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ENHANCED PHENYLPYRUVIC ACID PRODUCTION WITH *PROTEUS VULGARIS* BY OPTIMIZING OF THE FERMENTATION MEDIUM

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Alpha keto acids are important food additives, which commonly produced by microbial deamination of amino acids. In this study, production of phenylpyruvic acid (PPA), which is the alpha keto acid of phenylalanine was enhanced in 2-l bench scale bioreactors by optimizing of fermentation medium composition using the Box-Behnken Response Surface Methodology (RSM). Optimum glucose, yeast extract, and phenylalanine concentrations were determined to be 119.4 g l^{-1}, 3.7 g l^{-1}, and 14.8 g l^{-1}, respectively, for PPA production, and 163.8 g l^{-1}, 10.8 g l^{-1}, and 9.8 g l^{-1}, respectively, for biomass production. Under these optimum conditions, PPA concentration was enhanced to 1349 mg l^{-1}, which was 28% and 276% higher than the unoptimized bioreactor and shake-flask fermentations, respectively. Moreover, *P. vulgaris* biomass concentration was optimized at 4.36 g l^{-1}, which was 34% higher than under the unoptimized bioreactor condition. Overall, this study demonstrated that optimization of the fermentation media improved PPA concentration and biomass production in bench scale bioreactors compared to previous studies in the literature and sets the stage for scale up to industrial production.

Keywords: phenylpyruvic acid, *Proteus vulgaris,* optimization medium parameters, submerged fermentation

Phenylpyruvic acid (PPA) is the alpha keto acid form of phenylalanine and generally used in medicine, the food industry, and agriculture. In medicine, PPA is an important compound for the diagnosis of phenylketonuria, a genetic disease involving phenylalanine metabolism (Folling, 1994). Also, since PPA is a deaminated product, it is used in kidney patients' diets instead of amino acids to reduce urea accumulation in their body without changing the nutritional intake (Krause et al., 2010). Additionally, it was reported that compared with phenylalanine, PPA has important attributes in the development of aroma, taste, and texture in cheese and wine manufacturing (Casey et al., 2004). Another application of PPA and other keto acids is in poultry feed to reduce excessive nitrogen accumulation in the manure, which have adverse effects on the environment (Summer, 1993).

The commercial production of PPA is based on the removal of amino groups from phenylalanine via deaminase enzyme. Deaminase can be derived from several microorganisms, such as *Proteus*, *Morganella*, and *Provedincia* spp (Smit, 1966; Coban et al., 2014). There are a few studies in the literature conducted to produce PPA using microbial deaminases in shake-flask fermentations. *Eubacterium* species were used for the production of several alpha keto acids. In that study, 5.3 mg $l^{-1}$of PPA was produced by using *Eubacterium nodatum* (Itoh et al., 1994). Also, 15.8 mg l^{-1} of PPA production was reported by using black pigmented oral *Bacteroides* species (Tsuchiya et al., 1990). However, all PPA productions in the literature

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were performed in shake-flask fermentations without any optimization. Therefore, the aim of this study was to enhance PPA production by scaling up the production to bench-top bioreactors and optimizing the fermentation medium.

1. Materials and methods

1.1. Microorganisms and inoculum preparation

Proteus vulgaris (B-123) was obtained from the USDA Agricultural Research Service Culture Collection (Peoria, IL) and grown in 10 ml of trypticase soy broth (TSB) at 37 °C for 24 h. After incubation, cultures were stored at 4 °C as working cultures and regularly transferred to sterile fresh media every 2 weeks to maintain viability. Flasks containing 100 ml of TSB were inoculated with 1% (v/v) *P. vulgaris* and incubated at 37 °C with 150 r.p.m. agitation for 30 h until late log phase. Inoculums, which had ~10^6 cells/ml was used to inoculate the reactors.

1.2. Batch fermentation in bioreactors and optimization of the fermentation medium

Sartorius Biostat B Plus bioreactors (Sartorius, Allentown, PA) equipped with 2 l vessel with 1 l working volume were used in the experiments. The base fermentation medium consisted of 80 g of glucose, 8 g of yeast extract, 5 g of NaCl, 1 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 5 g of L-phenylalanine per litre of DI water. Temperature, pH, and aeration values were maintained at 34.5 °C, 5.12, and 0.5 vvm, as indicated by our previous optimizing study (Coban et al., 2014). Additionally, agitation was maintained at 300 r.p.m. for all fermentation runs. Fermentations were initiated by adding 1% (v/v) inoculum and samples were taken every 5 h until the 75th h, then analysed for biomass, PPA, and phenylalanine concentrations. Three medium nutrients, including glucose (80–180 g l^{-1}), yeast extract (1–15 g l^{-1}), and phenylalanine (5–20 g l^{-1}), were evaluated by Box-Behnken Response Surface Methodology (RSM) (Table 1). Biomass and PPA concentrations were the dependent variables of response, and analysed to optimize the fermentation medium composition to maximize the concentrations of each outcome using MINITAB statistical software (Version 15, Minitab Inc., State College, PA).

1.3. Validation of the model

Fermentations were run under the conditions identified as optimum with three replications to validate the RSM. Experimental data was compared with the predicted values from the model.

1.4. Analysis

Sample replicates collected during the fermentations were first subsampled to determine biomass content, then centrifuged (Model Galaxy, VWR, Radnor, PA) at 5 200 ×*g* for 15 min to remove the biomass. The supernatant was used for analysis of PPA and phenylalanine concentrations. Samples without centrifugation were used to determine biomass concentrations. All procedural details are indicated below.

1.4.1. Biomass. Absorbance of sample was measured at 620 nm by using a spectrophotometer (Beckman Coulter, Fullerton, CA) and biomass concentration was calculated using the standard curve equation (Biomass g l^{-1}=(0.4499×Absorbance–0.0075)×Dilution rate) to determine microbial concentration in the broth. This equation was obtained from preliminary experiments by measuring dry weight of the biomass versus absorbance.

1.4.2. Phenylpyruvic acid. Cell-free samples were obtained after centrifuging at 5200 × *g* for 15 min followed by filtering through 0.22 μm pore size syringe filters, which were then derivatized with 2,4-dinitrophenylhydrazine (DNPH) and analysed with HPLC as described in the literature (Elias et al., 2008).

1.4.3. Phenylalanine. Samples were derivatized with *o*-phthalaldehyde (OPA). The cell-free sample (0.1 ml) was mixed with 1 ml OPA and filtered into HPLC vials using 0.2 μm PTFE filters. Phenylalanine concentrations were measured at 230 nm by using the same HPLC system with 1 ml min^{-1} gradient flow of A: 50 mM sodium acetate (pH 7.2) and solvent B: 25% 50 mM sodium acetate (pH 7.2) and 75% methanol.

1.4.4. Statistical analysis. MINITAB Statistical Software package was used for statistical analyses. Analysis of variance (ANOVA) was performed for investigating statistically significant differences between PPA and biomass concentrations (P-value=0.05).

2. Results and discussion

2.1. Phenylpyruvic acid production in bioreactors

PPA was mostly produced in higher concentrations when the fermentations were run with either 80 or 130 g l^{-1} glucose concentrations compared to 180 g l^{-1} glucose runs. Maximum PPA concentration was measured as 1123 mg l^{-1} (run #12) and 1312 mg l^{-1} (run #2), when fermentations were run with 80 or 130 g l^{-1} glucose concentrations, respectively (Table 1). However, maximum PPA concentration was obtained as 1013 g l^{-1} (run #1) when fermentation was run with 180 g l^{-1}. It was also determined that PPA production increased when fermentations were run with low yeast extract concentrations. PPA concentration was always higher than 1000 mg l^{-1} and measured the highest as 1290 mg l^{-1} (run #3), when 1 g l^{-1} yeast extract was used in the fermentations. Moreover, the highest PPA concentration was measured as 1312 mg l^{-1}, when the fermentation was run with 12.5 g l^{-1} phenylalanine (run #2). However, PPA concentrations were measured as 1098 mg l^{-1} (run #10) and 1290 g l^{-1} (run #3), when the medium included 5 g l^{-1} and 20 g l^{-1} phenylalanine concentrations, respectively.

2.2. Biomass production in bioreactors

On the other hand, biomass concentration was also affected by the different concentrations of evaluated medium parameters. Overall, biomass concentration was measured the lowest as 2.19 g l^{-1} and the highest as 4.09 g l^{-1} in the runs (Table 1). Higher biomass concentrations were obtained when high glucose concentrations were used in the fermentation medium. The highest biomass concentrations were measured as 4.09 g l^{-1} (run #9) and 3.99 g l^{-1} (run #6), when 130 g l^{-1} and 180 g l^{-1} glucose was used in the runs, respectively. However, maximum

biomass concentration was measured as 2.98 g l^{-1} when 80 g l^{-1} glucose was used in the bioreactors (run #4). Also, high yeast extract concentrations increased biomass concentrations in the productions. The biomass concentration was measured as 3.12 g l^{-1} (run #1) when 1 g l^{-1} yeast extract was used in the productions. However, maximum biomass concentration was increased to 3.99 g l^{-1} (run #6 and run #8) and 4.09 g l^{-1} (run #9) when 15 g l^{-1} and 8 g l^{-1} yeast extract was used in the fermentations, respectively. However, different phenylalanine concentrations had no significant effect on the biomass production (Table 1).

Table 1. Box-Behnken response surface design for phenylpyruvic acid and biomass concentrations

Run order	Glucose (g l^{-1})	Yeast extract (g l^{-1})	Phenylalanine (g l^{-1})	Measured PPA (mg l^{-1})	Predicted PPA (mg l^{-1})	Measured biomass (g l^{-1})	Predicted biomass (g l^{-1})
1	180	1	12.5	1013	1018	3.12	2.97
2	130	8	12.5	1312	1308	4.05	4.07
3	130	1	20.0	1290	1258	2.39	2.59
4	80	8	5.0	911	884	2.98	3.03
5	80	1	12.5	1165	1199	2.19	2.15
6	180	15	12.5	991	957	3.99	4.02
7	130	15	20.0	1039	1046	3.26	3.27
8	130	15	5.0	917	949	3.99	3.79
9	130	8	12.5	1304	1308	4.09	4.07
10	130	1	5.0	1098	1091	2.75	2.74
11	180	8	20.0	922	949	3.75	3.70
12	80	8	20.0	1123	1121	2.88	2.71
13	130	8	12.5	1308	1308	4.08	4.07
14	180	8	5.0	921	923	3.88	4.05
15	80	15	12.5	912	907	2.68	2.83

2.3. Optimization of phenylpyruvic acid production

A second order polynomial equation (Eq. 1) was created and ANOVA was performed by MINITAB software to show the predicted values, effects of glucose, yeast extract, and phenylalanine concentration on PPA productions.

$$PPA(mg\,l^{-1}) = -563.358 + 20.8123\times(G) - 1.98997\times(YE) + 90.4033\times(P) - 0.0809\times(G\times G) - 1.7449\times(YE\times YE) - 2.42667\times(P\times P) - 0.165\times(G\times YE) - 0.140667\times(G\times P) - 0.333333\times(YE\times P) \quad (1)$$

where "G" is glucose, "YE" is yeast extract, and "P" is phenylalanine concentration.

The model has a high R^2 value (0.9828) indicating that the model fits the experimental data well. To show the good fit, experimental and predicted values were plotted (not shown) and the slope of the best fitted line was determined as 0.9832, which is very close to "1". ANOVA showed that glucose, yeast extract, and phenylalanine concentrations have significant

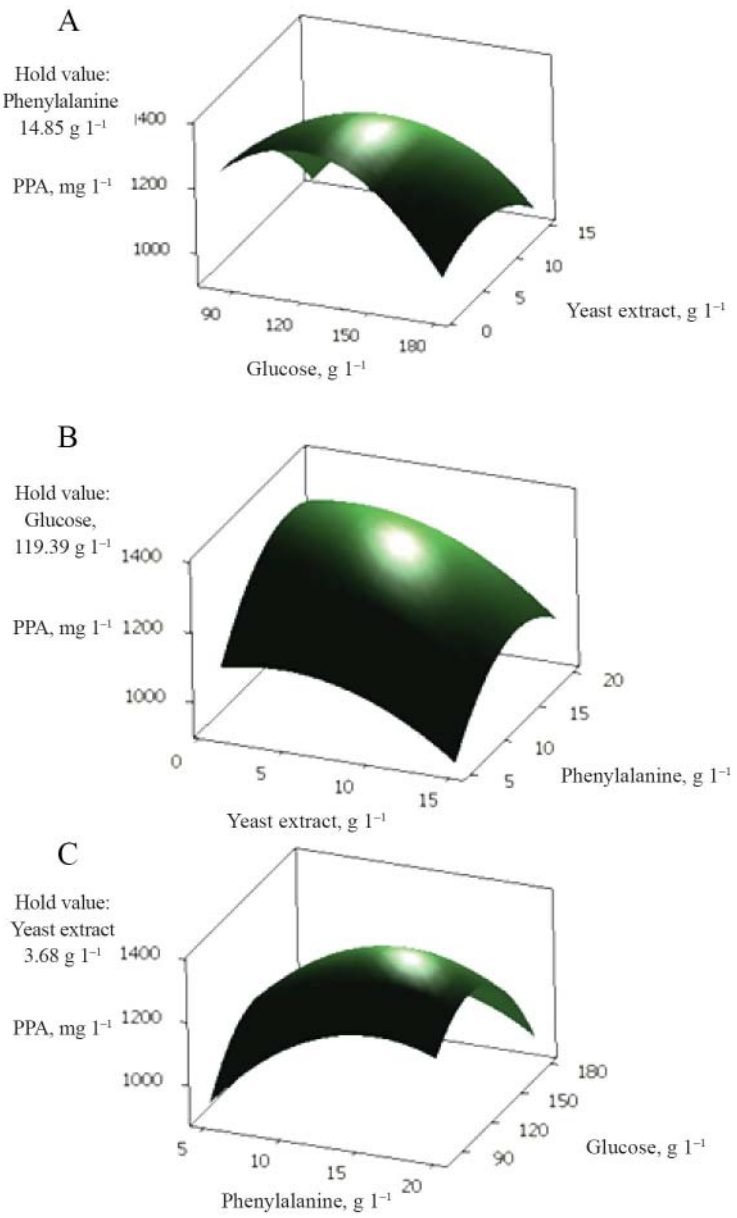

Fig. 1. Phenylpyruvic acid production profile under various medium conditions

effect ($P<0.05$) on PPA production (data not shown). Also, optimization results suggested that the maximum PPA concentration can be obtained as 1349 mg l^{-1} with 119.4 g l^{-1} glucose, 3.68 g l^{-1} yeast extract, and 14.85 g l^{-1} phenylalanine concentrations (data not shown). Additionally, PPA concentration trends with changing medium parameters are shown in Fig. 1. PPA production was increased when glucose concentration increased until 120 g l^{-1}. However, production decreased remarkably at higher glucose concentrations (Fig. 1A). Figure 1B clearly shows that PPA was produced in higher concentrations when low yeast extract concentrations were used in the fermentations. When yeast extract concentration was increased to more than 5 g l^{-1}, PPA production decreased significantly. Also, there is a direct relationship with PPA concentration and phenylalanine concentration in the fermentations. As it is shown in Fig. 1C, PPA production increased remarkably by increasing phenylalanine concentration till 15 g l^{-1}, and remained almost constant at higher phenylalanine concentration values.

2.4. Optimization of biomass production

Another second order polynomial equation (Equation 2) was created and ANOVA was performed by MINITAB for biomass production in the fermentation runs.

$$\text{Biomass (g l}^{-1}) = -1.80713+0.0498769\times(G)+0.269655\times(YE)+0.127658\times(P)-0.000160667\times(G\times G)-0.0138095\times(YE\times YE)-0.00531852\times(P\times P)-0.000271429\times(G\times YE)-0.00002\times(G\times P)-0.0017619\times(YE\times P) \quad (2)$$

where "G" is glucose, "YE" is yeast extract, and "P" is phenylalanine concentration.

The biomass prediction model has also a high R^2 value (0.9698), which shows that the process was represented successfully. Fitted slope between measured and predicted biomass values was determined as 0.9697 (not shown). Moreover, the model has a good R^2 adjusted value (0.9153), which also shows the model can also predict biomass concentrations successfully out of the studied growth parameter ranges. ANOVA showed that all main factors, expect for phenylalanine concentration, are significant on *P. vulgaris* biomass production ($P<0.05$). Optimization results showed that maximum biomass concentration can be obtained as 4.35 g l^{-1} at 163.8 g l^{-1} glucose, 10.75 g l^{-1} yeast extract, and 9.84 g l^{-1} phenylalanine concentrations (data not shown). It is reasonable that higher glucose concentrations are needed for the biomass growth comparing to PPA production, since carbon source directly affects the microbial growth. Similarly, optimum yeast extract concentration for biomass growth is higher than for PPA production. It was also shown that alpha keto acids are produced in higher concentrations under low initial nitrogen concentrations in the fermentation medium (Raunio, 1966; Nesbakken et al., 1988; Chernyavskaya et al., 2000). It can be explained that microorganisms use more amino acid in the fermentation medium to maintain nitrogen intake in the low yeast extract concentration conditions. This can be explained by that, high PPA production needs low yeast extract and high phenylalanine concentration, whereas opposite conditions are needed for biomass production.

Effects of medium ingredient concentrations on *P. vulgaris* biomass concentration are shown in Fig. 2. Biomass production increased with the glucose concentration until 160 g l^{-1} and remained almost the same at higher concentrations (Fig. 2A). Yeast extract concentration remarkably increased biomass concentration until 10 g l^{-1} (Fig. 2B). Figure 2C shows that phenylalanine concentration had no significant effect on biomass formation.

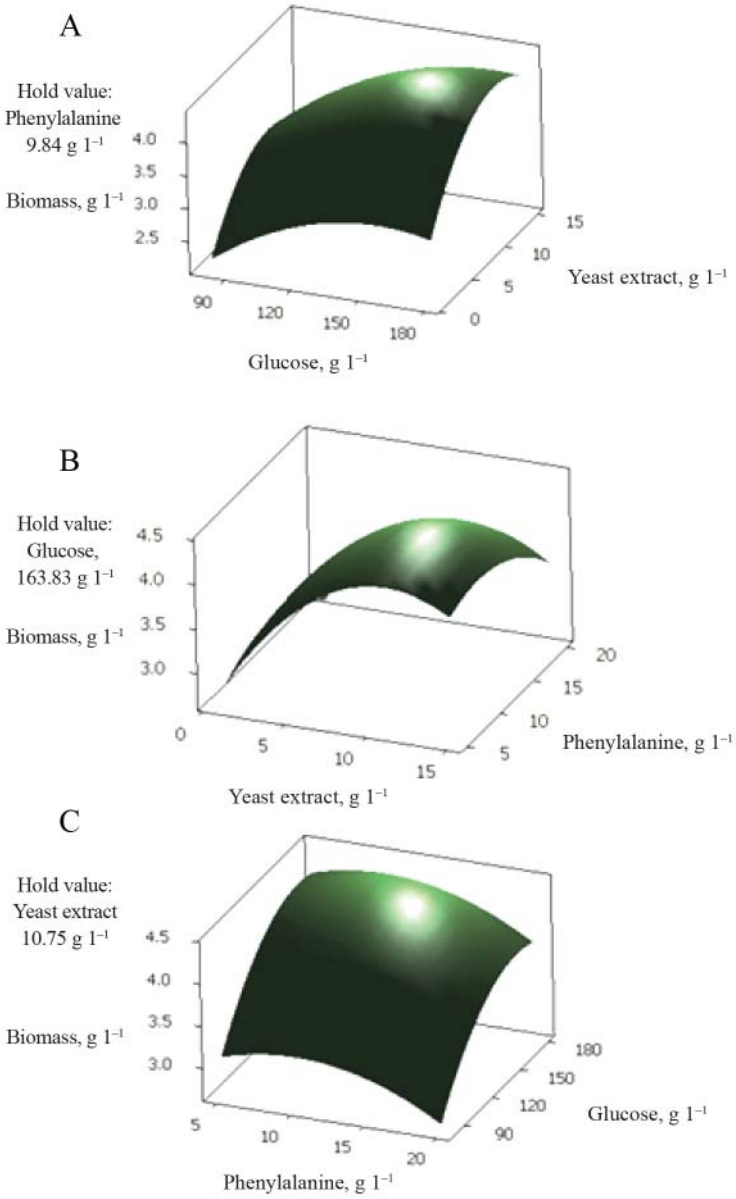

Fig. 2. P. vulgaris biomass production profiles under various medium conditions

2.5. Validation of the optimum conditions

Batch fermentations were conducted in triplicate at the conditions identified as optimum for both PPA and biomass productions. The maximum PPA and biomass concentrations were measured as 1350 mg l^{-1} (Fig. 3) and 4.35 g l^{-1} (Fig. 4), respectively, under their specific optimum conditions. These results were very close to the estimated values. PPA and biomass concentration increased in the fermentation medium with a same pattern. However, almost 54% of produced PPA was lost in the broth at the 75th hour of fermentation, whereas biomass concentration decreased slightly. This may be explained by usage of PPA by microorganisms as a nutrient, further degradation of PPA to aromatic compounds, breaking down of produced PPA by hydrogen peroxide effect, which was produced during microbial deamination reaction. It was reported that amino acid deamination reaction byproduct, which is hydrogen peroxide, can deactivate enzyme and/or substrate and causes low yields at the end of the process (Lafuente et al., 1988; Xu et al., 2008). Under the determined optimum fermentation medium for PPA production, maximum PPA production and biomass production rates were calculated as 48 mg PPA l^{-1} h^{-1}, and 175 mg biomass l^{-1} h^{-1}, respectively (Fig. 3). Also, under the determined optimum fermentation medium for biomass production, maximum PPA and biomass production rates were calculated as 42 mg PPA l^{-1} h^{-1} and 179 mg biomass l^{-1} h^{-1}, respectively (Fig. 4), which shows slightly higher biomass production but remarkably less PPA production.

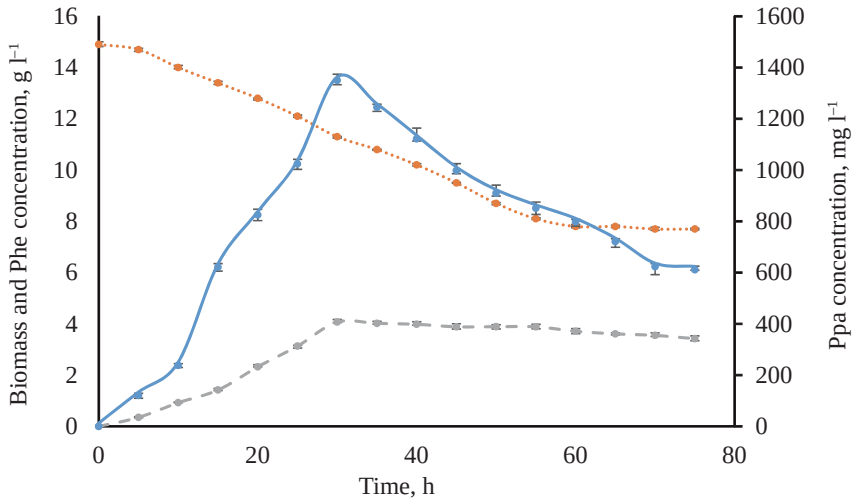

Fig. 3. Phenylalanine consumption, PPA and biomass accumulation under optimum medium conditions for PPA production (119.39 g l^{-1} glucose, 3.68 g l^{-1} yeast extract, 14.84 g l^{-1} phenylalanine).
•●•: Phe (g l^{-1}); • •●• •: Biomass (g l^{-1}); —●—: Ppa (g l^{-1});

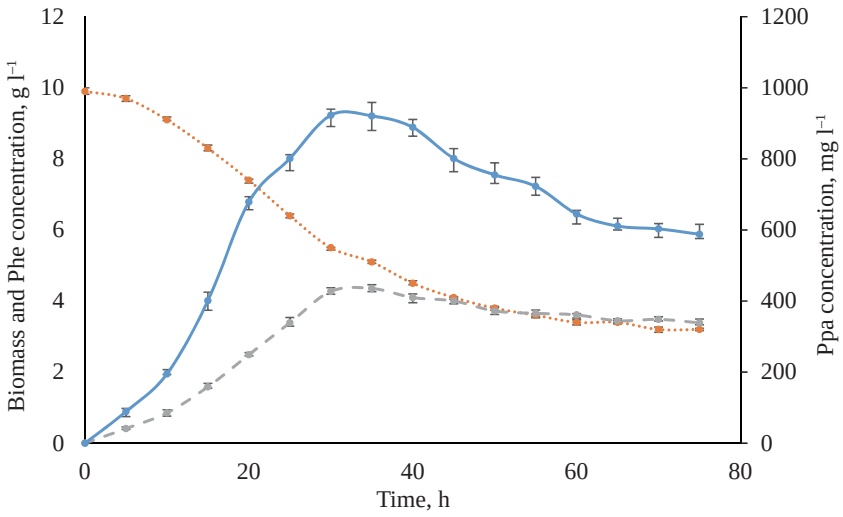

Fig. 4. Phenylalanine consumption, PPA and biomass accumulation under optimum medium conditions for biomass production (163.83 g l^{-1} glucose, 10.75 g l^{-1} yeast extract, 9.84 g l^{-1} phenylalanine). ●: Phe (g l^{-1}); ●: Biomass (g l^{-1}); ●: Ppa (g l^{-1})

3. Conclusions

In conclusion, optimum medium conditions for PPA production were determined as 119.4 g l^{-1} of glucose, 3.68 g l^{-1} of yeast extract, and 14.85 g l^{-1} of phenylalanine, whereas 163.8 g l^{-1} glucose, 10.75 g l^{-1} yeast extract, and 9.84 g l^{-1} phenylalanine for biomass production. Under the specific optimum conditions maximum PPA and biomass concentrations were measured as 1350 mg l^{-1} and 4.35 g l^{-1}, respectively. PPA production was increased by 2.8-fold compared to shake-flask fermentations and 0.3-fold compared to bioreactor productions under optimized growth parameters. Additionally, it was shown that glucose, yeast extract, and phenylalanine concentrations had significant effect on PPA production. However, phenylalanine concentration was found to be insignificant on biomass production (P-value: 0.063). This study clearly demonstrated that PPA production has been enhanced, which can be a step forward for commercial production.

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