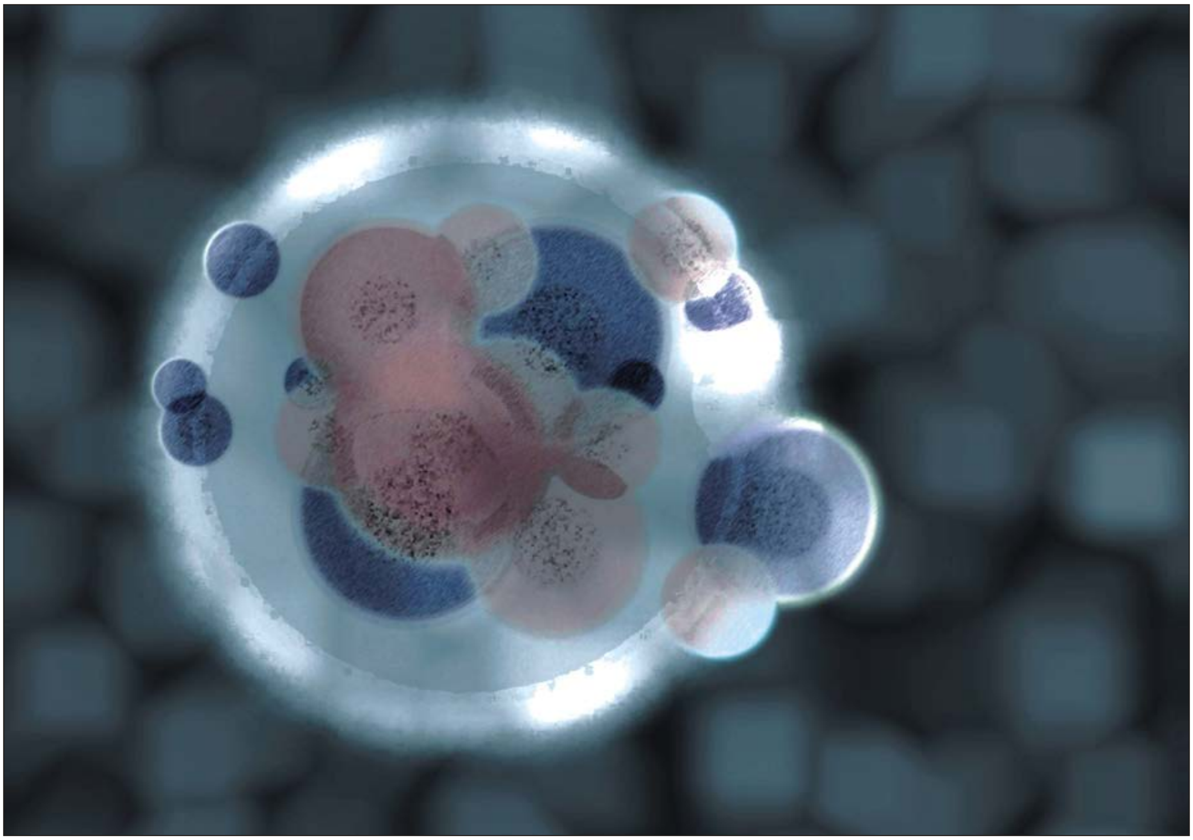

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Optimization of Parameters for Citric Acid Production from Cheddar Cheese Whey Using *Metschnikowia pulcherrima* NCIM 3108

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Abstract: The present study was carried out at flask level for the fermentative production of citric acid from cheddar cheese whey using a yeast culture *Metschnikowia pulcherrima* NCIM 3108. Clarified and unclarified whey with and without nutrient supplementation was screened for citric acid production. The order of citric acid production was supplemented unclarified whey>supplemented clarified whey>unclarified whey>clarified whey. The effect of various process parameters was investigated to maximize the citric acid production from supplemented unclarified whey. The lactose concentration of 6.0% (w/v) at pH 6.0 with an inoculum size 10% (v/v) of 48 h old culture at 30°C supported the maximum concentration of citric acid production after 5 days of incubation period. The agitation of the yeast culture suppressed the citric acid production to a little extent as compared to stationary conditions.

Key words: Citric acid, cheese whey, *Metschnikowia pulcherrima*

INTRODUCTION

Cheese whey is the watery portion remaining after the precipitation and removal of milk casein during cheese manufacture. The composition and type of whey varies considerably depending upon the source of milk, type of cheese manufactured and on the process of technology employed for its production. Whey is produced in huge amount in dairy industry and it is loaded with high organic matter content exhibiting high biological oxygen demand and chemical oxygen demand[1]. Its disposal as waste poses serious pollution problems for the surrounding environment. It affects the physical and chemical structure of soil resulting in a decrease of crop yield and when released in water bodies, reduces the aquatic life by depleting the dissolved oxygen[2].

Whey represents about 85-95% of the milk volume and retains 55% of milk nutrients[3]. Among the most abundant of these nutrients are lactose, soluble proteins, lipids and mineral salts etc.. It is also a very good source of vitamins of B-complex group, riboflavin and pantothenic acid[4]. Based on the nutritional value of whey number of commercial uses like production of ethanol, organic acids, non-alcoholic beverages, single cell proteins, whey protein concentrate, lactose hydrolysed products etc. have been discussed earlier[3,5-9]. Although, novel approaches for the utilization of whey have been developed, but half of the total whey produced is still discarded[10]. The dumping of surplus whey constitutes a significant loss of potential food and energy.

Whey is a rich and inexpensive raw material which is available all year round and can be utilized for the production of various valuable products. In most of the reports on citric acid production from whey *Aspergillus niger* is the organism of choice[11-14]. Citric acid fermentation by *Aspergillus niger* is considered as a problematic process, since it is influenced by a number of variables which can not be accurately controlled[15]. Keeping this in view yeast strain *Metschnikowia pulcherrima* NCIM 3108 has been selected and used for the production of citric acid from cheese whey.

MATERIALS AND METHODS

Yeast culture: *Metschnikowia pulcherrima* NCIM 3108 was procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The culture was maintained on agar plants containing malt extract (0.3%), glucose (1.0%), yeast extract (0.3%), peptone (0.5%) and agar-agar (2.5%) adjusted to pH 6.5, by subculturing fortnightly.

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Procurement of cheddar cheese whey: The fresh cheddar cheese whey were periodically collected in airtight plastic containers (5 L) from Verka Milk Plant, Patiala, India. The samples were stored at 4± 1°C, until further use.

Clarification of whey: The clarification of whey was carried out by the method of Capoor and Singh[16]. Nutrient supplementation of whey: The clarified and unclarified whey was supplemented with nutrients (g L^{-1}): $(NH_4)_2SO_4$ 1.0, Mg_2SO_4 0.25; $CaCO_3$ 5.0; KH_2PO_4 1.0 and (μg L^{-1}): $CuSO_4$. $5H_20$ 40.0; $ZnSO_4$. 10.0; $MnSO_4$ 5.0.

Preparation of starter: Yeast culture was grown in 100 mL of broth in 250 mL capacity of conical flasks. The composition of media was same as of maintenance media except agar. The culture was incubated at 30°C for 48 h, under stationary conditions. An inoculum concentration of 10%, v/v (unless otherwise specified) were used for citric acid production.

Citric acid production from cheddar cheese whey using *Metschnikowia pulcherrima* NCIM 3108: The lactose and pH of the clarified and unclarified whey with and without nutrient supplementation was adjusted to 5.5% (w/v) and 6.0, respectively, unless otherwise, specified. 90 mL of each type of whey was taken in 250 mL capacity conical flask and inoculated with 10% (v/v) of yeast culture and incubated at 30°C for 72 h, under stationary conditions (unless otherwise inoculum size, temperature, incubation period and conditions are mentioned). The samples were analyzed for change in pH, lactose utilization and citric acid production.

Optimization of process parameters: Clarified and unclarified cheese whey with and without nutrient supplementation were screened for citric acid production potentials by *Metschnikowia pulcherrima* NCIM 3108. To maximize the production of citric acid, samples of unclarified way with nutrients were supplemented with different concentrations (5.0, 6.0, 7.0, 8.0 and 9.0%, w/v) of lactose. The initial adjustment of pH (5.0-7.0) was made, to study its influence on citric acid production. To optimize the temperature the yeast cultures were incubated at 20-40°C. To investigate the effect of inoculum age on citric acid production from cheese whey 24-96 h old yeast cultures were used. To determine the effect of inoculum size on citric acid production cell concentration of 5-15% (v/v) were used. The incubation period of 2-6 days were used to determine the effect of retention time on citric acid production. To investigate the influence of agitation on citric acid production the flasks were incubated under stationary conditions in a BOD incubator and shaking conditions at 100 rpm on a rotary shaker (Certomat M, Germany).

Analytical techniques

Titratable acidity: The method of AOAC[21] was followed to estimate the acidity of whey samples.

Total solids: The procedure of Indian Standards Institution[17] was employed to calculate the contents of total solids in whey samples.

Lactose: The lactose content was estimated according to the method of Perry and Doan[18]

Citric acid: Citric acid was measured by the spectrophotometric method of Marier and Boulet[19]

Protein: The protein content of whey was estimated by the method of Lowry *et al.*[20]

Total nitrogen: The quantitative determination of total nitrogen was carried out by Kjeldahl method[21].

Fat: The fat content in whey was measured by Rose-Gottlieb method[21].

Biochemical Oxygen Demand (BOD_5): The biochemical oxygen demand was determined by the method of APHA[22].

Chemical Oxygen Demand (COD): The chemical oxygen demand was determined by closed reflux method[22] using Hach COD reactor and Hach DR- 2000 spectrophotometer system (Hach Company, USA).
Three replicates were carried out and analysed for each experiment.

RESULTS AND DISCUSSION

Cheddar cheese whey was analysed for its physico-chemical characteristics and the results are presented in Table 1. The whey was pale-yellow in colour and sticky in appearance. The stickiness may be attributed to the presence of proteins and fats in the

Table 1: Characteristics of cheddar cheese whey*

pH	5.50-6.00
Acidity	0.15-0.22
Total solids	6.15-6.80
Lactose	5.00-5.80
Proteins	0.45-0.60
Total nitrogen	0.70-0.85
Fat	0.28-0.40
BOD_5 (mg L^{-1})	48,700-50,225
COD (mg L^{-1})	72,400-74,325

*All parameters except pH, BOD_5 and COD are in%

whey. The amounts of total solids, biochemical oxygen demand and chemical oxygen demand of the tested samples were much higher than the federal permissible limits governing the discharge of industrial effluents in India. The high biochemical oxygen demand is mainly due to the lactose which is present at concentration of between 4.5-5.0%[23]. The composition of whey varies according to its origin, type of cheese produced and to the cheese making technique employed. The results showed the same trend as reported by a number of workers[1,5,7,23]

Screening of clarified, unclarified and supplemented cheese whey for citric acid production by *Metschnikowia pulcherrima* NCIM 3108: The clarified and unclarified whey with and without nutrient supplementation were screened for citric acid production by *Metschnikowia pulcherrima* NCIM 3108. The order of citric acid production was supplemented unclarified whey>supplemented clarified whey>unclarified whey>clarified whey (Table 2). The citric acid production was more in unclarified whey as compared to clarified whey. This may be due to the presence of proteins in unclarified whey which were utilized by yeast cultures. However, the supplemented unclarified whey supported the maximum (2.5 g L^{-1}) citric acid production. The corresponding lactose utilization was 82.50% (w/v). It was noticed from these observations that the nutrient supplementation in whey has a significant effect on the citric acid production. The presence of inorganic phosphate has been reported to favour the citric acid production by yeast cultures[24]. The supplementation of ammonia salts [$(NH_4)_2SO_4$] has also been recommended for citric acid production for yeast strains[15]. Keeping these observations in view the supplemented unclarified whey was selected for further experimentation to optimize the process parameters.

Table 2: Citric acid production from clarified and unclarified cheddar cheese whey [a] using *Metschnikowia pulcherrima* NCIM 3108

Types of whey	pH after fermentation	Lactose utilization (%, w/v)	Citric acid production (g L^{-1})
Clarified whey	5.2	66.18	1.6
Unclarified whey	5.0	72.02	2.0
Clarified whey supplemented with nutrients[b]	4.8	76.29	2.2
Unclarified whey supplemented with nutrients[b]	4.5	82.50	2.5

[a]The lactose content of cheese whey was 5.5% (w/v)

[b]Clarified and unclarified whey was supplemented with nutrients (g L^{-1}): $(NH_4)_2SO_4$ 1.0; $MgSO_4$ 0.25; $CaCO_3$ 5.0; KH_2PO_4 1.0 and (μg L^{-1}): $CuSO_4.5H_20$ 40.0; $ZnSO_4$. 10.0; $MnSO_4$ 5.0. Fermentation conditions: Temperature: 30°C; Incubation period: 72 h; Age of inoculum: 48 h; Inoculum concentration: 10% (v/v); pH: 6.0; Condition: Stationary

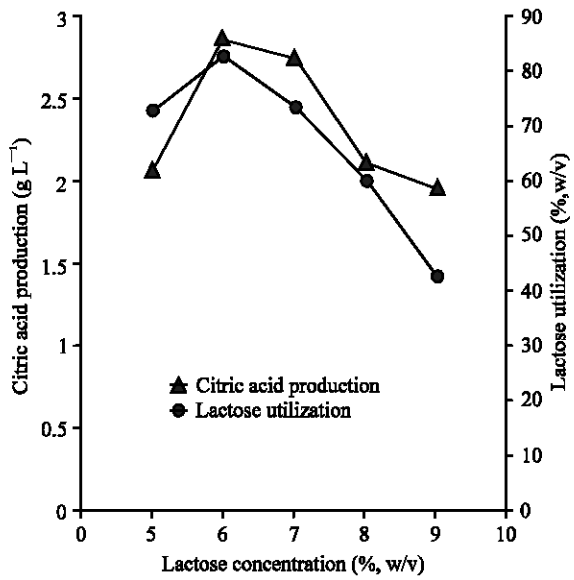

Fig. 1: Citric acid production from cheddar cheese whey by *Metschnnikowia pulcherrima* NCIM 3108 with lactose concentation as a function

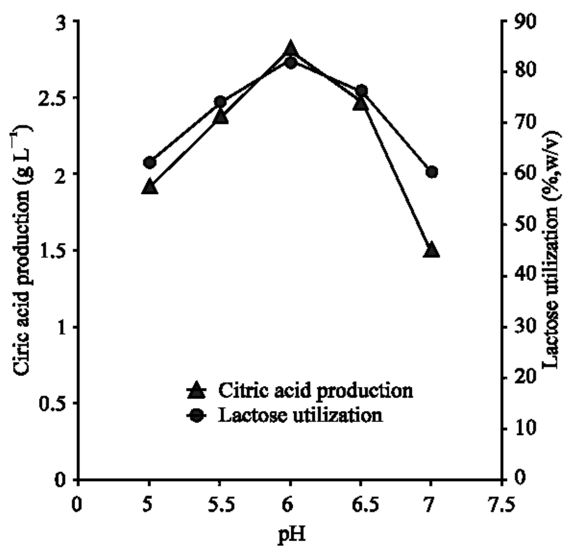

Fig. 2: Citric acid production from cheddar cheese whey by *Metschnnikowia pulcherrima* NCIM 3108 with pH as a function

Citric acid production from supplemented unclarified whey using *Metschnikowia pulcherrima* NCIM 3108 with lactose concentration as a function: Various concentrations of lactose (5-9%, w/v) were tested to evaluate their effect on citric acid production. The maximum citric acid production (2.85 g L^{-1}) was observed at 6.0% (w/v) lactose concentration (Fig. 1). The corresponding lactose utilization was 82.65% (w/v). The

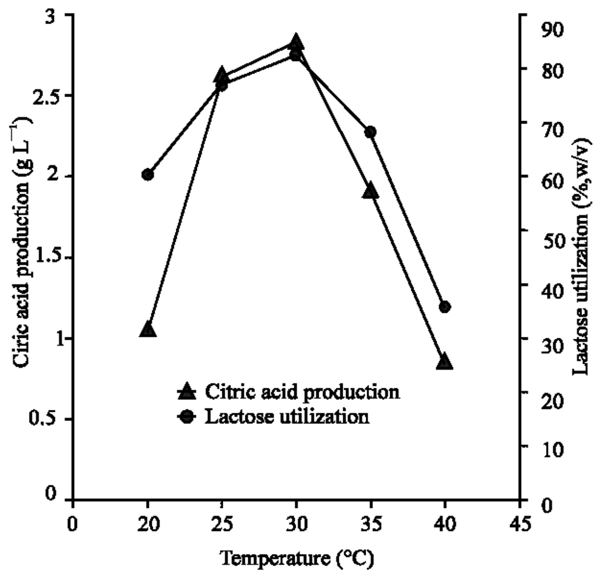

Fig. 3: Citric acid production from cheddar cheese whey by *Metschnnikowia pulcherrima* NCIM 3108 with temperature as a function

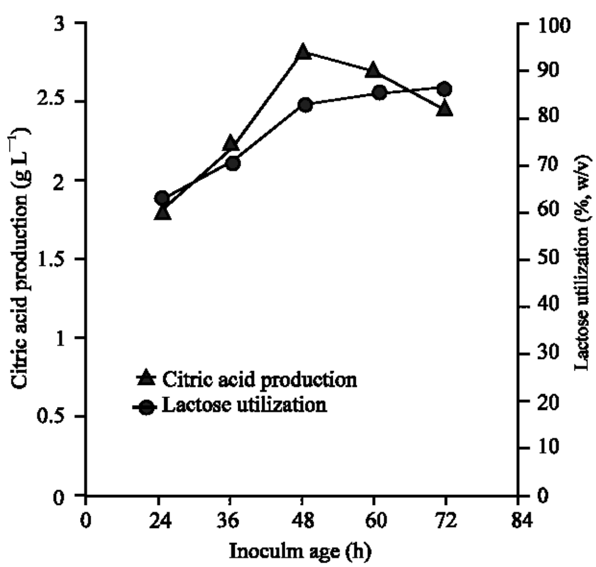

Fig. 4: Citric acid production from cheddar cheese whey by *Metschnnikowia pulcherrima* NCIM 3108 with inoculum age as a function

degree of citric acid production and lactose utilization was increased up to 6.0% (w/v) of lactose concentration. Thereafter, a decline in both functions was observed. The increase in lactose concentration above 6.0% (w/v) has an inhibitory effect on citric acid production and lactose utilization by yeast cultures. Therefore, 6.0% (w/v) lactose concentration was selected for further experimentation.

Citric acid production from supplemented unclarified whey using *Metschnikowia pulcherrima* NCIM 3108 with pH as a function: The pH of supplemented unclarified whey was adjusted (5-7) to determine its effect on citric acid production. The citric acid production and lactose utilization was increased with increase in pH up to 6.0. Thereafter, a decline in both the functions was observed. The maximum citric acid production and lactose utilization at pH 6.0 was 2.81 (g L^{-1}) and 82.31% (w/v),

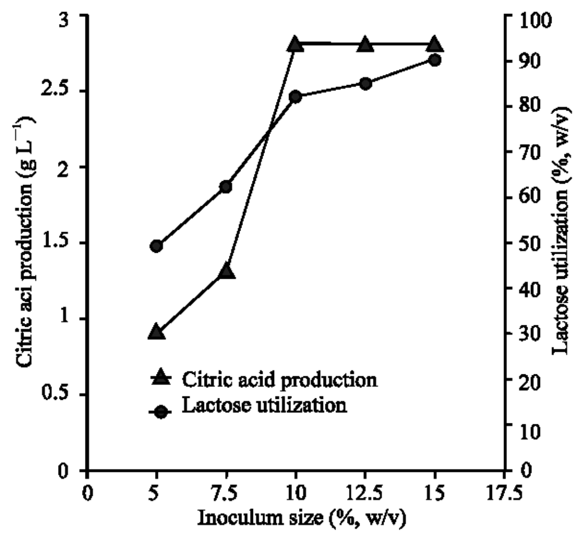

Fig. 5: Citric acid production from cheddar cheese whey by *Metschnnikowia pulcherrima* NCIM 3108 with inoculum size as a function

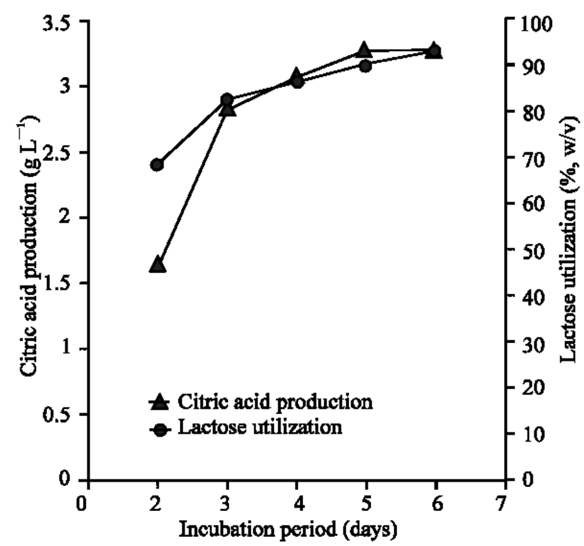

Fig. 6: Citric acid production from cheddar cheese whey by *Metschnnikowia pulcherrima* NCIM 3108 with incubation period as a function

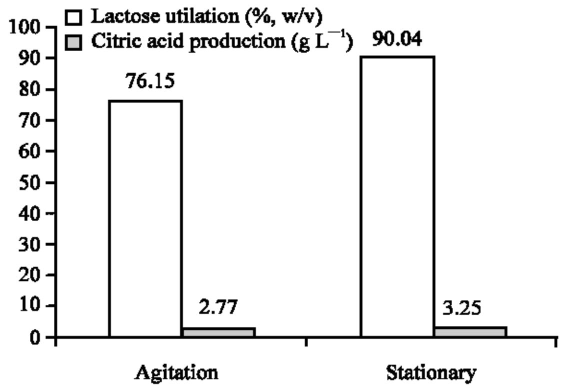

Fig. 7: Citric acid production from cheddar cheese whey by *Metschnnikowia pulcherrima* NCIM 3108 with agitation as a function

respectively (Fig. 2). The minimum citric acid production (1.5 g L^{-1}) was observed at pH 7. The pH 4.5-6.5 has been recommended optimal for citric acid production from n-alkanes by yeast[15]. From these observations pH 6 was considered optimal for citric acid production from whey and was selected for further studies.

Citric acid production from supplemented unclarified whey using *Metschnikowia pulcherrima* NCIM 3108 with temperature as a function: To study the effect of temperature on citric acid production, whey samples were incubated at 20-40°C. An increase in citric acid production and lactose utilization was observed up to 30°C (Fig. 3). The maximum citric acid production and lactose utilization was recorded at 30°C and was 2.82 (g L^{-1}) and 82.50% (w/v), respectively. The temperature of 28°C has been reported optimal for citric acid fermentation by yeasts from n-alkanes[15]. Since 30°C supported the maximum citric acid production and lactose utilization, it was selected for further studies.

Citric acid production from supplemented unclarified whey using *Metschnikowia pulcherrima* NCIM 3108 with age of inoculum as a function: The data represented in Fig. 4 depicts the influence of age of inoculum on the ability of the yeast culture for citric acid production from whey. The maximum citric acid production 2.81 (g L^{-1}) was observed when 48 h old culture of yeast was used. However, the maximum utilization of lactose 86.06% (w/v) was observed when 72 h old culture of yeast was used. The lactose utilization was progressively increased with increase in age of inoculum. However, slight reduction in citric acid production was observed when more than 48 h old culture was used. Since the increase in age of culture beyond 48 h depresses the citric acid production. Therefore, 48 h old culture was selected for further studies.

Citric acid production from supplemented unclarified whey using *Metschnikowia pulcherrima* NCIM 3108 with inoculum size as a function: To study the effect of cell biomass on citric acid production, the inoculum concentrations of 5-15% (v/v) were used. A progressive increase in citric acid production with an increase in concentration of inoculum was observed up to a biomass concentration of 10% (v/v). At this inoculum level citric acid production was 2.80 (g L^{-1}). The corresponding lactose utilization was 81.98% (Fig. 5). The higher levels of inoculum concentration (12.5-15.0%, v/v) tested did not show further improvement in the citric acid production. However, lactose utilization increased with an increase in the cell load. It was maximum (90.01%, w/v) when 15% (v/v) concentration of inoculum used. Therefore, 10% (v/v) inoculum level was selected for further experimentation.

Citric acid production from supplemented unclarified whey using *Metschnikowia pulcherrima* NCIM 3108 with time-course as a function: To determine the effect of time (48-144 h) on citric acid production from supplemented unclarified whey, 10% (v/v) of inoculum level was employed. The results depicted a progressive increase in citric acid production up to 120 h of incubation period (Fig. 6). However, after this no further improvement in citric acid production was recorded, but increase in the lactose utilization was observed. Keeping this in view, an incubation period of 120 h was considered optimal for citric acid production.

Citric acid production from supplemented unclarified whey using *Metschnikowia pulcherrima* NCIM 3108 with agitation as a function: The maximum citric acid production and lactose utilization under stationary conditions was 3.25% (g L^{-1}) and 90.40% (w/v), respectively. The agitation of the culture supported the poor production of citric acid and lactose utilization as compared to stationary conditions (Fig. 7). The effect of aeration on citric acid production by yeast is yet controversial[15]. Some of the yeast strains require higher aeration for citric acid production while others supported the non-agitated mode of fermentation.

The above experimentation carried out in flask culture at laboratory scale would provide useful guidelines for further investigations on citric acid production from whey using the yeast culture.

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