Article

Immobilization of *Aspergillus oryzae* DSM 1863 for L-Malic Acid Production

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Abstract: Whole-cell immobilization by entrapment in natural polymers can be a tool for morphological control and facilitate biomass retention. In this study, the possibility of immobilizing the filamentous fungus *Aspergillus oryzae* for L-malic acid production was evaluated with the two carbon sources acetate and glucose. *A. oryzae* conidia were entrapped in alginate, agar, and κ-carrageenan and production was monitored in batch processes in shake flasks and 2.5-L bioreactors. With glucose, the malic acid concentration after 144 h of cultivation using immobilized particles was mostly similar to the control with free biomass. In acetate medium, production with immobilized conidia of *A. oryzae* in shake flasks was delayed and titers were generally lower compared to cultures with free mycelium. While all immobilization matrices were stable in glucose medium, disintegration of bead material and biomass detachment in acetate medium was observed in later stages of the fermentation. Still, immobilization proved advantageous in bioreactor cultivations with acetate and resulted in increased malic acid titers. This study is the first to evaluate immobilization of *A. oryzae* for malic acid production and describes the potential but also challenges regarding the application of different matrices in glucose and acetate media.

Keywords: malate; organic acid; acetate; whole-cell immobilization by entrapment; alginate; carrageenan; agar; filamentous fungi

1. Introduction

Malic acid, a C4-dicarboxylic acid, is mainly used as acidulant and taste enhancer in the food industry. It can furthermore be applied for the synthesis of bio-based polymers and the production of pharmaceuticals as well as personal care and cleaning formulations [1,2]. Malic acid is an intermediate of the tricarboxylic acid cycle and as such can be produced by various microorganisms. However, biological malic acid production is currently not able to compete with low-cost chemical production methods based on fossil resources. For the economic production of bio-based chemicals, the utilization of low-cost substrates is essential. Therefore, various side or waste streams are being evaluated for their suitability for microbial malic acid production. These include hydrolysates of lignocellulosic residues such as corncob or soybean hull [3,4], crude glycerol from biodiesel production [5,6] or thin stillage from ethanol production [7]. In our previous study, we evaluated acetate as alternative carbon source for L-malic acid production with the filamentous fungus *Aspergillus oryzae* [8]. Acetate is a main component of the aqueous pyrolytic condensate, a side stream of fast pyrolysis, and a product of syngas fermentation. While acetate is a suitable substrate for *A. oryzae*, several challenges accompany its utilization. These include substrate inhibition and the dependence of the morphology on the substrate concentration. Filamentous fungal morphology in submerse culture can range from dispersed hyphae to compact biomass pellets [9]. As a pelleted morphology ensures a lower viscosity of the fermentation broth, leading to a better heat, oxygen, and nutrient transfer, it is often preferred. Furthermore, it facilitates the separation and retention of the biomass which allows

for a continuous process management. While acetate concentrations of 45 g/L to 50 g/L were shown to be necessary for an efficient malic acid production with *A. oryzae*, these high concentrations induced a filamentous morphology in shake flask cultivations [8]. One possibility for morphological control could be immobilization which allows for physical confinement of the biomass in a defined pelleted morphology. As malic acid production from acetate with *A. oryzae* is limited to concentrations below 10 g/L in a batch process due to substrate inhibition, immobilization could enable a continuous operation of bioreactors and allow for higher productivities.

A common method for whole-cell immobilization is entrapment in a polymer matrix. Solutions of polymers such as agar, carrageenan and alginate, or the monomer acrylamide are mixed with biomass and solidified by cooling, ionic gelation, or polymerization. Immobilization of *Aspergillus* species for malic acid production was not described so far, except for the production of other organic acids and enzymes. For citric acid production, *Aspergillus niger* was immobilized in alginate [10–17], agarose [18], κ-carrageenan [11], and polyacrylamide [15,19]. Further reports describe the immobilization of *Aspergillus terreus* in alginate, agar, and polyacrylamide for itaconic acid production [20,21]. With *A. oryzae*, immobilization was performed in a sol-gel matrix consisting of tetraethylorthosilicate, starch and alginate for alpha-amylase production [22], alginate for the biotransformation of L-tyrosine to dopamine [23], or agar as well as alginate for phytase production [24]. Entrapment of *Aspergillus* species proved to improve the organic acid production process. Compared to free biomass, immobilized cells of *A. niger* were reported to perform better in repeated-batch cultures for citric acid production as the productivity was maintained at a high level for an increased number of cycles [13,18,19]. Similar observations were made for *A. terreus* and itaconic acid production [21]. It was furthermore shown that entrapment can decrease byproduct formation [25] and increase the tolerance of microorganisms to certain inhibitors such as ethanol, acetic acid, and phenol, which is especially interesting when using side or waste streams as substrates [26–28].

Reports on the production of malic acid using immobilized microorganisms are scarce. The fumarase activity of *Brevibacterium flavum* and *Brevibacterium ammoniagenes* immobilized in κ-carrageenan and polyacrylamide was utilized for the conversion of fumaric to malic acid [29–31]. Fumarate was also used as substrate for malic acid production with *Saccharomyces cerevisiae* immobilized in polyacrylamide [32,33] and a copolymeric hydrogel consisting of alginate and polyvinyl alcohol [34]. However, fumaric acid is currently produced from fossil resources and therefore not a renewable carbon source.

The aim of this study was to evaluate L-malic acid production with immobilized *A. oryzae* in batch cultivations. Immobilization was performed by entrapment of conidia in the natural polymers alginate, agar, and κ-carrageenan. The performance of the immobilized particles was assessed with the two carbon sources acetate and glucose in shake flask and 2.5-L bioreactor cultivations and compared to that of fermentations with free biomass.

2. Materials and Methods

2.1. Microorganism and Media

Aspergillus oryzae DSM 1863 was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany).

For propagation through spore formation the fungus was grown on a minimal medium for *Aspergillus* species [35] containing 15 g/L glucose monohydrate, 6 g/L $NaNO_3$, 0.52 g/L KCl, 0.52 g/L $MgSO_4 \cdot 7H_2O$, 1.52 g/L KH_2PO_4, 15 g/L agar and 2 mL/L 1000 × Hutner's Trace Elements solution. The pH was set to 6.5 with NaOH and the medium was autoclaved for 20 min at 121 °C. 1000 × Hutner's Trace Element solution consists of 5 g/L $FeSO_4 \cdot 7H_2O$, 50 g/L EDTA-Na_2, 22 g/L $ZnSO_4 \cdot 7H_2O$, 11 g/L H_3BO_3, 5 g/L $MnCl_2 \cdot 4H_2O$, 1.6 g/L $CoCl_2 \cdot 6H_2O$, 1.6 g/L $CuSO_4 \cdot 5H_2O$, and 1.1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ with a pH of 6.5 [36]. After 7–10 days of incubation on agar plates at 30 °C, conidia were harvested with 50% glycerol, filtered through Miracloth (Merck KGaA, Darmstadt, Germany) and stored in aliquots at −80 °C.

The preculture medium contained either 45 g/L acetic acid or 40 g/L glucose monohydrate as carbon source, 4 g/L $(NH_4)_2SO_4$, 0.75 g/L KH_2PO_4, 0.98 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, and 5 mg/L $FeSO_4 \cdot 7H_2O$. For the cultivation of free biomass with acetate both in shake flasks and bioreactors, furthermore 2 mL/L Hutner's Trace Element solution was added. The main culture medium was composed of 45 g/L acetic acid or 120 g/L glucose monohydrate, 1.2 g/L $(NH_4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.17 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 5 mg/L NaCl, 60 mg/L $FeSO_4 \cdot 7H_2O$ and 90 g/L $CaCO_3$. The pH of the preculture and main culture medium containing acetic acid was adjusted to a value of 6.5 and 5.5 with NaOH, respectively, while the pH of the glucose-containing media remained unadjusted. The preculture medium without carbon source was adjusted to a pH value of 6.5. All pre- and main culture media containing either glucose or acetate were sterilized by autoclaving for 20 min at 121 °C.

2.2. Immobilization

Immobilization was performed by entrapment of *A. oryzae* conidia in the natural polymers sodium alginate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), agar (European agar Difco TM, BD, Germany), and κ-carrageenan (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Polymer solutions were prepared by dissolving 0.75–6.00 g polymer powder in a final volume of 100 g double distilled water and autoclaved for 20 min at 121 °C. For the alginate beads 4 mL spore suspension ($c = 2 \times 10^7$/mL) was mixed with 16 mL alginate solution and extruded through a canula with a diameter of 0.80 mm into a gently stirred 0.2 M $CaCl_2$ solution which was cooled in an ice bath. After 2 h of solidification in the cooled 0.2 M $CaCl_2$ solution the beads were washed with double distilled water and stored at 4 °C overnight until the inoculation of the preculture on the next day. The 0.75% κ-carrageenan beads were prepared in the same way as the alginate beads, but solidification was performed with a 0.1 M KCl solution. The agar and 1.5% κ-carrageenan solutions were brought to a temperature of 45 °C, then 16 mL of the respective solution was mixed with 4 mL spore suspension ($c = 2 \times 10^7$/mL). Subsequently, the mixtures were poured in petri dishes and solidified at room temperature. The petri dishes were stored at 4 °C overnight. Before inoculation of the precultures the films were cut in cubes of about 2–3 mm in length.

2.3. Preculture Conditions

For the cultivations with free biomass, 100 mL preculture medium in 500-mL shake flasks was inoculated with 1 mL of a spore suspension containing 3×10^7 conidia/mL. For the precultures with immobilized biomass, 20 mL of immobilized material was distributed equally by weight to three shake flasks containing 100 mL of preculture medium. Precultures with glucose were incubated for 24 h at 30 °C and 100 rpm and those with acetate for 48 h under the same conditions. The carbon source remained the same in pre- and main culture, hence biomass cultivated in preculture medium containing glucose was used for the inoculation of main cultures with glucose medium, the precultures with acetate were used for the main cultures with acetate as carbon source.

2.4. Main Culture for Acid Production in Shake Flasks

Main culture cultivations were performed in 500-mL shake flasks containing 100 mL main culture medium and 90 g/L $CaCO_3$. The free biomass and immobilized particles grown in the preculture were separated from the preculture medium by filtration and thoroughly washed with distilled water to remove medium residues. For the cultures with free *A. oryzae*, 0.75 g biomass was used for the inoculation of one shake flask. For all immobilized cultures, the content of one preculture triplicate was distributed equally by weight to three shake flasks. Main cultures were incubated at 120 rpm and 32 °C for 168 h (glucose media) or 240 h (acetate media). All experiments were performed as biological triplicates. Samples were taken at the indicated time points and analyzed for pH and glucose, organic acid, and ammonium concentration.

2.5. Main Culture for Acid Production in Bioreactors

For bioreactor cultivations 2.5-L stirred tank reactors (Minifors, Infors HT, Bottmingen, Switzerland) filled with 1.4 L main culture medium were used. Before inoculation 0.1 mL of the antifoaming agent Contraspum A 4050 HAc (Zschimmer und Schwarz GmbH und Co KG, Lahnstein, Germany) was added. To the bioreactors containing acetate medium, 17.5 mL of a sterile glucose solution (c = 400 g/L) was added before inoculation to reach a concentration of 5 g/L glucose. This additional carbon source was used to decrease the duration of the lag phase. One bioreactor was inoculated with four precultures. The temperature was controlled at 32 °C and the aeration rate was 0.5 vvm. Two Rushton turbines with a distance of 8 cm were used at a stirrer velocity of 300 rpm. All experiments were performed as biological duplicates. The pH value was recorded via online measurements. Samples were taken every 24 h for offline determination of glucose, organic acid, and ammonium concentration.

2.6. Analytics

For organic acid and glucose quantification a standard HPLC device (Agilent 1100 Series, Agilent, Germany) equipped with a Rezex ROA organic acid H+ (8%) column (300 by 7.8 mm, 8 m, Phenomenex) and a Rezex ROA organic acid H+ (8%) guard column (50 by 7.8 mm) was used. For glucose analysis, 1 mL of the sample was centrifuged for 10 min at $20,000 \times g$. The supernatant was diluted appropriately and analyzed under isocratic conditions using 5 mM H_2SO_4 as eluent with a flow rate of 0.5 mL/min at 50 °C. The injection volume was 10 μL. Detection was performed by means of a refractive index detector. For organic acid determination 1 mL of the sample was added to 3 mL of distilled water and 1 mL of 3 M H_2SO_4. The suspension was incubated for 20 min at 80 °C and subsequently centrifuged for 10 min at $20,000 \times g$. The supernatant was diluted appropriately and analyzed under isocratic conditions using 3 mM H_2SO_4 as eluent with a flow rate of 0.5 mL/min at 60 °C. The injection volume was 10 μL. Organic acids were detected with a UV detector at 220 nm.

Ammonium concentration was quantified photometrically using a Spectroquant assay kit (114752, Merck KGaA, Darmstadt, Germany). The assay was scaled down to a volume of 200 μL and sample supernatants were measured in duplicate in microtiter plates according to the manufacturer's instructions.

Fungal morphology was characterized by light microscopy using a Nikon Eclipse E200 equipped with a DFK 23U274 camera (Imaging Source, Bremen, Germany) and the software NIS-Elements D ver. 4.50.

3. Results

3.1. Malic Acid Production in Shake Flask Cultivations

Malic acid production with immobilized *A. oryzae* cells was first studied in shake flasks with acetate or glucose as substrate. When grown in medium with 45 g/L acetic acid, *A. oryzae* develops a dispersed filamentous structure whereas in glucose medium biomass pellets are formed naturally. Therefore, the term "free biomass" refers to either of these morphological forms depending on the carbon source. In the cultures with free biomass, the acid production was completed within 144 h. For this reason, all values summarized in Table 1 are based on the measurements of this sampling point.

With acetate as carbon source, four alginate concentrations (3–6%), two agar concentrations (1.5% and 3%) and two κ-carrageenan concentrations (0.75% and 1.5%) were evaluated for the immobilization of *A. oryzae*. Compared to that of the cultures with free biomass, which produced 7.96 ± 0.26 g/L malic acid within 144 h, the concentration in all immobilized cultures was lower (Figure 1). With *A. oryzae* immobilized in alginate and agar, an increase of the polymer concentration resulted in a lower malic acid production. While *A. oryzae* entrapped in beads prepared with 3% alginate produced 6.34 ± 0.72 g/L malic acid within 144 h, the utilization of 6% alginate resulted in 1.30 ± 0.43 g/L which was the lowest concentration measured of all tested conditions. With 1.5% agar the second

highest concentration of 5.53 ± 0.52 g/L malic acid was detected which was reduced to 3.68 ± 0.21 g/L with 3% agar. With κ-carrageenan, on the other hand, beads produced with 0.75% of the polymer reached a malic acid concentration of 2.98 ± 0.34 g/L compared to 4.72 ± 0.39 g/L obtained with 1.5% κ-carrageenan. Yields obtained after 144 h ranged between 0.08 ± 0.02 g/g with 6% alginate and 0.20 ± 0.01 g/g with free biomass. Regarding the productivity, the highest value was obtained with free biomass (0.055 ± 0.002 g/L*h) and the lowest with 6% alginate beads (0.009 ± 0.003 g/L*h). For all conditions, ammonium was depleted or close to depletion within 96 h of cultivation and the pH increased to values around 9.0 within 144 h (Figure S1). Besides malic acid, *A. oryzae* produces several other organic acids. For the total acid concentration with the substrate acetate, values between 3.71 ± 0.61 g/L for cultures with *A. oryzae* immobilized in 6% alginate and 15.69 ± 0.67 g/L with free biomass were calculated. Malic acid is usually the main product with around 50% of the organic acids produced when acetate is used as carbon source (Table A1, see Appendix A). Malic and succinic acid percentages tended to decrease in favor of oxalic acid with an increase of density of the immobilization matrix. This is especially illustrated with 6% alginate (26.4 ± 8.1% oxalic acid). Cultures with 5% alginate, 3% agar, and 0.75% κ-carrageenan also showed a considerably higher oxalic acid concentration than that of the control.
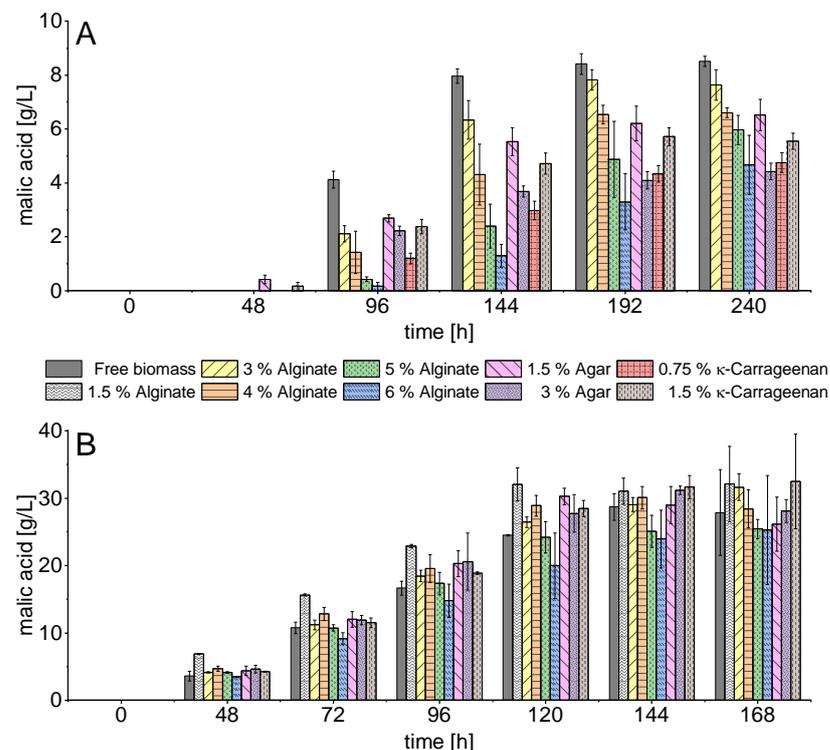
Figure 1. Malic acid production in shake flask cultivations with *A. oryzae* immobilized in different natural polymers using acetate (**A**) or glucose (**B**) as carbon source. Values are means of triplicates with standard deviation.

With glucose as carbon source, five alginate concentrations (1.5–6%), two agar concentrations (1.5% and 3%) and 1.5% κ-carrageenan were tested as immobilization matrices. Different than with acetate, the majority of the immobilized conditions performed slightly better or similar compared to the control with free biomass (Figure 1). Only the cultures with 5% and 6% alginate showed lower acid concentrations after 144 h. With 1.5% alginate the production was accelerated compared to that of the control, reaching its peak concentration at 120 h with 32.06 ± 2.44 g/L malic acid compared to 24.49 ± 0.10 g/L with free biomass. At the start of the cultivation, the 1.5% alginate beads featured the smallest diameter of about 0.3 cm, compared to approximately 0.4 cm for the other con-

centrations. Productivities calculated for the sampling point at 144 h ranged between 0.166 ± 0.030 g/L*h obtained with 6% alginate and 0.220 ± 0.012 g/L*h for cultures with 1.5% κ-carrageenan (Table 1). The highest yield was calculated for the cultures with free biomass (0.59 ± 0.05 g/g) and the lowest for the cultures with 6% alginate (0.40 ± 0.03 g/g). Ammonium was depleted for all conditions within 48 h and the pH decreased to values of around 6.7 during cultivation (Figure S2). Total acid concentrations ranged between 33.34 ± 6.81 g/L which was detected for cultures with 6% alginate and 44.63 ± 3.10 g/L with free biomass pellets. The proportion of malic acid regarding the acid spectrum was not largely influenced by the immobilization and amounted to about 70–75% (Table A1). Generally, the differences amongst the performance of *A. oryzae* in the tested matrices compared to that of the free biomass culture was less pronounced than with acetate as carbon source.

Table 1. Fermentation results for shake flask cultivations of *A. oryzae* after 144 h. Values are means of triplicates with standard deviation.

Substrate	Immobilization Matrix	Consumed Substrate (g/L)	Malic Acid (g/L)	$Y_{P/S}$ [1] (g/g)	Productivity (g/L*h)	Total Acids (g/L)
	Free biomass	38.90 ± 1.09	7.96 ± 0.26	0.20 ± 0.01	0.055 ± 0.002	15.69 ± 0.67
	3% Alginate	32.15 ± 3.27	6.34 ± 0.72	0.19 ± 0.00	0.044 ± 0.005	12.03 ± 1.12
	4% Alginate	26.91 ± 5.35	4.31 ± 1.13	0.16 ± 0.02	0.030 ± 0.008	8.88 ± 2.12
	5% Alginate	20.30 ± 3.44	2.40 ± 0.82	0.12 ± 0.02	0.017 ± 0.006	5.26 ± 1.59
Acetic acid	6% Alginate	16.56 ± 1.73	1.30 ± 0.43	0.08 ± 0.02	0.009 ± 0.003	3.71 ± 0.61
	1.5% Agar	30.71 ± 1.28	5.53 ± 0.52	0.18 ± 0.01	0.038 ± 0.004	11.40 ± 1.00
	3% Agar	25.72 ± 0.66	3.68 ± 0.21	0.14 ± 0.00	0.026 ± 0.001	7.62 ± 0.74
	0.75% κ-Carrageenan	23.71 ± 2.50	2.98 ± 0.34	0.13 ± 0.02	0.021 ± 0.002	6.80 ± 0.48
	1.5% κ-Carrageenan	28.92 ± 0.99	4.72 ± 0.39	0.16 ± 0.02	0.033 ± 0.003	9.64 ± 0.84
	Free biomass	48.99 ± 0.97	28.69 ± 1.98	0.59 ± 0.05	0.199 ± 0.014	39.08 ± 1.26
	1.5% Alginate	69.80 ± 1.06	31.04 ± 1.95	0.45 ± 0.03	0.216 ± 0.014	44.63 ± 3.10
	3% Alginate	64.96 ± 2.20	29.05 ± 1.03	0.45 ± 0.03	0.202 ± 0.007	40.18 ± 0.36
	4% Alginate	66.81 ± 1.53	30.08 ± 1.66	0.45 ± 0.03	0.209 ± 0.012	41.31 ± 1.85
Glucose	5% Alginate	64.83 ± 2.98	25.08 ± 2.40	0.39 ± 0.04	0.174 ± 0.017	35.68 ± 2.74
	6% Alginate	59.29 ± 6.96	23.92 ± 4.30	0.40 ± 0.03	0.166 ± 0.030	33.34 ± 6.81
	1.5% Agar	65.96 ± 2.43	28.96 ± 2.74	0.44 ± 0.05	0.201 ± 0.019	40.75 ± 2.76
	3% Agar	64.96 ± 0.64	31.16 ± 0.63	0.48 ± 0.01	0.216 ± 0.004	42.21 ± 0.37
	1.5% κ-Carrageenan	61.62 ± 1.63	31.63 ± 1.74	0.51 ± 0.02	0.220 ± 0.012	41.81 ± 2.41

[1] $Y_{P/S}$ (g/g) = substrate specific malic acid yield calculated as g (malic acid)/g (consumed substrate).

For successful application of immobilized microorganisms, not only the production performance but also the matrix stability has to be considered. Ideally, the matrix is insoluble and chemically robust under the reaction conditions throughout the duration of the process. Differences in stability of the immobilized structures were observed between glucose and acetate-containing medium. As the cultivation progressed, the initially "fluffy" surface of the beads incubated in glucose medium and the free biomass pellets became smoother while all matrices remained intact with $CaCO_3$ adhering to the biomass layer (Figure 2).
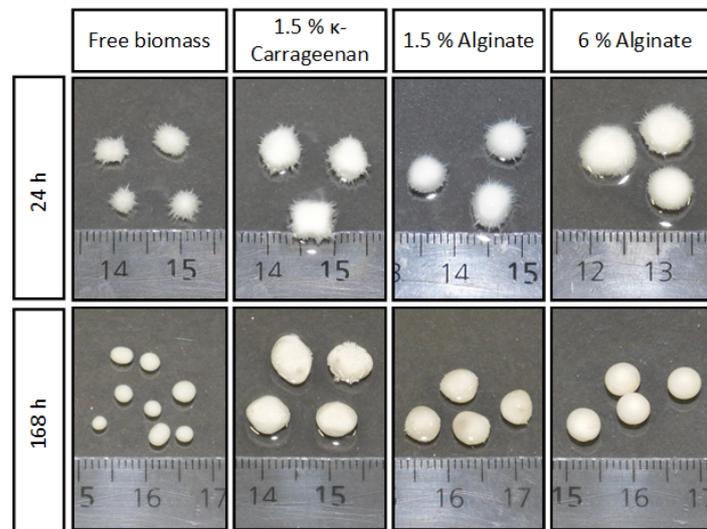
Figure 2. Appearance of free biomass pellets and immobilized *A. oryzae* during malic acid production in glucose medium.

In acetate medium, not all the immobilized structures were stable until the end of the fermentation. Between 96–144 h, the beads prepared with 3% and 4% alginate showed first signs of disintegration and were completely dissolved after 240 h. The alginate beads prepared with 5% and 6% of the polymer remained intact but gradually showed a softer consistency. Generally, with a higher concentration of alginate the amount of outgrowth from the beads decreased, reducing the amount of biomass surrounding the beads (Figure 3). On the other hand, no difference in growth behavior was noticed for the two agar and κ-carrageenan concentrations tested. While the agar and κ-carrageenan matrices were stable and did not show any signs of degradation, an abrasion of the biomass was observed starting between 96 h and 144 h. Exemplarily, the development of cuboids prepared with 3% agar is shown in Figure 4. In the preculture, a dense biomass layer was developed towards the outer region and surrounding the particles while the conidia located near the particle's cores remained unsporulated or in the early stages of growth (Figure 4A). In the main culture the cuboid structures were initially well covered with biomass which captured the $CaCO_3$ present in the main culture medium (Figure 4B). Towards the end of the fermentation, the biomass was stripped off and only a thin biomass layer remained on the surface (Figure 4C,D). This behavior was comparable for both agar and κ-carrageenan concentrations tested using acetate medium.
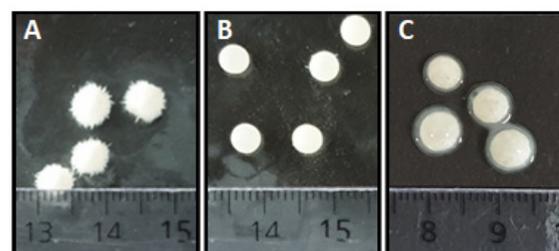
Figure 3. *A. oryzae* immobilized in 3% alginate after 48 h (**A**), 6% alginate after 48 h (**B**), and 6% alginate after 240 h (**C**) of incubation in main culture medium containing acetate.
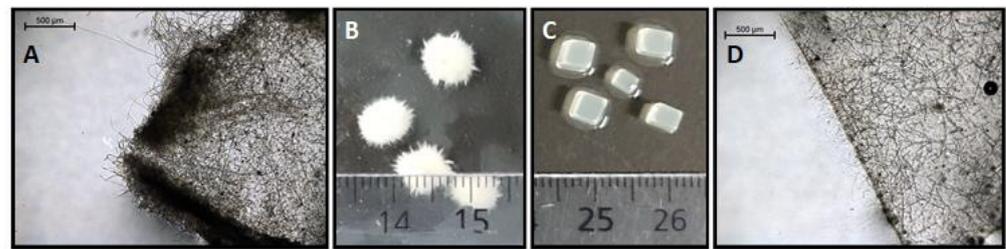
Figure 4. *A. oryzae* immobilized in 3% agar in the beginning (**A**), after 48 h (**B**), and after 240 h (**C**,**D**) of incubation in main culture medium containing acetate.

3.2. Malic Acid Production in Bioreactor Cultivations

2.5 L-bioreactor cultivations were carried out with *A. oryzae* immobilized in alginate and κ-carrageenan using glucose or acetate as the main carbon source. *A. oryzae* entrapped in agar and κ-carrageenan performed similarly regarding acid production and growth behavior in shake flasks and therefore only the latter was tested in bioreactor fermentations. For a better comparison to the shake flask cultivations, the values summarized in Table 2 are calculated for the 144 h-measurement point.

Table 2. Fermentation results for 2.5-L bioreactor cultivations of *A. oryzae* after 144 h. Values are means of duplicates with standard deviation.

Main Substrate	Immobilization Matrix	Consumed Substrate [1] (g/L)	Malic Acid (g/L)	$Y_{P/S}$ [2] (g/g)	Productivity (g/L*h)	Total Acids (g/L)
Acetic acid	Free biomass	HAc: 6.68 ± 4.91 Glc: 4.91 ± 0.54	0.73 ± 0.27	0.06 ± 0.00	0.005 ± 0.002	3.13 ± 0.64
	3% Alginate	HAc: 7.38 ± 0.29 Glc: 4.90 ± 0.03	2.60 ± 0.14	0.21 ± 0.02	0.018 ± 0.001	5.39 ± 0.07
	6% Alginate	HAc: 7.91 ± 0.76 Glc: 5.04 ± 0.02	1.70 ± 0.05	0.13 ± 0.01	0.012 ± 0.000	3.91 ± 0.06
	1.5% κ-Carrageenan	HAc: 6.82 ± 0.55 Glc: 4.67 ± 0.05	1.60 ± 0.09	0.14 ± 0.00	0.011 ± 0.001	4.23 ± 0.19
Glucose	Free biomass	39.09 ± 1.65	29.77 ± 2.70	0.76 ± 0.10	0.207 ± 0.019	36.91 ± 3.81
	1.5% Alginate	35.26 ± 1.36	25.23 ± 3.13	0.72 ± 0.12	0.175 ± 0.022	32.12 ± 1.42
	3% Alginate	35.35 ± 2.90	27.62 ± 0.05	0.78 ± 0.06	0.192 ± 0.000	33.66 ± 0.30
	1.5% κ-Carrageenan	42.40 ± 0.99	23.61 ± 4.68	0.56 ± 0.12	0.164 ± 0.033	31.42 ± 5.45

[1] HAc = acetic acid, Glc = glucose. [2] $Y_{P/S}$ (g/g) = substrate specific malic acid yield calculated as g(malic acid)/g(total consumed substrate(s)).

For the fermentations with glucose, *A. oryzae* immobilized in two different alginate concentrations (1.5% and 3%) and 1.5% κ-carrageenan was used. With the two alginate concentrations the progression of the fermentation was similar to the one with free biomass (Figure 5). Slightly lower malic acid concentrations were quantified compared to the free biomass fermentation, which yielded 29.77 ± 2.70 g/L of the product within 144 h (Table 2). Compared to that of shake flask cultivations, the malic acid concentrations obtained with free biomass and *A. oryzae* immobilized in alginate was similar. The yield, however, was increased compared to that of shake flask cultivations, which was associated with a decreased byproduct formation. While the malic acid percentage in shake flasks was around 70%, it increased to about 80% in the bioreactor cultivations with free biomass and *A. oryzae* immobilized in alginate (Table A2, see Appendix A). At the same time, especially the citric acid production decreased to values below 1%. With *A. oryzae* immobilized in 1.5% κ-carrageenan, malic acid production was lowest obtaining 23.61 ± 4.68 g/L within 144 h and the yield was reduced to a value of 0.56 ± 0.12 g/g. Using glucose as carbon source, the malic acid concentration usually peaks around 168 h hours of fermentation,

followed by a decrease. With κ-carrageenan, this peak apparently occurred earlier than in the other fermentations, leading to a lower maximum concentration. As in shake flask experiments, ammonium was depleted within 48 h for all conditions and the pH decreased to values of 6.7–7.0.
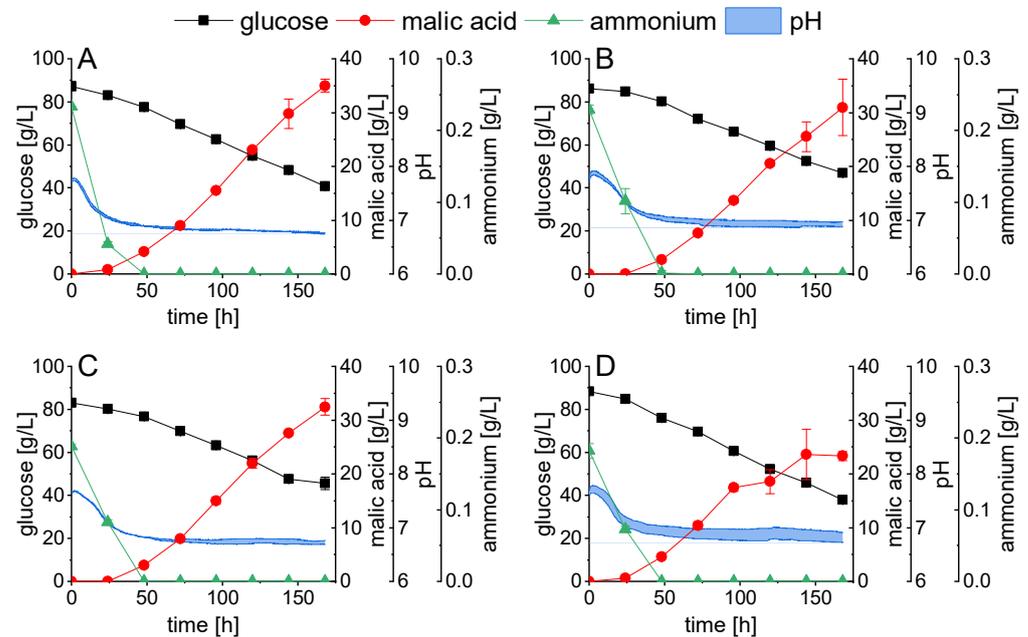
Figure 5. Malic acid production using glucose as carbon source in 2.5 L-bioreactors with free biomass (**A**) and *A. oryzae* immobilized in 1.5% alginate (**B**), 3% alginate (**C**), and 1.5% κ-carrageenan (**D**). Values are means of duplicates with standard deviation.

For the bioreactor cultivations with acetate as carbon source, immobilization was evaluated with 3% and 6% alginate as well as 1.5% κ-carrageenan using the same cultivation conditions as with glucose. Different than with glucose, the malic acid concentration obtained with free biomass in the bioreactor cultivation was much lower compared to the cultivation in shake flasks (Figure 6). Within 144 h, 0.73 ± 0.27 g/L malic acid was produced with free mycelium, representing only about 10% of the concentration obtained in shake flasks during the same time (Table 2). Even though substrate was present at concentrations above 30 g/L until the end of the fermentation, the malic acid concentration stagnated after 192 h and did not surpass 2 g/L. All fermentations with immobilized *A. oryzae* performed better. The highest malic acid concentration of 2.60 ± 0.14 g/L after 144 h was obtained with 3% alginate. Towards the end of the fermentation, this value further increased to a concentration of more than 4 g/L. However, this is still considerably lower than the malic acid concentration determined in shake flask cultivations. In the bioreactor fermentations with acetate, glucose was added at a concentration of 5 g/L to shorten the lag phase. The additional carbon source was depleted within 48 h (free biomass) or 72 h (immobilized cultures). The consumption of the main carbon source acetate within 144 h was below 10 g/L for all fermentations. Ammonium was depleted after 96 h in the cultivations with free biomass and after 120 h for the fermentations with immobilized *A. oryzae*. Regarding the matrix stability, similar observations were made as in the shake flask cultivations observing the disintegration of the alginate beads and an abrasion of the biomass for the κ-carrageenan particles. In the bioreactors, however, also the 6% alginate beads dissolved starting between 144 h and 168 h, which might explain the slightly higher malic acid concentration compared to that of the shake flask cultivations at the 144-h sampling point. Compared to that of the shake flask cultivations, the percentage of pyruvic acid was increased, especially for the cultivation with free biomass for which 37.0 ± 10.5% of the 3.13 ± 0.64 g/L total acids was apportioned to this product (Table A2).
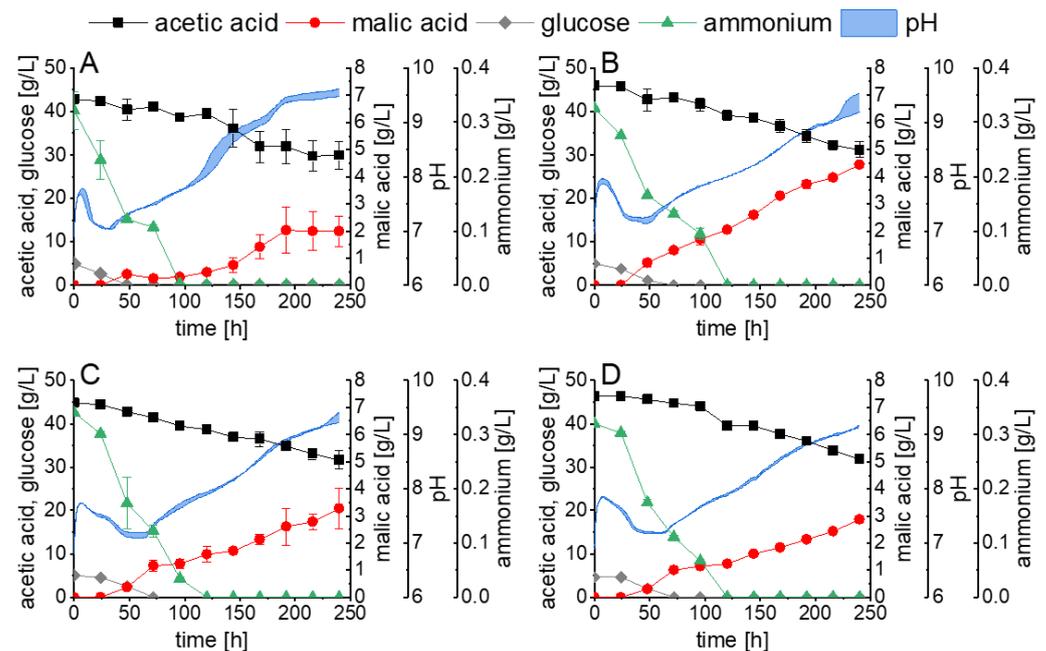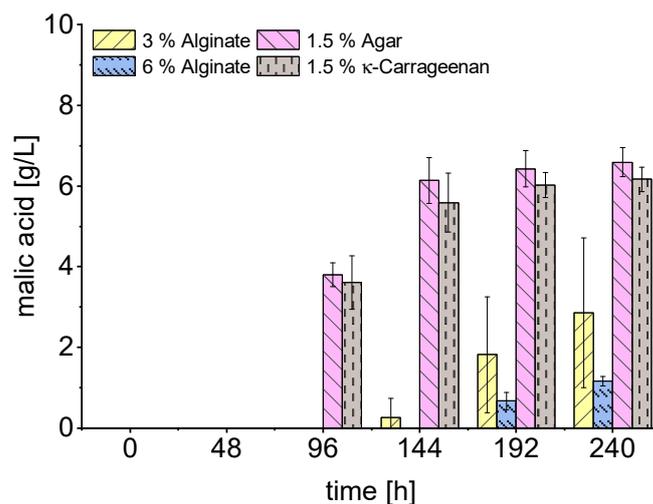
Figure 6. Malic acid production using acetate as carbon source in 2.5 L-bioreactors with free biomass (**A**) and *A. oryzae* immobilized in 3% alginate (**B**), 6% alginate (**C**), and 1.5% κ-carrageenan (**D**). Values are means of duplicates with standard deviation.

3.3. Shake Flask Cultivations without Carbon Source in the Preculture Medium

Due to the challenges described for the cultivations in acetate medium, possibilities to improve the growth characteristics were evaluated. Albeit the biomass was mainly located on the surface of the beads or cuboids in the cultivations described in the previous chapters before biomass detachment occurred, growth of free mycelium was also observed. To confine the growth of *A. oryzae* to the immobilization matrix and reduce extensive outgrowth, the carbon source was omitted in the growth medium. The growth behavior was evaluated with *A. oryzae* immobilized in 3% and 6% alginate, 1.5% κ-carrageenan, and 1.5% agar, and several differences were observed compared to that of the cultivations with a carbon source in the preculture medium. Regarding the alginate beads, *A. oryzae* grew throughout the matrix when the carbon source was absent instead of growing mainly on the bead's surface while growth for the other two polymers was still mainly located near or on the surface of the cuboids (Figure S3).

Subsequently, malic acid production was evaluated with these immobilized particles with a main culture containing acetate as carbon source. With *A. oryzae* immobilized in alginate, malic acid production was delayed compared to that of the cultures grown in a preculture medium containing acetate, and much lower concentrations were obtained (Figure 7). For the 6% alginate beads no malic acid was detected after 144 h of cultivation (Table 3). Still, 1.06 ± 0.13 g/L total acids were obtained of which 99% was oxalic acid (Table A3, see Appendix A). With the agar and κ-carrageenan particles, slightly higher concentrations were measured than after a preculture with carbon source within 144 h. As for the 1.5% agar cuboids, 6.14 ± 0.57 g/L compared to 5.53 ± 0.52 g/L were observed and with κ-carrageenan 5.59 ± 0.72 g/L compared to 4.72 ± 0.39 g/L, while the yields remained similar. Ammonium was depleted after 96 h in the cultures with agar and κ-carrageenan and depleted or very close to depletion after 144 h for the alginate beads (Figure S4). In the first 48 h of the acid production phase, the agar and κ-carrageenan particles developed a biomass layer on the surface of the beads, which was comparable to that of the particles grown with a carbon source in the preculture, whereas the alginate beads did not (Figure S3). Between 48 and 96 h, the alginate beads prepared with 3% of the polymer formed a thin, filamentous biomass layer around the particle's surface. The

formation of a biomass layer on the surface of the immobilization material seems necessary for an effective malic acid production. While showing a markedly reduced malic acid productivity, the infiltration of the alginate beads by *A. oryzae* resulted in their stabilization in acetate medium. The 3% alginate beads did not dissolve during the fermentation as observed for the cultivations with acetate in the preculture since they were probably stabilized by the fungal filaments. As observed for the cultivations with matrices grown in the presence of acetate, the biomass layer formed in the main culture was detached in later stages of the fermentation (Figure S3).

Figure 7. Malic acid production with immobilized *A. oryzae* grown in a preculture medium without carbon source. Acetate was used as carbon source for acid production in the main culture. Values are means of triplicates with standard deviation.

Table 3. Fermentation results for shake flask cultivations of *A. oryzae* after 144 h using acetate as carbon source in the main culture, while preculture was grown without carbon source in medium. Values are means of triplicates with standard deviation.

Substrate	Immobilization Matrix	Consumed Substrate (g/L)	Malic Acid (g/L)	$Y_{P/S}$ [1] (g/g)	Productivity (g/L*h)	Total Acids (g/L)
Acetic acid	3% Alginate	15.46 ± 1.25	0.27 ± 0.47	0.02 ± 0.03	0.002 ± 0.003	1.33 ± 0.59
	6% Alginate	15.37 ± 0.88	0.00 ± 0.00	0.00 ± 0.00	0.000 ± 0.000	1.06 ± 0.13
	1.5% Agar	34.28 ± 1.67	6.14 ± 0.57	0.18 ± 0.01	0.043 ± 0.004	10.85 ± 0.94
	1.5% κ-Carrageenan	32.04 ± 1.04	5.59 ± 0.72	0.17 ± 0.02	0.039 ± 0.005	10.51 ± 0.92

[1] $Y_{P/S}$ (g/g) = substrate specific malic acid yield calculated as g (malic acid)/g (consumed substrate).

4. Discussion

In this work, different concentrations of the natural polysaccharides alginate, agar, and κ-carrageenan were evaluated for the immobilization of *A. oryzae* for malic acid production in shake flask and bioreactor cultivations. A suitable immobilization material provides an adequate growth environment and is chemically and mechanically stable.

With alginate in shake flask cultivations, the utilization of higher polymer concentrations resulted in decreased productivities with both acetate and glucose as shown in Figure 1 and Table 1 due to enhanced diffusion limitations. Decreased activity with increasing alginate concentration was also observed by other authors [37–39]. These diffusion limitations affected the biomass growth and the subsequent production of malic acid especially for cultures with acetate. We observed that growth on the surface of the alginate beads was reduced with higher concentrations of the immobilization matrix especially in acetate medium (Figure 3), resulting in a lower amount of active biomass which was

associated with a reduced production of malic acid. Furthermore, the bead diameter was influenced by the alginate content, featuring slightly smaller diameters with lower polymer concentrations. This was particularly visible for beads prepared with 1.5% alginate which were about 1 mm smaller in diameter than the other alginate concentrations in the beginning of the fermentation. In immobilized structures, the mass transfer towards the center of the particles is restricted which creates a region that can be considered biologically inactive [40]. The smaller bead diameter and lower diffusion limitations associated with the low alginate content were probably the reasons for which cultures with 1.5% alginate beads featured the highest malic acid production in shake flask experiments (Figure 1, Table 1). However, the utilization of a low alginate concentration was associated with lower mechanical stability. Even though the 1.5% alginate beads were stable until the end of the fermentation in shake flasks, they were soft and easily deformable in a bioreactor environment. Therefore, 3% alginate beads were better suited for cultivation in the stirred tank reactor with glucose medium. Adjusting the alginate concentration offers the possibility of optimizing the characteristics of the beads, depending on the requirements of the microorganism and the process. Often, alginate concentrations between 2–3% seem to be a good balance between production performance and mechanical strength of the beads [37,38,41], which is consistent with the results presented here.

Whereas in glucose medium all alginate concentrations were stable, this was not the case for the cultivations using acetate. Beads with lower alginate concentrations seemed to be more favorable for malic acid production in shake flasks but the stability of the beads was poor. Only the beads prepared with 5% and 6% alginate remained intact in shake flask cultivations, although showing a softer consistency. Alginate is a linear copolymer consisting of D-mannuronate and L-guluronate which forms hydrogels by cross-linking the chains with divalent cations such as Ca^{2+} [42]. These divalent ions can be replaced by monovalent ions which gradually weakens the hydrogel by reducing the cross-linking, leading to swelling and subsequent disintegration [43]. As the acetate medium was rich in sodium ions due to the adjustment of the pH with sodium hydroxide, this explains the dissolution of the alginate beads while they were stable in glucose medium. Similar observations were reported with medium containing potassium acetate [11]. In 2.5-L bioreactor cultivations, even the beads prepared with 6% alginate disintegrated at around 168 h of fermentation due to increased mechanical stress compared to shake flask cultivations. The stability of alginate gels is affected by the polymer concentration, the molarity of the curing solution, the type of cross-linking ion and the curing time and temperature. Further stabilization of the matrix can be achieved by treatment with the cross-linker glutaraldehyde, which was reported to reduce the cell leakage of alginate-immobilized *E. coli* cells [44]. By preparing a mixed polymer gel composed of alginate and polyvinyl alcohol the mechanical stability of alginate beads could be increased considerably [45]. However, in both reported applications the increased stability was accompanied by a decrease in relative activity. A tighter matrix created by an increased alginate concentration as presented in this work was also accompanied by a reduced productivity. Coating of the beads might be a possibility to improve their stability without altering the properties of the alginate core. By coating alginate beads with a chitosan layer, the surface properties of the beads could be improved while maintaining similar catalytic activity with optimized coating conditions, resulting in an increased number of reuse cycles [41]. Chitosan coating furthermore resulted in reduced swelling of the alginate beads by improving the surface characteristics [46]. Other authors, however, did not find an improved stability of alginate core beads with a chitosan layer and disintegration was still observed [47].

Regarding the immobilization in agar and κ-carrageenan, no disintegration of the matrices was found as these polysaccharides feature different gelling characteristics. Agar is composed of agaropectin and agarose, a linear polysaccharide consisting of D-galactose and 3,6-anhydro-L-galactose, which is mainly responsible for the gelling properties by forming a 3D structure of aggregated helixes upon cooling [48]. κ-Carrageenan is structurally similar to agarose consisting of D-galactose and 3,6-anhydro-D-galactose units, with the

difference of the D-galactose residue being sulfated. The temperature-dependent gelation is assumed to be the same as for agarose, transitioning from a random coil structure at high temperatures to an ordered helical structure upon cooling in the presence of cations such as potassium, sodium, and calcium [49]. The cations shield the sulfate groups and reduce repulsion forces. The gelation temperature of κ-carrageenan solutions is therefore dependent on the cation concentration [50]. For this reason, the 0.75% κ-carrageenan solution was in a sol state at room temperature and beads were prepared by dripping the suspension in a KCl solution. The 1.5% κ-carrageenan solution, however, was in a gel state at ambient temperature and a film was prepared which was cut into cuboids after cooling. Due to the additional ions, beads prepared with 0.75% κ-carrageenan probably featured a higher gel strength than the 1.5% κ-carrageenan cuboids which were not further solidified by ion treatment. This likely resulted in the reduced malic acid production due to increased diffusion limitations even though the polymer concentration was lower (Figure 1, Table 1).

The diffusivity in the matrices was probably further influenced by the charged groups of the polymers. In acetate medium, the utilization of *A. oryzae* immobilized in 3% agar resulted in considerably reduced malic acid titers compared to the 1.5% agar cuboids as shown in Figure 1 and Table 1. With glucose, by contrast, no difference in malic acid production between *A. oryzae* immobilized in agar and κ-carrageenan was noticed, and titers were similar to the control with free biomass. Potentially, the diffusion of glucose, an uncharged monosaccharide, in the polymer matrices is easier than the diffusion of the negatively charged acetate anion. κ-Carrageenan and agar, particularly the agaropectin portion, feature sulfate groups and the alginate chains carboxyl groups. Especially in alginate beads, the diffusivity could be further hindered by the high pH values reached during cultivation in acetate, enforcing deprotonation. This possibly contributed to the larger difference in malic acid production between the tested alginate concentrations compared to fermentations with glucose (Figure 1, Table 1). Furthermore, it could explain the increased proportion of oxalic acid accompanied by a higher polymer concentration or enhanced growth within the matrix as observed for the alginate beads grown without carbon source (Table A1). The increased mass transfer limitations probably resulted in an undersupply of nutrients which likely caused a change in the metabolism. In our previous study, we observed an increased oxalic acid production associated with a low initial acetate concentration in the medium [8]. Oxalic acid can potentially be produced from oxaloacetate by oxaloacetate hydrolase as demonstrated for *A. niger* [51,52]. Another difference in the side product spectrum between the carbon sources was the increased succinic acid percentage observed with acetate (Tables A1 and A2). This is likely due to the utilization of the glyoxylate cycle as main pathway towards malic acid production with acetate, which we further discussed previously [8].

While the agar and κ-carrageenan matrices were stable in acetate medium, biomass detachment was observed in later stages of the fermentation as depicted in Figures 4 and S3. A possible explanation for this observation is fragmentation of the hyphae due to mechanical stress [53,54]. As fragmentation was observed in later fermentation stages, it was possibly associated with physiological changes caused by aging or nutrient limitation such as increased vacuolation which is a characteristic feature of the basal, older region of the hyphae [55,56]. It might be that increased vacuolation weakened the older or nutrient-deprived hyphae, making them susceptible to fragmentation which contributed to the observed biomass abrasion [57,58]. However, the involvement of vacuolation needs to be confirmed in further experiments. With glucose, complete biomass abrasion was not observed but the particles transitioned from a fluffy to a dense and smooth biomass layer, which was maintained until the end of the cultivation as pictured in Figure 2. Fragmentation of the long filaments also happened but still, a major part of the biomass was located around the surface of the particles. Potentially, the osmotic stress *A. oryzae* experiences in acetate medium contributes to these differences. Overall, malic acid production with immobilized *A. oryzae* was more successful in glucose media, as the immobilization matrices were stable and malic acid production was similar to the control except for the cultures with high

alginate concentrations. This suggests their suitability for prolonged use in repeated-batch or continuous processes which needs to be verified in further experiments.

Although challenges regarding the stability of the immobilization matrices were observed in acetate media, all immobilized particles showed improved malic acid production compared to the fermentation with free biomass in a bioreactor environment (Figure 6, Table 2). Compared to that of shake flask cultivations, the bioreactor fermentation with free biomass resulted in considerably reduced malic acid titers. For the fermentations with acetate the same cultivation conditions were chosen as for cultivations with glucose. While the scale-up worked well for the saccharide (Figure 5), the conditions were not suited for the process using acetate. Literature about malic acid production with *A. oryzae* using acetate is yet scarce and only Oswald et al. performed bioreactor fermentations with acetate derived from syngas fermentation, observing malic acid concentrations below 2 g/L [59]. Especially for the fermentation with free mycelium we observed an increased proportion of pyruvate (Table A2). This is unlikely to be linked with the addition of glucose as additional substrate since shake flask cultivations with 45 g/L acetate plus 5 g/L glucose did not show increased pyruvic acid production [8]. The accumulation of pyruvic acid might be associated with gas transfer limitations. *A. oryzae* presumably experiences oxygen limitation, reducing its ability to replenish redox equivalents required for the tricarboxylic acid cycle. We assume that the process needs to be optimized especially regarding the stirrer velocity and aeration rate. During the fermentation, we observed attachment of the filamentous biomass to the bioreactor installations such as baffles and the Rushton turbines. The improved performance of the immobilized structures can be explained by the fact that the entrapment in a defined matrix ensured a proper dispersion of the biomass in the bioreactor as extensive attachment was not observed. However, increased shear forces in the bioreactor setting caused an earlier disintegration of the alginate matrices and biomass detachment of the κ-carrageenan cuboids. Testing the κ-carrageenan and agar cuboids for the possibility of catalyst recycling and regrowth of the biomass layer could still be interesting. This would enable a repeated-batch or continuous process management, which is challenging with dispersed filaments due to difficulties in biomass retention. Possibly, performing surface immobilization on a synthetic material instead of polymer entrapment might be better suited for the fermentation in acetate media. Materials described for surface immobilization of *Aspergillus* species include macro porous polymeric sponge [60], polyurethane foam [61], nylon mesh [62], or celite beads [63].

The possibility of limiting the biomass formation to the beads was evaluated by performing experiments without carbon source in the preculture medium, and growth was observed with all matrices (Figure S3). This could be explained by either the consumption of the polymer or the glycerol in which the spores were suspended. Each 20 mL of the immobilization matrix, i.e., the amount for one triplicate, was prepared with 4 mL spore suspension consisting of 50% glycerol. Cultivation without an external carbon source was efficient in limiting the amount of free mycelium to a minimum. Interestingly, growth throughout the entire matrix was only observed with alginate. This might be explained by an enhanced diffusivity of nutrients and gas in this material. Growth throughout the matrix was also reported for *A. niger* immobilized in alginate upon nitrogen limitation to a value of 0.05 g/L NH_4NO_3 [11]. With a slightly higher concentration of 0.2 g/L NH_4NO_3, the formation of a biomass layer near the surface of the beads was observed. The citric acid concentration obtained with *A. niger* grown with 0.05 g/L NH_4NO_3, however, was considerably lower than with 0.2 g/L NH_4NO_3 [12]. This is in accordance with the results presented in this work as the infiltration of the alginate beads was associated with considerably reduced malic acid productivities (Figure 7, Table 3). Determining the optimum nutrient concentrations especially regarding the nitrogen content in both pre- and main culture can maximize acid production and limit the growth of free mycelium wherefore it should be further investigated for malic acid production with immobilized *A. oryzae* [16,64]. The occurrence of free biomass during the cultivation of entrapped

filamentous fungi can most likely not be prevented completely and some outgrowth seems to be necessary for efficient acid production.

5. Conclusions

This study investigated the production of L-malic acid with *A. oryzae* immobilized by entrapment in different natural polymers. Large differences were observed regarding the performance of the immobilization matrices with the two tested carbon sources acetate and glucose. Cultivation of immobilized *A. oryzae* in acetate medium was accompanied by several challenges, due to the low stability of the alginate beads and the abrasion of the biomass from the surface of the agar and the κ-carrageenan structures. Optimization regarding the medium composition and growth conditions can potentially improve the growth characteristics and malic acid production. However, immobilization was able to improve malic acid production with acetate in bioreactor cultivations by ensuring a thorough distribution of the biomass. Generally, further research is required regarding the optimization of malic acid production with acetate in a bioreactor setting. In contrast, all immobilization matrices were stable in glucose medium, and productivities of the immobilized cultures were similar to that of the control with free biomass, except for high alginate concentrations. In further experiments, the reusability and long-term production potential in repeated-batch or continuous processes should be evaluated.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation8010026/s1, Figure S1: Acetic acid and ammonium concentration as well as progression of the pH value for shake flask experiments using acetate as carbon source; Figure S2: Glucose and ammonium concentration as well as progression of the pH value for shake flask experiments using glucose as carbon source; Figure S3: *A. oryzae* immobilized in different matrices in the pre- and main culture. The preculture was grown without carbon source in the medium while the main culture medium contained acetate; Figure S4: Acetic acid and ammonium concentration as well as progression of the pH value for main culture shake flask experiments using immobilized *A. oryzae* grown without external carbon source in the preculture.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Acid distribution for shake flask cultivations of *A. oryzae* after 144 h. Values are means of triplicates with standard deviation.

Substrate	Immobilization Matrix	Organic Acid [%]						
		Malate	Succinate	Fumarate	Pyruvate	Ketoglutarate	Oxalate	Citrate
Acetic acid	Free biomass	50.8 ± 0.7	41.8 ± 0.7	4.2 ± 0.5	0.8 ± 0.1	0.3 ± 0.1	1.3 ± 0.3	0.7 ± 0.3
	3% Alginate	52.6 ± 1.2	38.9 ± 0.7	4.6 ± 0.2	1.0 ± 0.3	0.4 ± 0.1	2.1 ± 0.8	0.5 ± 0.4
	4% Alginate	48.4 ± 1.1	41.8 ± 1.0	5.1 ± 0.3	0.9 ± 0.3	0.5 ± 0.0	3.2 ± 1.9	0.1 ± 0.2
	5% Alginate	45.1 ± 2.5	36.3 ± 4.4	4.6 ± 1.0	0.6 ± 0.6	0.4 ± 0.4	12.5 ± 9.1	0.4 ± 0.7
	6% Alginate	34.4 ± 5.5	34.5 ± 1.8	3.1 ± 1.0	1.3 ± 0.2	0.3 ± 0.3	26.4 ± 8.1	0.0 ± 0.0
	1.5% Agar	48.5 ± 0.7	41.4 ± 1.4	5.1 ± 0.4	1.3 ± 0.1	0.4 ± 0.1	2.9 ± 1.2	0.4 ± 0.3
	3% Agar	48.5 ± 2.2	34.6 ± 5.7	5.6 ± 0.2	1.3 ± 0.2	0.3 ± 0.2	9.2 ± 2.6	0.6 ± 0.6
	0.75% κ-Carrageenan	43.8 ± 2.1	41.0 ± 2.8	4.8 ± 0.3	1.0 ± 0.2	0.6 ± 0.2	8.0 ± 1.1	0.8 ± 0.9
	1.5% κ-Carrageenan	49.0 ± 0.9	38.9 ± 1.0	5.4 ± 0.2	1.1 ± 0.1	0.4 ± 0.0	4.5 ± 0.6	0.7 ± 0.3
Glucose	Free biomass	73.3 ± 2.7	18.4 ± 1.4	1.7 ± 0.2	1.5 ± 0.6	0.4 ± 0.0	0.2 ± 0.0	4.6 ± 1.8
	1.5% Alginate	69.6 ± 1.6	19.1 ± 1.9	1.1 ± 0.1	2.5 ± 0.3	0.4 ± 0.0	0.2 ± 0.0	7.1 ± 0.5
	3% Alginate	72.3 ± 1.9	15.0 ± 1.8	1.7 ± 0.0	2.3 ± 0.4	0.3 ± 0.0	1.4 ± 0.5	7.0 ± 0.4
	4% Alginate	72.8 ± 0.8	15.0 ± 0.5	1.5 ± 0.2	2.8 ± 0.3	0.3 ± 0.0	0.8 ± 0.2	6.8 ± 0.4
	5% Alginate	70.2 ± 1.5	16.0 ± 0.7	1.9 ± 0.1	2.6 ± 1.0	0.3 ± 0.1	1.3 ± 0.7	7.7 ± 1.1
	6% Alginate	72.0 ± 1.8	14.7 ± 1.8	2.0 ± 0.2	2.2 ± 1.7	0.2 ± 0.1	1.8 ± 1.0	7.0 ± 0.7
	1.5% Agar	71.0 ± 2.1	16.3 ± 0.9	1.5 ± 0.2	3.0 ± 0.3	0.3 ± 0.0	0.8 ± 0.2	7.1 ± 1.6
	3% Agar	73.8 ± 0.9	15.6 ± 1.6	1.4 ± 0.0	2.7 ± 0.5	0.3 ± 0.0	0.6 ± 0.1	5.6 ± 0.4
	1.5% κ-Carrageenan	75.7 ± 0.5	13.8 ± 0.2	1.4 ± 0.1	2.9 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	5.3 ± 0.4

Table A2. Acid distribution for 2.5-L bioreactor cultivations of *A. oryzae* after 144 h. Values are means of duplicates with standard deviation.

Main Substrate	Immobilization Matrix	Organic Acid [%]						
		Malate	Succinate	Fumarate	Pyruvate	Ketoglutarate	Oxalate	Citrate
Acetic acid	Free biomass	23.1 ± 4.0	34.1 ± 3.9	1.4 ± 0.5	37.0 ± 10.5	2.1 ± 0.5	1.2 ± 0.9	1.1 ± 1.6
	3% Alginate	48.1 ± 2.0	35.0 ± 2.1	5.1 ± 0.6	10.6 ± 0.5	0.6 ± 0.0	0.6 ± 0.0	0.0 ± 0.0
	6% Alginate	43.6 ± 2.0	36.5 ± 1.4	5.9 ± 1.3	10.6 ± 0.3	1.0 ± 0.7	2.4 ± 0.3	0.0 ± 0.0
	1.5% κ-Carrageenan	37.9 ± 0.5	33.7 ± 0.0	5.1 ± 0.1	21.5 ± 0.7	1.1 ± 0.1	0.7 ± 0.1	0.0 ± 0.0
Glucose	Free biomass	80.7 ± 1.0	15.2 ± 1.5	1.6 ± 0.0	2.2 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.4
	1.5% Alginate	78.4 ± 6.3	17.7 ± 5.8	1.9 ± 0.1	1.2 ± 0.6	0.1 ± 0.1	0.2 ± 0.2	0.5 ± 0.8
	3% Alginate	82.0 ± 0.6	13.7 ± 1.1	2.0 ± 0.1	1.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.7 ± 0.4
	1.5% κ-Carrageenan	75.0 ± 1.9	21.1 ± 1.6	1.9 ± 0.2	1.5 ± 0.5	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.5

Table A3. Acid distribution for shake flask cultivations of *A. oryzae* after 144 h using acetate as carbon source in main culture, while preculture was grown without carbon source. Values are means of triplicates with standard deviation.

Substrate	Immobilization Matrix	Organic Acid [%]						
		Malate	Succinate	Fumarate	Pyruvate	Ketoglutarate	Oxalate	Citrate
Acetic acid	3% Alginate	13.5 ± 23.4	12.3 ± 21.3	2.9 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	71.4 ± 44.7	0.0 ± 0.0
	6% Alginate	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	99.1 ± 0.1	0.0 ± 0.0
	1.5% Agar	56.6 ± 1.3	34.0 ± 1.3	5.0 ± 0.0	1.2 ± 0.1	0.6 ± 0.2	1.4 ± 0.6	1.3 ± 0.4
	1.5% κ-Carrageenan	53.0 ± 2.4	35.8 ± 1.0	5.1 ± 0.2	1.2 ± 0.1	0.5 ± 0.2	2.1 ± 1.0	1.3 ± 0.5

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