pelletization diminished at nitrogen conditions higher than 1 g/L. This is understandable since higher nitrogen concentration might have altered the cellular metabolism to prevent the lipid accumulation. The low nitrogen conditions reflect the commercially relevant conditions for lipid production.

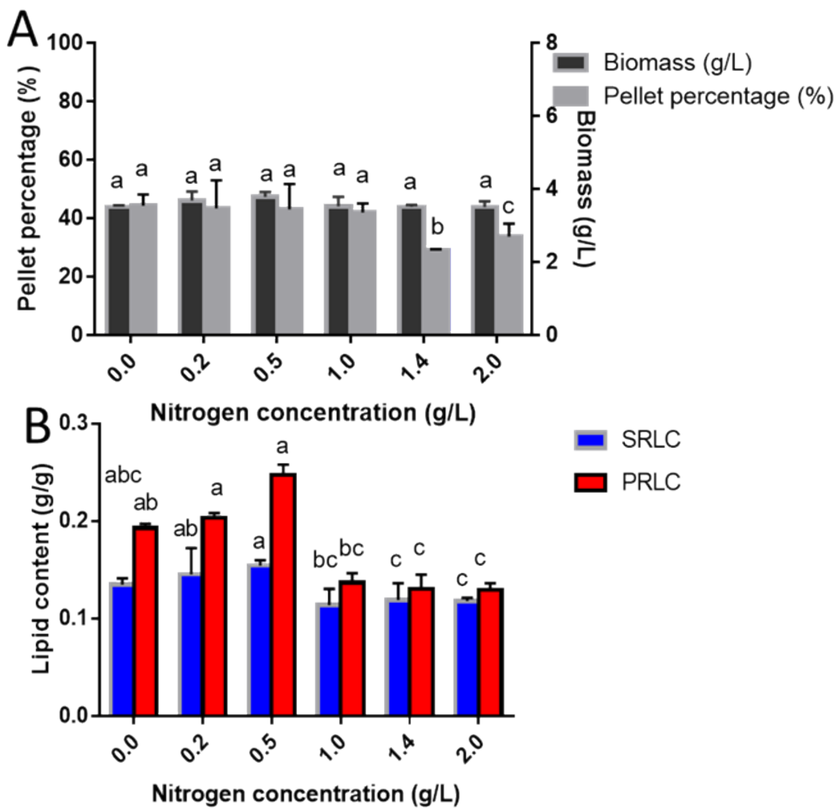

Fig. 4 Effect of nitrogen concentration on lipid fermentation by using APL as substrate. (A) Effect on biomass growth and pellet formation. (B) Effect on lipid content in suspended biomass and pellets. SRLC: Suspended *R. opacus* PD630 lipid content, PRLC: Pellet *R. opacus* PD630 lipid content.

The initial OD_{600} influences pellet formation and lipid production

The initial OD_{600} influences the inoculum to substrate ratio and is one of the most important factors in fermentation systems [14]. Theoretically, at high inoculum to substrate ratios, the productivity should increase, as the toxic effects of some APL compounds such as furfural and weak acids would be minimized. Fig. 5 shows that the cell biomass at the end of fermentation increased from 3.6 g/L to 6.7 g/L when the initial OD_{600} was increased from 5 to 20. It has been widely reported that alkaline pretreatment of biomass such as NaOH also leads to some degree of degradation of polysaccharides and further formation of weak acids and furans [37]. These compounds alongside the phenolic compounds from the degradation of lignin may exert some degree of inhibition over R. *opacus* PD630. Such effect was probably minimized by increasing the inoculum to substrate ratio. Interestingly, the pellet formation also increased from 24% to 45% when the OD_{600} was increased from 5 to 10. However, no significant difference was found when the OD_{600} was further increased to 15 and 20. We hypothesize that at low OD_{600} values longer fermentation times are needed to grow enough R. *opacus* PD630 and aggregate in the form of pellets. However, at high OD_{600} values, more substrate (lignin) is probably needed to increase the pellet formation. Fig. S6 also shows that the size and form of the pellets are highly variable at high OD_{600} values, 15 and 20.

Regarding the lipid content in biomass, it increased almost linearly for both pellets and suspended biomass from 0.13 to 0.18 g/g biomass when the OD_{600} was increased from 5 to 20. As an exception, the lipid content in pellets reached 0.21 g/g biomass at 10 OD_{600}. Overall, these results are in accordance to our previous report where biomass growth and lipid content were enhanced at high OD_{600} values [14]. This previous study demonstrated that lignin consumption is also improved at high OD_{600} values, which is expected since more biomass is produced and therefore more APL is degraded.

As the main focus of the present study is the pellet formation, we decided to use an OD_{600} of 10 for the last experiment to study the effect of the centrifugation speed. Centrifugation is commonly used to harvest planktonic bacteria and it mainly involves compacting bacteria by centrifugal force. This forced compaction causes collisions of bacteria against each other that result in shear forces. These shear forces may lead to cell surface damage with a potential effect on the outcome of surface-sensitive experiments, such as microbial biofilm or pellet formations. Previous reports have found that centrifugation was related with the viability, surface adhesion and change of the cell surface charge of the bacteria [38-39].

In the present study, we centrifuged the pre-culture to harvest *R. opacus* PD630 and then inoculated the different experiments. The bacterial collisions caused during *R. opacus* harvesting and the presumptive cell surface damage was hypothesized to be a factor that potentially influence the pellet formation. However, Fig. 6 shows that even though a slightly higher pellet percentage was achieved at centrifugation speeds of 6000 and 8000 rpm compared to 4000 rpm, such difference was not statistically significant. Moreover, as expected no difference was found for biomass growth and lipid content in biomass. The pellet distribution and photographic evidence can be observed in Fig. S7.

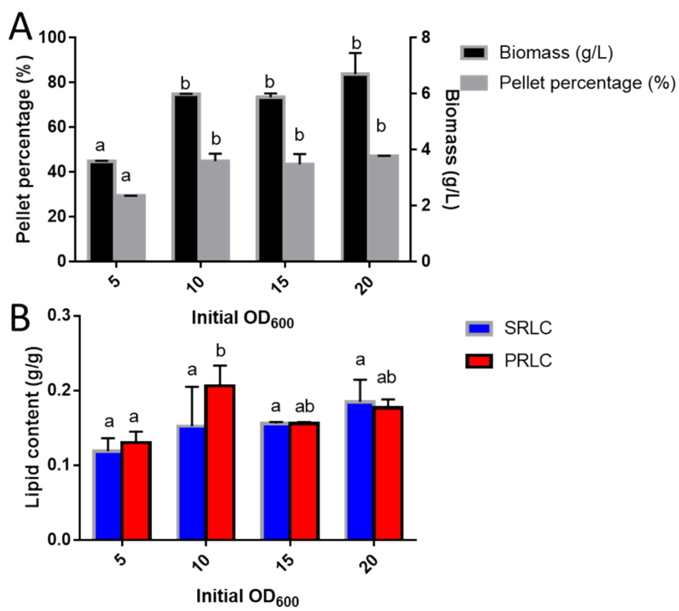

Fig. 5 Effect of initial OD_{600} on lipid fermentation by using APL as substrate. (A) Effect on biomass growth and pellet formation. (B) Effect on lipid content in suspended biomass and pellets. SRLC: Suspended *R. opacus* PD630 lipid content, PRLC: Pellet *R. opacus* PD630 lipid content.

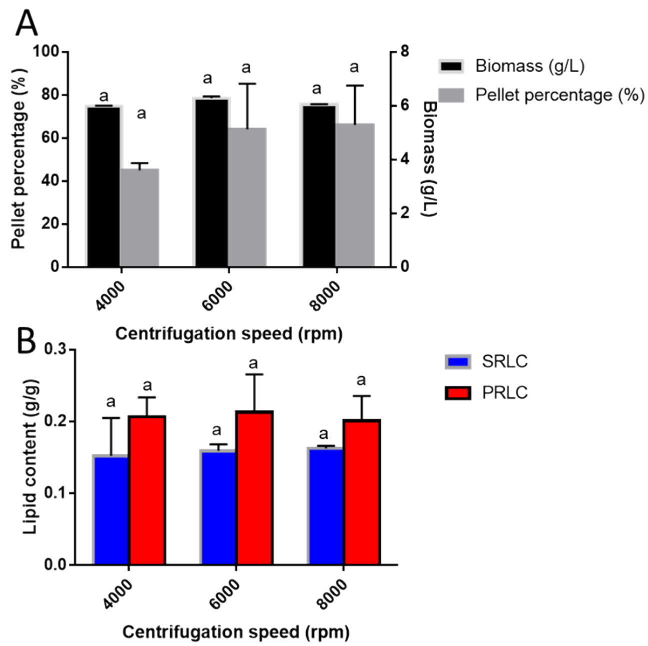

Fig. 6 Effect of centrifugation speed to obtain the inoculum on lipid fermentation by using APL as substrate. (A) Effect on recovered pellets and biomass. (B) Effect on lipid content in suspended biomass and pellets. SRLC: Suspended *R. opacus* PD630 lipid content, PRLC: Pellet *R. opacus* PD630 lipid content.

Pellet structure and possible scenarios of formation

To the best of our knowledge, pelletized cultivation is reported for the very first time in non-filamentous organisms without the addition of external coagulant agents. Traditionally, aluminum salts and organic polymers were added to promote the microorganism pelletization. However, the high cost and difficulties in subsequent removal have prevented the broad application of these technologies. The pelletization platform in this study thus has unique advantages. We attributed this phenomena to the unique structure of lignin which could not only serve as the carbon source of *R.opacus* but also interact with the surface of cell wall and exopolysaccharides, probably contributing to pelletization. Fig. 7 shows SEM micrographs of the pellets obtained at the best culture conditions. The diameter of the pellets was around 0.8 mm, while the structure seems to be compact and constituted of *R. opacus* embedded in another substance. In this context, fractionated lignin from alkaline or Kraft treatments have been reported to induce the formation of lignin-based polymers and coagulants[24]. Nonetheless, *R. opacus* has also been reported to produce exopolymers and to possess a negatively charged cell surface with high hydrophobicity [40]. These characteristics are widely recognized as adjuvants for the initial aggregation of non-filamentous microorganisms. On one hand, the hydrophobic interaction and local dehydration model proposes that irreversible aggregation could occur, when microbial surfaces are hydrophobic. On the other hand, the extracellular polymer bonding model suggests that exopolysaccharides bridge and embed cells to start granule formation [41-42]. In this sense, we extracted exopolysaccharides from *R.opacus* grown on glucose and APL stream and characterized them by using 2D HSQC NMR. As shown in the Fig. S8 and Table S1, some peaks of alginate and exopolysaccharides were clearly determined for the bacteria incubated in different fermentation conditions, indicating the existence of these exocellular polymers. When lignin was used as the substrate, these exopolysaccharides were also found (Fig. S8B), however, lignin polymer might not exist along with the exopolysaccharides, because peaks of lignin linkages were not found in the HSQC spectrum, especially the most abundant β-*O*-4 links (Fig. S8A). Alternatively, some aromatic compounds with significant methoxyl groups on the aromatic rings have been characterized. These data suggested that lignin-derived aromatic monomers instead of lignin polymer might exist in the pellets. These lignin-derived monomers were most likely guaiacol, creosol, and vanillin as assigned in the Fig. S8B, which could derive from lignin depolymerization during the alkaline pretreatment of the corn stover. Overall, these and other hypothesis such as the multilayer, inert nuclei or quorum sensing have been proposed to explain the aggregation of non-filamentous microorganisms in wastewater treatment systems [43-44]. However, the mechanisms for these and other theories are yet to be fully elucidated, especially in single-bacterial strain pelletization.

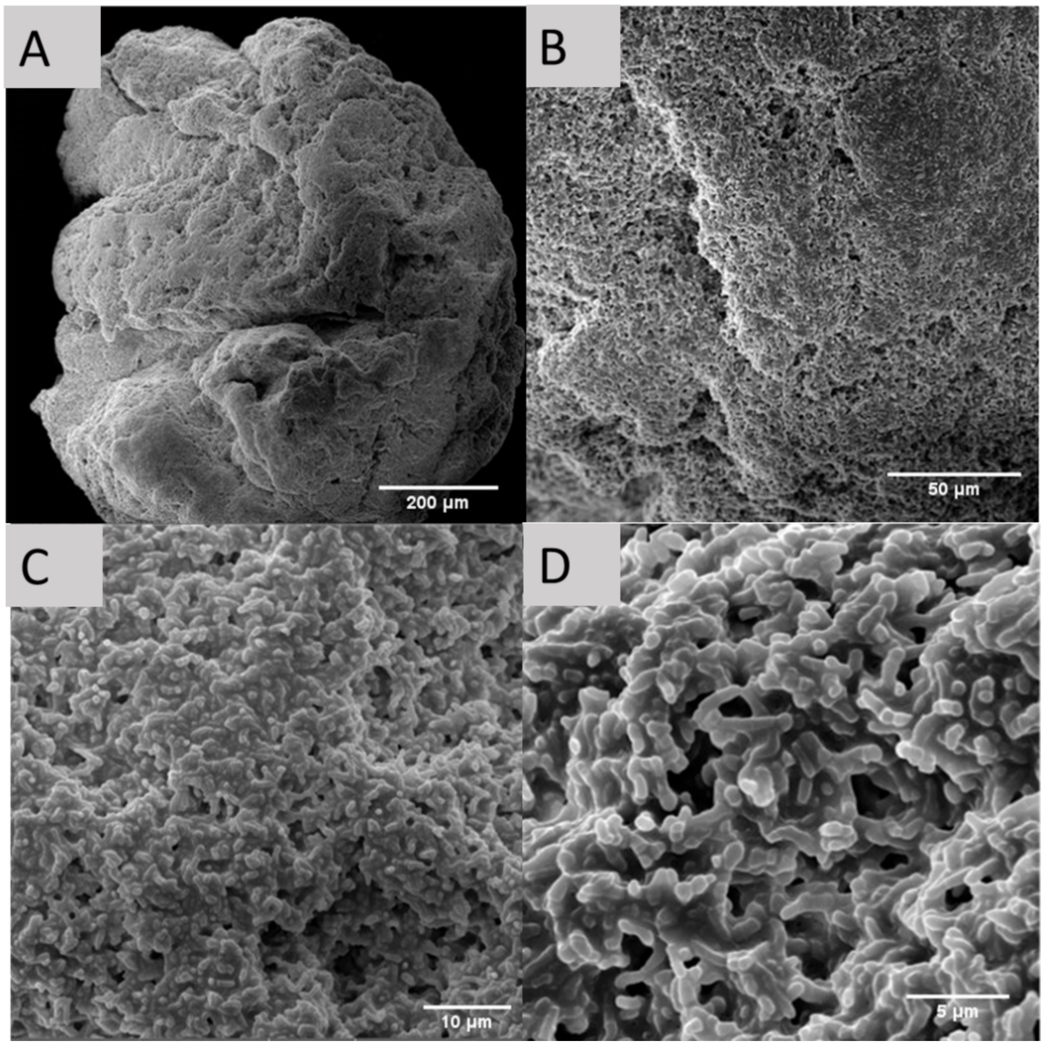

Fig. 7 Scanning electron micrographs for pellets obtained at the best conditions: 30 g/L SSC, 180 rpm, 1.4 g/L NH_4NO_3, 10 initial OD_{600} and 6000 rpm of centrifugation speed. A), B), C) and D) pictures of the pellets obtained at different magnification.

Discussion

Overall, a novel system to form pellets from non-filamentous bacteria (*R. opacus* PD630) has been developed by using lignin as substrate. As compared to previous studies in filamentous species (Table 2), the study revealed potential new mechanisms for bacterial pelletized cultivation. We propose that the properties of *R. opacus* such as its high hydrophobicity, negative charge and potential excretion of exopolysaccharides could have initiated the formation of small bacterial clusters that later interacted with lignin to form compact pellets. Lignin or its derived molecules may have induced the formation of pellets through hydrophobic interaction, non-charged interactions and charged-π interactions. This proposed mechanism is supported by our observations in Fig. 1 where pellets were not formed with glucose as the only carbon source even though *R. opacus* has attractive properties for bacterial aggregation. Furthermore, it is in accordance to previous research where lignin was used as an external agent to induce granulation during wastewater treatment [26]. Interestingly, the overall lipid content in pellets was higher than that in the suspended biomass during the different culture conditions. This observation is also in accordance to multiple studies that have suggested that the aggregation of microorganisms can improve product formation [1-6]. Lipid production in pellets was probably enhanced by the syntrophy among bacteria or by the low oxygen concentration inside the compact structure of the pellets, since it has been suggested that the content of some types of lipids is improved at low oxygen levels [45-46].

Table 2. Works reported on the pelletization of filamentous microorganisms

Strain	Type of microorganism	Culture type	External agent addition	Reference
Penicillium ochrochloron	fungus	Pure culture	No	3

Aspergillus niger	fungus	Pure culture	Yes	15
Aspergillus niger	fungus	Co-culture microalgae	No	16
Aspergillus niger	fungus	Co-culture microalgae	No	17
Rhizopus	fungus	Pure culture	No	47
Penicillium chrysogenum	fungus	Pure culture	No	2
Rhizopus oryzae	fungus	Pure culture	Yes	18
Rhizopus oryzae	fungus	Pure culture	No	48
Neurospora intermedia	fungus	Pure culture	Yes	5
Rhizopus sp.	fungus	Co-culture yeast	No	7
Mucor circinelloides	fungus	Pure culture	No	49
Streptomyces lividans	Filamentous bacteria	Pure culture	No	21
Streptomyces tendae	Filamentous bacteria	Pure culture	No	20

The study represents the first report of spontaneous pelletized cultivation without addition of chemical coagulants, and could lead to broad impacts. First, the new cultivation strategy can guide the design of pelletized cultivation of various species to reduce the harvesting cost. One of the significant advantages of pelletized cultivation is the low harvesting cost with simple filtration. Such advantage is particularly important for biofuel production as the end product has lower price. The similar principle can be used to engineer a broad range of microorganisms for pelletized cultivation to reduce the harvesting cost. Second, the pelletized cultivation could reduce the viscosity and subsequently energy consumption of the cultivation system. For large scale cultivation in biofuel production, palletization will improve energy efficiency of the entire system. Third, the increased lipid production at nutrient deficiency in pelletized cells represents a significant advantage of the system. Even though lipid production is generally higher in all cells under nitrogen starvation, the increase is particularly significant for the pelletized cells. The platform provides a new strategy to improve lipid production. With the low harvesting cost, high energy efficiency and product yield, the new bacterial pelletized cultivation platform can significantly reduce the bioproduction cost and improve sustainability.

The mechanistic study and condition optimization will further extend the technical benefits. Several culture strategies were designed to enhance the formation of pellets and the accumulation of lipids that may further facilitate biomass harvesting and lipid extraction. Moreover, the study suggested that lignin played a key role in pelletization. HSQC NMR analysis of lignin before and after fermentation revealed its structural changes. As shown in Fig. S9, lignin linkages were decreased after the fermentation, especially the *β-O*-4 linkage decreased significantly from 32.2% to 24.8%, indicating that *Rhodococus* could break down lignin by cleaving its interunitary linkage. The fermentation may also alter lignin units, since *ρ*-hydroxyphenyl (H) unit has been increased along with the decrease in the *ρ*-coumaric acid (*ρ*CA) units (Fig. S9). The mechanisms of pelletization seem to be different from those of oleaginous fungus and filamentous bacteria, where cell morphology seems to play a role for pellet formation. This new discovery could empower the design of pelletized cultivation in a broad range of microorganisms either through using lignin as a co-substrate or engineering the altered cell surface polysaccharide. The research thus could have a broad impact.

CONCLUSIONS

Microbial pellet formation from non-filamentous bacteria was explored for the very first time without the addition of external coagulants by using alkaline lignin from corn stover as substrate. The results showed that pellet formation without the addition of external agents is possible in *R.*

opacus PD630 when lignin is used as substrate. The unique characteristics of *R. opacus* PD630 to convert lignin into lipids as well as the coagulant properties of lignin led to pellet formation under specific culture conditions. The lignin concentration as well as the shaking speed and the OD_{600} showed significant differences among groups. Overall, the pellet formation was favored at high lignin concentrations, low shaking speeds and moderate OD_{600} values. The microbial pellets showed compact structures that were probable induced by the cell surface characteristics of *R. opacus* and the presence of lignin in the substrate. More importantly, such microbial growth in the form of pellets has the potential to reduce the harvesting cost and improve the lipid production under low nitrogen (high C/N ratio) conditions positively impacting the biofuel industry.

MATERIALS AND METHODS

Strain and culture medium

R. opacus PD630 (DSMZ 44193) was purchased from the DSMZ-German Collection of Microorganisms and Cell Cultures at the Leibniz Institute (Braunschweig, Germany). Engineered *R. opacus* PD630_FA was used in this study to overexpress lipid production [14]. To obtain the engineered strain, the fatty acid synthase I *(PD630_LPD05549)* along with the Pben promoter was cloned into pBSNC9031 vector to overexpress lipid production. The competent cell preparation and transformation by electroporation was carried out as previously described [13]. Engineered *R. opacus* PD630 was grown on minimal medium modified from Kurosawa et al (2010) [50] as reported by Xie et al (2017) [51].

Lignin-rich liquid stream preparation from corn stover

Lignin was obtained from corn stover by sodium hydroxide pretreatment. The conditions of this alkaline treatment were as follows: 50 g of corn stover, 10% (w/w) solid loading, and 1% (w/w) sodium hydroxide solution. The reaction was carried out in a 1 L screw bottle and heated for 1 hour at 121°C in an Amsco® LG 250 Laboratory Steam Sterilizer (Steris, USA). After pretreatment, the pretreated liquid stream was filtered by vacuum. The liquid stream containing lignin was collected for fermentation experiments and characterized as reported in the analytical methods and statistical analysis section. The lignin-rich stream is identified as alkaline pretreatment liquor (APL).

Lipid fermentation of the lignin-rich stream

Prior to fermentation of APL, a single colony of *R. opacus* was pre-cultured in 10 mL of Tryptic Soy Broth (TSB) medium at 28 °C until stationary stage. Then, 1 mL of this culture was transferred into 500mL of TSB with 10g/L of glucose aiming to reach a high OD_{600}. The culture was incubated at 28 °C in a 1 L flask at 200 rpm for 48 h. The cells were harvested by centrifugation.

Centrifuged cells were inoculated in 50 mL of minimal medium at different culture conditions as shown in Table 1. Fermentation experiments were carried out in 250 mL Erlenmeyer flasks at pH 7.0 and 28 °C. APL containing lignin derivatives from corn stover was sterilized at 121 °C for 15 min before fermentation; then, diluted to a particular SSC using ddH_2O. Each experiment was carried out in duplicate and photographic evidence of pellet formation was taken at the end of fermentation. Sodium benzoate was added as an inducer after 24 hours into the fermentation medium to obtain a final concentration of 200µM.

Cell harvesting

Cells were harvested after 48 h of fermentation. Briefly, the fermentation medium was gently poured into a 50 mL Falcon tube avoiding the transfer of biomass pellets. The pellets in the bottom were transferred into another 50 mL Falcon tube. Both suspended bacteria and pellets were harvested by centrifugation at 8000 rpm for 10 min and then cell biomass was lyophilized for 24 h. The weight of both dried biomasses was recorded to calculate biomass production and pellet percentage as regards to the total biomass.

Measurement of lipid content

Lipids were extracted from *R. opacus* after lignin fermentation in the form of fatty acid methyl ester (FAME) according to the method described in previous works [51-53]. Briefly, *R. opacus* in the form of pellets and suspended cells were harvested by centrifugation. The cell biomass was dried in the lyophilizer and then incubated with 20 mL of methanol at 65 °C for 30 min. Then, 1 mL of 10 M KOH was added and incubated at 65 °C for 2 h. After incubation, the sample was moved from the water bath and cooled to room temperature. Then, 1 mL of concentrated sulfuric acid was added drop by drop and incubated at 65 °C for another 2 h. Subsequently, 8 mL of hexane was added and incubated with periodic shaking for 5 min to extract the lipid. The hexane layer was collected in a pre-weighted glass tube after centrifugation, and this hexane extraction step was repeated once. The hexane was evaporated under a nitrogen gas stream, and the lipid content was calculated according to the weight change. The lipid content is expressed as g lipid/g dry biomass.

Extraction of exopolysaccharides for NMR analysis

The extraction of exopolysaccharides was carried out as described by Felz *et al 2016* [54]. Briefly, 2.5g cell biomass (wet weight) was transferred into 50mL ddH$_2$O with addition of 0.25g Na$_2$CO$_3$.

The mixture was heated and stirred for 35min at 400rpm and 80°C in a 200mL flask. The mixture then was centrifugated at 4,000 × g and 4 °C for 20 min. The collected supernatant was dialyzed for 24 h using 1,000 ml ultrapure water (dialysis bag with 3,500 Da molecular weight cut off). Then, the supernatant was lyophilized to obtain the cell wall polymers. The cell wall polymers were subjected to 2D HSQC NMR analysis as described below for lignin.

2D HSQC NMR analysis

Lignin before and after fermentation was recovered by acid precipitation by adding sulphuric acid until reaching pH 2. The acidified medium was centrifugated at 8000 rpm for 20 min, which was followed by lyophilization for lignin powders. Lignin samples (30–50 mg) and the polymers extracted from the pellets were subjected to 2D HSQC NMR analysis as described by Shawn D Mansfield *et al* [55]. The frequencies of lignin linkages and lignin units were quantified as we reported before[56-57], and lignin linkage were expressed as per 100 aromatic rings.

Analytical methods and statistical analysis

Sugar concentration before and after fermentation was analyzed by HPLC as previously reported [14]. The compositional analysis of corn stover and lignin concentration in APL was conducted following the Laboratory Analysis Protocols of the National Renewable Energy Laboratory (NREL), Golden CO, USA. SSC in APL was determined by the dry weight method, filtering the sample through 0.22µm membrane and using a 105 °C oven. The pellet structure was analyzed by SEM using a TESCAN VEGA microscope as reported elsewhere [58]. The pellet area was calculated by using imageJ software. Finally, the statistical significance was determined by one-way analysis of variance (ANOVA) followed by Tukey test. P-values < 0.05 were considered statistically significant. All the statistical analysis were carried out using GraphPad Prism 7.0.

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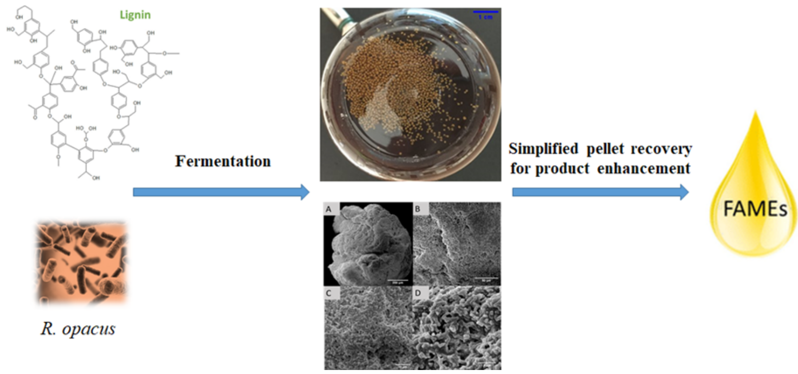

Pelletized cultivation of *Rhodococcus opacus* PD630 in the presence of lignin opens new avenues for bacterial bioproduction to improve yield and conversion efficiency.