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Scaling Up for the Industrial Production of Rifamycin B Fed-Batch Production Mode in Shake Flasks and Bench-Scale Fermentor

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Abstract

The production of rifamycin B using the gene amplified variant of *Amycolatopsis mediterranei* (NCH) was initially optimized in shake flasks through medium modifications and fed-batch addition of uracil. The yield was increased by 21.7% (from 11.7 to 14.3 g/l) when F2m1 medium was used. The production was further verified and optimized in fed-batch-mode in a laboratory fermentor using F2m3 medium and the optimized conditions (agitation 500 rpm, aeration; 1.5 for 3 days then control DO at 30% thereafter, pH; 6.5 for 3 days then 7 thereafter and control temperature at 28°C). Fed-batching of glucose syrup (5% v/v at day 3) and glucose (1% at days 6 and 8) increased the yield from 17.8 to 20.9 g/l (17.3%) at day 10. A yield of upto 20 g/l was recorded when 0.1% uracil was fed-batched at day 2. Integration of the most optimum conditions for fed-batching glucose syrup, glucose and uracil further increased the yield from 17.8 to 24.8 g/l (39%) in 10 days. The overall optimization of rifamycin B production increased the yield almost 2 folds. Statistical analysis revealed that there is a significant increase in rifamycin B production by using One-Way ANOVA at $p<0.05$ in all the tested fed-batch addition regimes.

Keywords: Carbon sources; Fermentor; Nitrogen sources; Productivity; Rifamycin B; Scaling up

Introduction

Fermentation media, cultural conditions and operation modes are the most important factors in a production process, providing the main environment of the organism. In the particular case of secondary metabolites, the interaction between growth metabolism and product secretion is often critically influenced by growth limiting nutrient concentrations [1]. Hence, media composition especially with the critical components shall be optimized to produce the maximum yield of product and minimal yield of undesired products. In these regards, fed-batch fermentation is an important operation mode to optimize and increase the productivity of secondary metabolites, such as antibiotics [2]. Fed-batch cultivation is generally superior to batch processing when changing nutrient concentrations affect the productivity and yield of the desired product [2].

In case of rifamycin B production, the consumption of the reducing sugar is common in the stationary phase although the mycelium growth almost stopped. This consumption is usually directed for rifamycin B biosynthesis and mycelium maintenance; therefore, fed-batching of carbon source such as glucose to maintain the reduced sugar at 1-1.5% is recommended [3]. Besides, relatively large amounts of nitrogen are necessary to stimulate rifamycin B production [4]. In addition, nitrate stimulates rifamycin production by its regulatory effect on lipid and rifamycin B biosynthetic pathways [5]. The addition of organic nitrogen compounds is generally recognized to be essential for high yields of rifamycin B production by serving either as a precursor or stimulant for antibiotic biosynthesis [4].

Enlarging the production scale is usually associated with reduction in the productivity due to the complexity of fermentation process [3]. Therefore, it is of great importance to study the scaling-up of the fermentation process and adopt suitable strategy in order to increase the productivity of the desired product on the industrial level. In this perspective, the current study aimed to improve the fermentation process parameters and achieve better yield of rifamycin B. Thus, further optimization of the process was carried through fed-batch addition of different carbon and nitrogen sources in shake flasks. Scaling up of the process previously developed in shake flasks on fermentation experiments was carried out in bench-scale laboratory fermentor. Integration of the optimum physical conditions previously obtained in the fermentor and the fed-batch additions of some carbon and nitrogen sources were studied.

Materials and Methods

Bacterial strains

The amplified variant NCH of *Amycolatopsis mediterranei*–RCP 1001, previously obtained by [5], was maintained on Q/2 agar slants and stored at 4°C to be used within 27 days. The strain was propagated on Bennett's agar. For long-term storage, the surface growth on Q/2 agar slants was harvested in 10% skim milk and lyophilized.

Chemicals

Chemicals used throughout this work were of laboratory reagent grade, unless otherwise indicated. Glucose monohydrate, sucrose, NH_4NO_3, $NaNO_2$ were the products of ADWIC, Egypt. Uracil, leucine, methionine, cystine, glycine, Para-amino benzoic acid (PABA) and phenylalanine, were the products of Sigma, St. Louis, USA. Sodium diethyl barbiturates (SDB) were the product of Grindsted vaerket A/S, Denmark. Potassium sodium tartarate tetrahydrate, 3,5-dinitrosalicylic acid, $CaCO_3$, $MgSO_4.7H_2O$, KH_2PO_4, $(NH_4)_2SO_4$, NaOH and HCl were

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the products of E. Merck, Darmstadt, Germany. Glacial acetic acid was the product of Aldrich Ltd, England.

Media

Yeast extract, malt extract, beef extract, tryptone, soytone, skim milk and bacto agar were the products of Difco Laboratories, Detroit, USA. Oat flakes and sunflower oil were obtained from commercial suppliers. Corn-steep liquor and glucose syrup were obtained from the Egyptian Co. for Production of Starch and Glucose, Cairo. Media used for propagation, selection and maintenance as well as the vegetative medium V2 were those previously reported by [5,6]. The modified fermentation medium F2m1 and F2m3 were previously reported [4,7].

Inoculum Preparation and Analytical Regime

The methods used for maintenance, propagation, selection and preparation of inoculums as well as determining of remaining glucose or reducing sugars concentration, biomass and assay of rifamycin B were those previously reported by [4,6].

Rifamycin B Production in Shake Flasks

Initial optimization on the shake flasks was conducted as previously reported by [5,6]. For fed-batch experiments, aliquots of 0.5 ml of corn-steep liquor, uracil, leucine, methionine, cystine, glycine, phenyl-alanine, para aminobenzoic acid (PABA), sodium diethyl barbiturate (SDB) and NH_4NO_3 were taken from sterile stock solutions containing suitable concentrations, and added to the fermentation culture at the indicated times to give the required final concentrations (Table 1).

Variable Factors	Concentration (g%)	Rifamycin B conc. at day 8 (g/l)	% of change
Control*		11.75	
Carbon source Sucrose [1]	5.0	4.0	-66
	7.5	3.5	-70
	10	3.0	-74
	12	2.7	-77
Organic Nitrogen Corn-steep liquor[2]	0.5	11.2	-4.5
	1.0	13.82	+17.5
	1.5	12.8	+9
	2.0	12.5	+6.5
Phenyl alanine[3]	0.1	6.12	-48
Leucine[3]	0.1	6.18	-47
Glycine[3]	0.1	5.5	-53
Methionine[3]	0.1	9.15	-22
Cystine[3]	0.1	3.1	-74
PABA	0.1	7.79	-34
	0.1	5.1	-56
Nucleotide base Uracil[6]	0.05	11.7	-0.5
	0.10	14.28	+21.5
	0.15	12.33	+5
	0.10	12.09	+3
Barbiturates SDB	0.1 [7]	10.44	-11
	0.1[8]	9.35	-20

[1]Sucrose was used instead of glucose.
[2]Corn-steep liquor was added on days 2, 3 and 4. The presented results were only for day 3 (the one with the highest antibiotic productivity).
[3]Phenyl alanine, leucine, glycine, cystine or methionine were added on day 2.
[4]Para-aminobenzoic acid (PABA) was added at day zero.
[5]Para-aminobenzoic acid (PABA) was added at day 2.
[6]Uracil was added on days 0, 1, 2, 3 or 4. The presented results were of only day 2 addition (the one with the highest antibiotic productivity).
[7]Sodium diethyl barbiturate (SDB) was added at day 2 instead of day zero.
[8]Sodium diethyl barbiturate (SDB) was further added at day 2.
*The following are the main constituents of F2m1 medium: dextrose: 120 g, soytone: 30.0 g, NH_4NO_3: 1.0 g, KH_2PO_4: 1.0 g, $CaCO_3$: 8.5 g, $MgSO_4.7H_2O$: 0.8 g, sodium diethyl barbiturate: 1.0 g and distilled water to 1000 ml.

Table 1: Effect of different modifications in F2m1 medium on rifamycin B production by NCH strain in shake flasks on day 8.

Rifamycin B Production in Laboratory Scale Fermentor

The production of rifamycin B was carried out in a 6.6 liter laboratory glass fermentor (Bioflo 3000 Benchtop fermentor, New Brunswick Scientific Co., NJ, USA) as previously described by [7]. For fed-batch experiments, aliquots of 100 ml of glucose, 150 ml of glucose syrup, 50 ml of NH_4NO_3, 50 ml corn-steep liquor and 25 ml uracil were taken from sterile stock solutions containing suitable concentrations, and added to the fermentation culture at the indicated times to give the required final concentrations (Tables 2-4).

Samples were periodically withdrawn every 24 hours for analysis of rifamycin B concentration, remaining reducing sugars concentration and dry cell weight. These analysis regimens were conducted as previously reported by [5,6]. Unless otherwise specified, the fermentation medium used was the F2m3 medium with the following adjustment; 5% glucose syrup was added after 3 days of incubation and sunflower oil as antifoam. The pH of the medium was automatically controlled at 6.5 for 3 days then 7 thereafter by the fermentor's control unit which applied 1N HCL and 1N NaOH actuated by peristaltic pumps. The agitation was 500 rpm, the aeration was 1, 1.5 or 2 vvm for 3 days then the dissolved oxygen concentration (DO) was controlled at 30% of saturation thereafter. The temperature was controlled at 28°C, unless otherwise indicated.

Results

Rifamycin B production in shake flasks

In general substitution of the carbon source with sucrose or addition of different nitrogen sources such as leucine, methionine, cystine, glycine, phenyl-alanine and para aminobenzoic acid (PABA) failed to increase the yield (Table 1). However, addition of corn-steep liquor at all tested concentrations and different times of the fermentation process fairly increased rifamycin B production (Table 1). The highest improvement (17%) was obtained when 1% corn-steep liquor was added after 3 days of incubation.

Moreover, batch-wise addition of 0.1, 0.15 and 0.2% of the nucleic acid uracil to F2m1 medium at different times of the fermentation process increased the yield (Table 1). The highest antibiotic production was obtained upon the addition of 0.1% uracil at day 2, where the yield increased from 11.75 to 14.28 g/l (21.5 %). Interestingly, the change of addition time of SDB to day 2 or further addition of SDB after 2 days of incubation decreased the yield (Table 1).

Rifamycin B production in laboratory scale fermentor

The addition of glucose syrup (GS) at day 3 instead of day 4 at aeration rate of 1 vvm had almost no effect on the production yield however; successive addition of GS at day 4 then at day 7 decreased the production using 1 vvm (Table 2). Addition of GS at day 3 instead of day 4 at aeration rate of 1.5 vvm slightly increased the yield to 17.8 g/l (Table 2). Furthermore, application of the last mentioned regime using aeration rate of 2 vvm also increased the yield (data not shown).

Similarly, the addition of GS at day 3 followed by addition of glucose at day 6 using 1 vvm aeration had no effect on the production yield while when this addition regimen was applied at aeration rate of 1.5

Set No.	Variable factor	Final conc. (g%)	Time of addition (days)	Aeration (vvm)	Rifamycin B conc. at day 10(g/l)
Control	**Glucose syrup**	33.3	4	1	17.10 ± 0.20[a]
C1	**Glucose syrup**	33.3	3	1	16.87 ± 0.351[a]
				1.5	17.80 ± 0.30[X]
			4 then 7[1]	1	14.95 ± 0.301[a]
C2	**Glucose syrup**	33.3	3	1	16.87 ± 0.351[b]
	Glucose	1	6		
C3	**Glucose syrup**	33.3	3	1.5	19.07 ± 0.305[y]
	Glucose	1	6		
C4	**Glucose syrup**	33.3	3	1	18.53 ± 0.251[c]
	Glucose	1	6 then 8[1]		
C5	**Glucose syrup**	33.3	3	1.5	20.83 ± 0.20[z]
	Glucose	1	6 then 8[1]		

[1] The glucose or glucose syrup was added successively during the same fermentation.
The different letters mean that there is a significant difference by using Tukey HSD multiple comparison test at $p<0.05$
a, b, c showed significant increase using aeration rate of 1 vvm x, y, z showed significant increase using aeration rate of 1.5 vvm.
SD=Standard Deviation
F-value and p-value for aeration rate of 1 vvm is 192.25 and 0.000, respectively.
F-value and p-value for aeration rate of 1.5 vvm is 153.75 and 0.000, respectively.

Table 2: The recorded yield of rifamycin B production (mean ± SD) by NCH strain using F2m3 medium during optimization by fed-batching of glucose syrup with or without glucose using aeration rates of 1 and 1.5 vvm in 6.6 l fermentor.

Variable factor	Final conc. (g%)	Volume added (ml)	Time of addition (days)	Feed rate (l/day)	Feed rate (g/day)	Rifamycin B conc. at day 10 (g/l)
Control	33.3	150	3	.....		17.8*
Glucose syrup	33.3%	150	3	0.504	36	15.5**
Glucose syrup	4%	1500	3	0.24	12	18.5[(1)]
Glucose syrup	4%	1500	3	2.88	144	17.0[(2)]
Glucose syrup	4%	1500	3	6.768	338.4	16.5[(2)]
						14.8[(2)]

(*) The control at the following conditions, which were kept constant in all investigations: the fermentation medium, F2m3 with 5% glucose syrup was added at day 3, unless otherwise indicated; working volume, 2.5 L; incubation temperature, 28°C, unless otherwise indicated; inoculum size, 5%; aeration was 1.5 vvm, agitation; 500 for 3 days then the DO was controlled at 30% of saturation thereafter; the incubation period, 240 hours and the fermentation process was pH controlled at 6.5 for 3 days then 7 thereafter.
(**) The control at all previously mentioned conditions with minor change where the incubation temperature was 30°C for 3 days then 28°C thereafter.
[(1)]The initial volume: 2.5 l, the final volume: 2.56 l and the temperature: 28°C.
[(2)]The initial volume: 1.5 l, the final volume: 3.0 l and the temperature: 30°C for 3 days then 28°C thereafter.

Table 3: Effect of rate of addition of glucose syrup on rifamycin B production by NCH strain 345 using F2m3 medium using aeration rate of 1.5 vvm in 6.6 l fermentor.

vvm (set C3), the yield was significantly increased to 19 g/l. When the GS was added at day 3 then glucose was added at day 6 and then at day 8 using 1 vvm aeration rate 155 (Set C4) the production yield increased to 18.5 g/l. The highest production yield (20.8 g/l) was obtained when the last regimen was applied at 1.5 vvm (set C5) (Table 2). Interestingly, when the same regime (C3 or C4) was tried using aeration rate of 2 vvm an increase in the yield (up to 15.5%) was recorded (data not shown).

Effect of the rate of fed-batch of the carbon source

The fed-batch of GS at a rate of 36 g/day (0.504 l/day) increased the rifamycin B production from 17.8 to 18.5 g/l at day 10 (Table 3 and Figure 1A). However, the dry cell weight stayed at almost the same order of magnitude of the control (Figure 1B). Another approach of fed batch was applied through continuous addition of 1.5 L of 5% GS, starting from day 3, to 1.5 liter of the fermentation medium, with different feed rates (12,144 and 338.4 g/day) at different temperature control regimes (Table 3). The yield increased from 15.5 to 16.5 and 17 g/l upon using the rate of 144 and 12 g/day, respectively (Table 3). However, when this fed batching rate increased to 338.4 g/day, the production yield decreased to 14.8 g/l (Table 3 and Figure 1A). Interestingly, the highest biomass was recorded for rate of 338.4 g/day while using 144 and 12 g/day (2.88 and 0.24 l/day) decreased the biomass (Figure 1B). In all tested fed rate of continuous fed batching of GS, the remaining reducing sugar were almost at the same order of magnitude (Figure 1C)

Fed-batching of nitrogen sources

The batch-wise addition of all tested nitrogen sources improved the production yield of rifamycin B (Table 4). The highest impact was recorded when 0.1% of uracil at day 2 where the production yield increased by 12.5% (20 g/l). This was followed by NH_4NO_3 and Corn-steep liquor (Table 4). Interestingly, at all applied fed batching regimen, the recorded dry cell weight and remaining reducing sugars' concentration were comparable with those of control (data not shown).

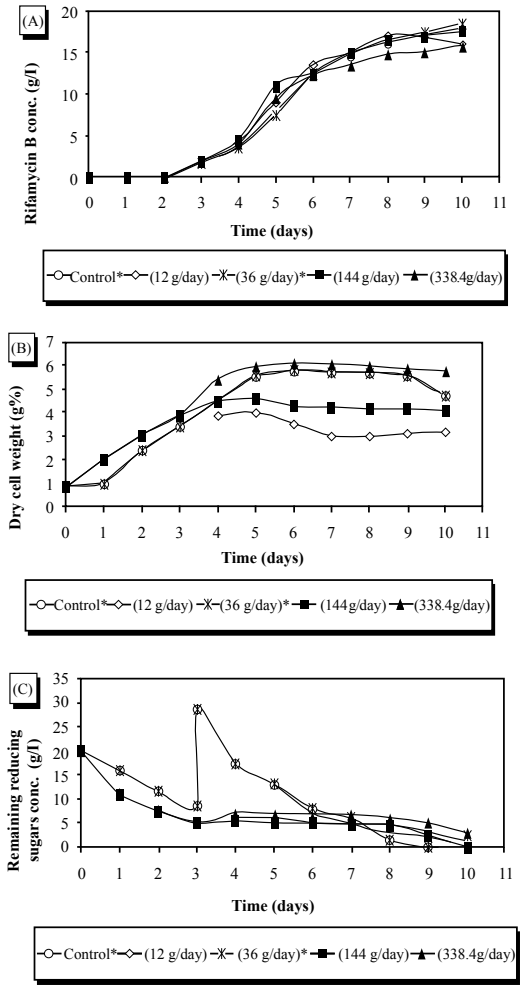

Figure 1: Effect of the feed rate of addition of 5% glucose syrup at day 3 on **(A)** rifamycin B production, **(B)** dry cell weight and **(C)** remaining reducing sugars concentration using NCH strain in 6.6 liters fermentor under the following conditions: aeration, 1.5 vvm for 3 days then DO control at 30% of saturation thereafter; agitation, 500 rpm; pH, control at 6.5 for 3 days then 7 thereafter; working volume, 2.5 l (*) or 1.5 l; medium used, F2m3; temperature, 28°C (*) or 30°C for 3 days then 28°C thereafter.

Integration of optimum conditions

Generally, integrated batch-wise addition of both carbon and nitrogen sources resulted in a significant increase in the production yield (Table 4). The addition of corn step liquor had the lowest improvement effect (9.5%) on the production yield (Set N2; Table 4). The highest improvement effect (40%) was recorded when uracil was fed-batched at day 2 then GS was added at day 3 followed by glucose addition at days 6 and 8 (Set N7; Table 4). This was comparable to the results recorded when the last regimen was applied but with glucose addition at day 6 only (Set N3; Table 4). On the other hand, when NH_4NO_3 was fed-batched at day 3 with GS addition at day 3 and then glucose addition at day 6, the yield increased only by 14%. This improvement reached 30% when the NH_4NO_3 was fed-batched with GS at day 3 followed by 195 glucose addition at days 6 and 8 (Set N6; Table 4). Almost the same pattern for dry cell weight and remaining reducing sugars concentrations was recorded for applied regimens (data not shown).

Discussion

The fed-batch addition of either inorganic or organic nitrogen sources in shake flasks had an improvement effect on the production yield. Previously, El-Tayeb et al. [4] have reported an increase of the production yield by 47% through fed-batch addition of carbon, inorganic or organic nitrogen sources. The highest effect (17.5–21.5%) was recorded to addition of 1% corn steep liquor at day 3 and 0.1% uracil at day 2, respectively with preferable effect towards the addition of uracil. Similarly, Krishna et al. [8] reported that using 0.2% of uracil was favorable for maximum production of rifamycin SV. This stimulation could be due to the development of more active system for rifamycin production or less active system for rifamycin inhibition [8].

Scaling up and optimization of antibiotic production in the fermentor necessitates studying influences of different physical and physiological factors affecting its production; taking into concern that the optimal fermentation conditions are related to the strain used [9]. Fed-batch addition of 5% glucose syrup at day 3 or day 4 gives almost the same yield (17–17.5 g/l) and even further addition of 5% glucose syrup at day 7 decreased the yield to 14.9 g/l. This reduction could be due to the presence of undesirable ingredients, such as hydroxyl-methyl furfural, in glucose syrup which may lead to inhibition of rifamyin B biosynthesis and/or biomass formation [5]. Thus to increase the yield of rifamycin B, attempts were made to maintain the level of the carbon source, a key substrate for rifamycin production, by addition of 1% glucose.

The maximum yield (20.88 g/l) was obtained by using an aeration rate of 1.5 vvm and addition of 2 increments of 1% glucose at days 6 and 8. This yield was accompanied by a comparable biomass to that of control. Hence, it was presumed that glucose was mainly consumed to maintain the biomass and biosynthesis of the antibiotic [3]. The lowest yield was obtained upon using 2 vvm and was associated with the higher biomass and higher glucose consumption. Therefore, 1.5 vvm was selected for further optimization of rifamycin B production.

Interestingly, the highest effect of fed-batching rates was obtained upon fed-batching the GS at a rate of 12 g/day, however; when this rate increased to 144 g/day the yield slightly 230 decreased. Further increase in the fed-batching rate to 338.4 g/day had a significant negative impact of the production of rifamycin B. These results were in agreement with that reported by Jin et al. [3] as ideally, the feeding rate of glucose concentration was dependant on the glucose concentration in the fermentation broth. These should direct the mycelium growth rate and mycelium concentration in favor of rifamycin B formation. However, the use of higher feed rate (338.4 g/day) resulted in significant increase in the biomass. This cell mass may be exceed the level that can be supported by the optimum oxygen transfer rate (OTR) required for maximum antibiotic production in the fermentor [10]. Hence, the fed-batch method is favorable for rifamycin B production because rifamycin B production with *A. mediterranei* is non-growth associated process [11].

The highest improvement in the production yield was recorded at 0.1% of uracil (day 2). Addition of either NH_4NO_3 or corn step liquor also improved the yield but to a lesser extent. These were in agreement with the results generated on the shake flasks scale and also with [4,8].

In general, integrating the optimized conditions (fed-batch addition of carbon and nitrogen sources) significantly improved the production yield. The highest yield (24.8 g/l) was recorded by addition of 0.1% uracil at day 2, GS at day 3 and glucose at days 6 and 8. This still in agreement with previous results generated in the present study.

Set No.	Variable factor	Final conc. (g %)	Time of addition (days)	Rifamycin B conc. at day 10 (g/l)	% of increase
Control	Glucose syrup	33.3	3	17.80 ± 0.30[a]	-------
	Corn-steep liquor	1	3	18.53 ± 0.251	4
N1	NH_4NO_3	0.05	3	19.51 ± 0.19	10
	Uracil	0.1	2	20.0 ± 0.10	12.5
	Glucose syrup	33.3	3		
N2	Corn-steep liquor	1	3	19.70 ± 0.264[bc]	9.5
	Glucose	1	6		
	Glucose syrup	33.3	3		
N3	NH_4NO_3	0.05	3	20.40 ± 0.36[c]	14.5
	Glucose	1	6		
	Uracil	0.1	2		
N4	Glucose syrup	33.3	3	23.87 ± 0.35[d]	34
	Glucose	1	6		
F-value			**153.75**		
p-value			**0.000***		
	Glucose syrup	33.3	3		
N5	Corn-steep liquor	1	3	22.20 ± 0.26[b]	24.5
	Glucose	1	6 then 8[(1)]		
	Glucose syrup	33.3	3		
N6	NH_4NO_3	0.05	3	23.03 ± 0.25[c]	30
	Glucose	1	6 then 8[(1)]		
	Uracil	0.1	2		
N7	Glucose syrup	33.3	3	24.87 ± 0.35[d]	40
	Glucose	1	6 then 8[(1)]		
F-value			**101.07**		
p-value			**0.000***		

[(1)]The glucose was added successively during the same fermentation.
SD=Standard Deviation
*= there is a significant difference by using One Way ANOVA at p<0.05.
The different letters means that there is a significant difference by using Tukey HSD multiple comparison test at p<0.05. G: Glucose, GS: Glucose syrup

Table 4: The recorded yield of rifamycin B production (mean ± SD) by NCH strain using F2m3 medium during optimization by fed-batching different nitrogen sources with or without glucose using aeration rates of 1 and 1.5 vvm in 6.6 l fermentor.

Such combination could stimulate rifamycin B production through the development of either a more active system for rifamycin B production or a less active system for rifamycin B inhibition [4,8]. Additionally, this could provide a better availability of glucose during the idiophase [12].

This was confirmed by statistical analysis using One-Way ANOVA at p<0.05 as the most significant yield (20.83 ± 0.20z) is obtained upon fed-batch addition of 1% glucose at days 6 and 8 using aeration rate of 1.5 vvm. Evidentially, the most significant yield is obtained upon fed-batching glucose at days 6 and 8 and addition of uracil at day 2 (24.87 ± 0.40d).

Conclusion

Stepwise optimization increased the yield almost by 2 fold. The highest yield (24.8 g/l) was achieved in a lab scale ferementor under the following optimized conditions: fermentation medium; F2m3, (500 rpm), aeration; 1.5 vvm for 3 days then control DO at 30% of saturation thereafter, pH; control at 6.5 for 3 days then raised to 7 thereafter, process temperature; control at 28°C throughout the process, addition of glucose syrup (5%) at day 3, addition of 0.1% uracil at day 2, addition of glucose (1%) at days 6 and 8 and total fermentation time of 10 days.

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