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High-Temperature Citric Acid Production from Sugar Cane Molasses using A Newly Isolated Thermotolerant Yeast Strain, *Candida parapsilosis* NH-3

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Abstract

Citric acid is a commodity chemical mainly used as an acidulant in the food and beverage industry. In the present study, the potential use of sugar cane molasses as cheap agro-industrial waste carbon source for isolation and screening of citric acid producing microbes was investigated. Yeast isolates were obtained from soil samples, using serial dilution agar plating method and the most potent citric acid-producing isolate, coded NH-3, was identified based on macroscopic, microscopic and molecular characteristics. This strain was identified as *Candida parapsilosis* NH-3 and used for optimization of citric acid production using both classical and statistical methods. In classical method various parameters were investigated including: temperature, pH, molasses concentration, pre-treatment of molasses, nitrogen and phosphorus limitation and alcohol addition. Moreover, Plackett-Burman design was used for further optimization. In this assay, the maximum production of citric acid was obtained at temperature 40±1° C, pH 5.0, molasses concentration 30%, pre-treatment of molasses using EDTA, nitrogen and phosphorus limitation excluding dipotassium hydrogen phosphate, and alcohol addition of 1% methanol.

Keywords: Yeast, citric acid, optimization, Plackett-Burman design, sugar cane molasses

1. Introduction

Citric acid (2-hydroxy-1, 2, 3-tricarboxylic acid) is an organic acid naturally present in all aerobic organisms, as it is produced during the process of cellular respiration. It is a ubiquitous intermediate product of metabolism and its traces are virtually present in all plants and animals **[Papagianni, 2007]**. Citric acid has found various uses in food, beverage, pharmaceutical, chemical, cosmetic and other industries. It is used to impart a characteristic tart taste to foods and beverages. Citric acid is also used in industries for acidulation, anti-oxidation, emulsification, preservation, flavour enhancement and as plasticizer and synergistic agent **[Soccol *et al.*, 2006]**. This acid is biodegradable and palatable, highly soluble and low/non-toxic and classified Generally Recognized As Safe **[Ali *et al.*, 2002]**. Therefore, it is one of the most important organic acids produced by fermentation and is the most exploited biotechnological/biochemical product **[Yalcin *et al.*, 2009]**. It has an annual industrial production of 1.6 million tons [**Sauer *et al.*, 2008**] with an annual growth demand/consumption rate of 3.5-4% **[Nadeem *et al.*, 2010]**. Seventy percent of the total citric acid produced globally are utilized in food industry while about 12% used in pharmaceuticals and the remaining 18% in other industries **[Ali *et al.*, 2002]**. The natural

supply of citric acid is very limited as compared to its growing industrial demand. Thus, increasing demand can be only accomplished via biotechnological processes **[Lofty *et al.*, 2007]**.

Biotechnological production by microbial fermentation is the most common method for large scale production of citric acid. Many micro-organisms have been used for citric acid production by various workers including bacteria such as *Arthrobacter paraffinens*, *Bacillus licheniformis* and *Corynebacterium* spp, fungi such as *Aspergillus* spp., *Penicillium jenthinellum* and yeasts such as *Candida* spp, *Torula* spp, *Torulopsis* spp, *Sacharomyces* spp, *etc* **[Grewal and Kalra, 1995;Abad *et al.*, 2010]**. *Candida parapsilosis* has been previsously used for several applications including production of lipase/acyltransferase **[Rodrigues *et al.*, 2016; Tecelao *et al.*, 2010]** and lipid production **[El-Refai *et al.*, 1987]**, citric acid production from various carbon sources **[Omar and Rehm, 1980]** and xylitol **[Preziosi-Belloy *et al.*, 1997; Kim *et al.*, 1997]**. Different methods of fermentation have been employed by different workers for producing citric acid using specific substrates and microbial strains.

The yield of citric acid production by specific strain depends on various factors including substrate used, physical and nutritional fermentation conditions employed. Different types of fermentation methods are employed for citric acid production on mass scale, which include solid-state fermentation, submerged fermentation and surface fermentation. The most common method however, is the submerged fermentation method **[Assadi and Nikkhah, 2018]**.

Currently, researchers are engaged in exploring the suitability of various waste materials for bioconversions which might be the cheaper sources for the production of various fermented and value-added products **[Desouky *et al.*, 2014; Khattab, 2016]**. Among such wastes, the sugar cane molasses might be a desirable raw material for citric acid fermentation due to its availability at relatively low price and it can be the basic substrate for citric acid fermentation using submerged technique of fermentation **[Ikram-ul *et al.*, 2004]**. Besides, the productivity of fermentation products is greatly influenced by the type of substrate as well as the fermentation conditions like temperature, fermentation time and the type of culture/strain **[Ali *et al.*, 2002]**. Keeping in view the importance of citric acid and utilization of wastes to prevent from pollution, this study was carried out to isolate and screen the ability of citric acid production by Yeast strain and study the effect of various fermentation parameters on citric acid production using sugar cane molasses.

2. Materials and Methods

2.1 Isolation sources and medium: Various isolation sources were collected from different localities in Egypt. Solid samples include lemon pieces and agriculture soils of Lemon, Lareng and wheat; Liquid samples include algae and sea water; and compost samples include animal sludge, mixture of agricultural wastes and spoiled juices. Yeast isolate obtained on soil samples which were collected from Lareng Soil-El-Behirah District, Egypt in 2015. The soil samples were taken by means of sterilized spatulas and collected sterile sealed polythene bags. The samples were brought to the Research laboratory, Department of Botany and Microbiology, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt and stored at refrigerator for microbiological study. One gram or 1 ml was inoculated in a 250 ml conical flask containing 100 ml of sterile distilled water and shaken well for 20 minutes then various dilutions were prepared (10^{-2}, 10^{-4} and 10^{-6}) using sterile distilled water. 1 ml of the serially diluted sample was poured in the two sterile petri-dishes, one containing Potato Dextrose Agar (PDA) medium, and another contain Mineral salt media containing bromocresol green as an acid production indicator (yellow zone). After incubation for 3-5 days at 30° C, different colonies were grown on the both medium. The isolated Yeast spp were purified by re-streaking on the same medium and the pure cultures were maintained on the PDA slants and glycerol, then stored at 5°C in the refrigerator. The most potent isolate has been tentatively named as NH-3.

2.2 Basal Screening Fermentation Medium: Yeast spp isolated in this study were screened qualitatively using both Potato Dextrose Agar media (PDA) and Mineral salt media (MSM). The PDA contained the following (L^{-1}): Potato extract 250 ml; glucose 20.0 g; agar, 20.0g **[Shiriling and Gottlieb, 1966]**. The pH was adjusted at 7.0 before sterilization, Autoclaved at 121°C for 20 min. While MSM content was (g/L) carbon source, 30.0; $MgSO_4.7H_2O$, 0.7; $Ca(NO_3)_2$, 0.4; NaCl, 0.5; KH_2PO_4, 1; K_2HPO_4, 0.1; Yeast Extract, 0.5; $(NH_4)_2SO_4$, 0.3; 1ml of trace elements solution "Burkholder's Trace" containing (mg/L): I^{-1}, 0.1; B^{+3}, 0.01; Fe^{+2}, 0.05; Zn^{+2}, 0.04; Mn^{+2}, 0.01; Cu^{+2}, 0.01; Mo^{+2}, 0.01.**[Schlegel *et al.*, 1961]**.

Molasses was supplemented as sole carbon source at different concentrations. The pH of the medium was adjusted to 4.5-5.5 and autoclaved at 121°C for 20 minutes.

On the other side, quantitatively screening for the production of citric acid occurred by in MSM. The MSM was inoculated with one disc of fresh culture for the isolated Yeast strain. The study occurred using Sugar Cane molasses (SCM) from El Hawamdia factory for integrated sugar industry (Giza, Egypt). It was diluted with distilled water in order to obtain the target concentration.

2.3. Identification of the selected isolate.

Macroscopic and microscopic morphology properties were determined for the most potent isolate (NH-3). Molecular identification was done conducted based on amplification and sequencing of internal transcribed spacer (ITS) region. Genomic DNA was extracted using the protocol of Gene Jet genomic DNA purification Kit (Thermo). In the ribosomal DNA (rDNA) cluster, D1/D2 region was amplified in polymerase chain reaction (PCR) from a broad spectrum of yeast using the genomic DNA as template and primers of NL-1 (5`-**GCATATCAATAAGCGGAGGAAAAG**-3`) and NL-4 (5`-**GGTCCGTGTTTCAAGACGG**-3`). The PCR mixture (50µL) contained Maxima Hot Start PCR Master Mix (Thermo), 0.5µM of each primer, and 1µL of extracted fungal genomic DNA. The PCR was performed in a DNA Engine Thermal Cycler by Sigma Scientific Services Company (Cairo, Egypt) with a hot starting performed at 94ºC for 1 min, followed by 35 cycles of 94ºC for 5 min, 50ºC for 2 min, and 74ºC for 1.5 min, followed by a final extension performed at 74ºC for 5 min. The commercial sequencing was conducted using ABI 3730x1 DNA sequencer at GATC Company (Germany). The NL sequence was compared against the GenBank database using the NCBI BLAST program. Sequences were then compared with NL sequences in the GenBank database using BLASTN, and phylogenetic tree was conducted by bootstrap analysis.

2.4 Factors affecting CA production

The following factors were investigated for optimization of CA production. The deduced optimal conditions resulted from each experiment was taken in consideration in the next experiment.

Samples were withdrawn periodically and analyzed for pH, DCW, CA production and consumed sugar.

For optimal temperature, different incubation temperatures were applied (20, 25, 30, 35, 40, 45and 50 °C). Strain NH-3 was inoculated at 10 % (v/v) and incubated at 150 rpm for 9 days.

To determine the optimal initial pH value, sodium hydroxide (1N) and hydrochloric acid (1N) were used for medium preparation. Initial pH of the fermentation media was adjusted at different pH values (2, 2.5, 3, 3.5, 4, 4.5, 5, and 6). The strain was inoculated at 10 % (v/v) and incubated at 45°C and 150 rpm for 9 days.

To determine the best SCM concentration, five concentrations of SCM were used 10, 20, 30, 40 and 50% with sugar concentration 5, 10, 15, 20, 25 %, respectively. SCM with different concentrations were supplemented to MSM and sterilized. Strain was inoculated at 10 % (v/v), using initial pH 4.5, incubated at 45 °C and 150 *rpm* for 96 hrs. To investigate the optimal SCM pre-treatment, different pretreatment methods were used: ***Sulphuric acid treatment***, SCM solution was adjusted to pH 3.0 with 1N H_2SO_4. The liquid was allowed to stand for 24 h and then centrifuged at 5000 g for 15 min. The pH of the supernatant was adjusted to 5.5 with 10 N NaOH and the solution sterilized at 121°C for 15 min; ***Potassium ferrocyanide and Ethylene Diamine tetra Acetic acid (EDTA) treatment***, SCM solution was adjusted to pH 5.5 with 5 N HCI and heated at 100°C for 15 min. The liquid was treated while hot with 1ppm potassium ferrocyanide or EDTA to encourage the precipitation of heavy metals. The mixture was allowed to stand for 24h at room temperature and then centrifuged at 5000g for 20 min. The supernatant was used for fermentation **[Roukas, 1998]**; ***Activated charcoal treatment,*** SCM treated with activated carbon for three hours and filtration duplicate **[Djordjevic *et al.*, 2018]**. All pre-treatments were used in 20% concentration, adjusted pH at 4.5 and added to MSM before autoclaving, after inoculation were incubated at 45°C for 96 hrs.

To enhance CA production, nitrogen and phosphorus limitation effect were studied. MSM were prepared with limitation of one or more ingredient as following. Media prepared without $Ca(NO_3)_2$ [A-1]; Yeast Extract [A-2]; $(NH_4)_2SO_4$ [A-3]; $Ca(NO_3)_2$ and Yeast Ext [A-4]; $Ca(NO_3)_2$ and $(NH_4)_2SO_4$ [A-5]; Yeast Ext and $(NH_4)_2SO_4$ [A-6]; $Ca(NO_3)_2$, Yeast Ext and $(NH_4)_2SO_4$ [A-7]; KH_2PO_4 [B-1]; K_2HPO_4 [B-2];

KH_2PO_4 and K_2HPO_4 [B-3]; $Ca(NO_3)_2$, $(NH_4)_2SO_4$, Yeast Ext, KH_2PO_4 and K_2HPO_4 [C]. The production media contain 20% of SCM pretreated with EDTA, pH is adjusted at 4.5, and incubated at 45°C for 96 hrs.

To study the effect of auxiliary carbon sources; methanol, ethanol, amyl alcohol, propanol and ethyl acetate were supplemented separately at 1 and 2% to the previously optimized MSM. Media were supplemented with 20% SCM treated with EDTA, pH 4.5, and incubated at 45°C for 72 hrs.

Plackett-Burman design (PBD) was one of the statistical techniques used for the screening of the experimental factors. The purpose of using this technique was to identify the significant nutrients that had an impact on the amount of citric acid production in a minimum number of experiments. The statistical screening was based on the main effects of the experimental factors, but not on their interaction effects **[Plackett and Burman, 1946]**. Each factor was represented within 2 levels, high (+) and low (-). Table 1 represents the 7 selected media components that were evaluated. The design of the 7 media components as evaluated in the 12 experimental runs is illustrated in Table 8,9 and Figures 8. The factors that had a level above 95% ($P < 0.05$) were considered as significant and used for further optimization **[Vining *et al.*, 2005]**. The designing and analyzing of these experiments was carried out using Minitab Software v. 18.

2.5 Analytical methods

Cell growth was monitored by centrifuge the culture medium at 10,000 rpm, at 4°C for 5 min and the cell pellet was washed with distilled water. The cell pellet was harvested by centrifugation and dried at 105°C overnight till constant weight was obtained. CA concentration was assayed using primary and secondary screening, the primary screening was done by viable-colony staining method using bromo-Cresol Green stain as follows: 50.0 mg bromo-cresol Green/L of solid medium was prepared, sterilized and poured into Petri plates. The obtained isolates were streaked on agar plates and incubated at 37°C for 3 days then examined. The colonies with yellow zone were recorded as acid producer **[Auta *et al.*, 2014]**.For secondary screening, selected isolates were cultivated in mineral salt medium. The media were incubated for 72 h at 37°C and 150 rpm. Citric acid was estimated gravimetrically, using pyridine–acetic anhydride method as reported by Marier and Boulet **[1958]**.

One ml of the diluted culture filtrate along with 1.30 ml of pyridine was added in the test tube and swirled briskly. Then 5.70 ml of acetic anhydride was added in the test tube. The test tube was placed in a water bath at 32 °C for 30 min. The optical density was measured on a spectrophotometer (M-ETKAL 721 Spectrophotometer) at 405 nm) and citric acid contents of the sample were estimated with reference (run parallel, replacing 1.0 ml of the culture filtrate with distilled water) to the standard. The standard curve was established with citric acid concentrations ranging from 25-200 µg/ml. The readings were taken at regular intervals of 24 hours from the time of inoculation of the medium with the fungal spores **[Marier and Boulet, 1958]**.

The consumed sugar was determined using phenol-sulfuric acid method that is the most widely used colorimetric method to date for determination of carbohydrate concentration in aqueous solutions. The basic principle of this method is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid, produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectible color. The standard procedure of this method is as follows. A 2 mL aliquot of a carbohydrate solution is mixed with 1 mL of 5% aqueous solution of phenol in a test tube. Subsequently, 5 mL of concentrated sulfuric acid is added rapidly to the mixture. After allowing the test tubes to stand for 10 min, they are vortexed for 30 s and placed for20 min in a water bath at room temperature for color development. Then, light absorption at 490 nm is recorded on a spectrophotometer (M-ETKAL 721 Spectrophotometer). Reference solutions are prepared in identical manner as above, except that the 2 mL aliquot of carbohydrate is replaced by DDI water. The phenol used in this procedure was redistilled and 5%phenol in water (w/w) was prepared immediately before the measurements **[Du Bios *et al.*, 1956; Masuko *et al.*, 2005]**.

3. Results

3.1 Isolation and screening of CA-producers

Two hundred and nine microbial isolates were obtained from 45 isolation sources. Out of these isolates, 173 isolates showed yellow color on PDA and MSM. This indicate that these isolates have ability to produce acids. therefore, selected for further quantification screening by cultivation in MSM.

CA content was estimated and compared. As shown in Table (1), 26 isolates showed CA production higher than 0.14 g/L with the highest concentration of 1.21 g/L with yield of 0.07 g/g by isolate NH-3. Therefore, this strain was considered as the most potent isolate and used in further studies.

Table 1: Qualitative and quantitative screening test by 25 selected isolates for CA production

Sample code	***NH-3***	NH-1	PH-2	PM-1	NP-2	PQ-3	PQ-4	PS-1	PV-3	NA-1	PZ-3	PBA-1	PBA-4	NCA-1	NHA-5	NLA-4	PMA-1	POA-1	POA-2	POA-3	PPA-2	PQA-2	PQA-3	H Hemi	S2I
Bromo Cresol	++	++	+	+	+++	+	+	++	++	+++	+++	++	++	++++	++	++	+	++	+++	+++	+	++	++	++	++
Citric acid; g/L	***1.15***	0.72	0.70	0.03	0.25	0.35	0.30	0.69	0.48	1.21	0.33	0.19	0.95	0.15	0.19	0.48	0.36	0.13	0.12	0.33	0.16	0.17	0.11	0.28	0.52
Consumed sugar; (g/L)	***16.85***	22.38	23.81	26.74	25.68	25.14	25.22	23.05	25.19	12.14	24.97	25.49	17.59	23.14	19.69	21.81	26.05	25.61	27.71	25.78	25.59	25.49	26.13	26.77	22.13
Citric acid Yield (g/g)	***0.07***	0.03	0.03	0.001	0.01	0.01	0.01	0.03	0.02	0.10	0.01	0.01	0.05	0.01	0.01	0.02	0.01	0.01	0.004	0.01	0.01	0.01	0.004	0.01	0.02

3.2. Identification of isolate NH-3

Identification of isolate NH-3 was performed by studying its morphological, with macroscopic and microscopic examination (Table 2, plate 1). Yeast isolate NH-3 grow rapidly on PDA with white, creamy and smooth or wrinkled colonies when incubated for 4 days at 28°C. The microscopic examination showed oval, round, or cylindrical shapes with pseudo hyphae.

Table 2: Culture and microscopic characteristics of yeast isolate NH-3 growing on PDA.

Character	**Examination**
Culture Examination	White and the reverse is white also
Growth characteristics	
Microscopic Examination	
Cell shape	Oval, round, or cylindrical

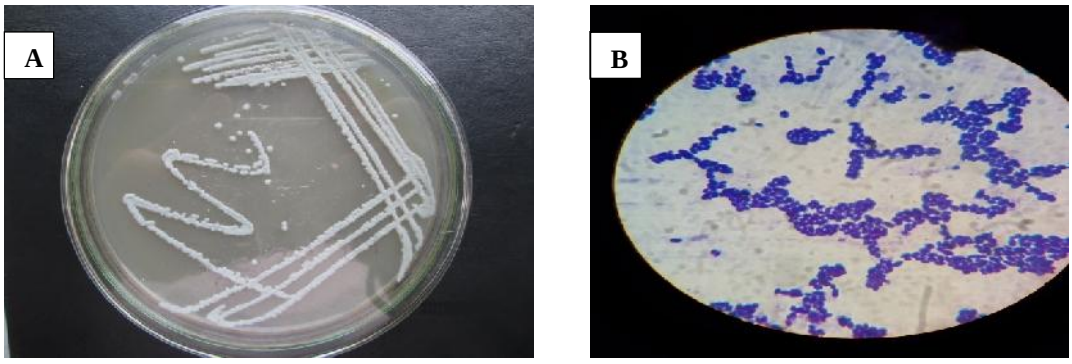


Plate 1 . Morphological and microscopic characteristic of yeast isolate NH-3. (A) colony of Yeast isolate NH-3 on PDA, (B) bright field microscope (X=10x40).

The gene sequence of the *NH-3* isolate showed the highest similarity at 99.0 % with *C. parapsilosis* isolate CP54 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence. The phylogenetic tree constructed using strain *NH-3* and other closely related type strains is depicted in Figure.1 and plate 2. The strain showed 99 % identity to *C. parapsilosis* isolate CP54 (MF 462160.1). Therefore, the strain was identified as *C. parapsilosis* NH-3.

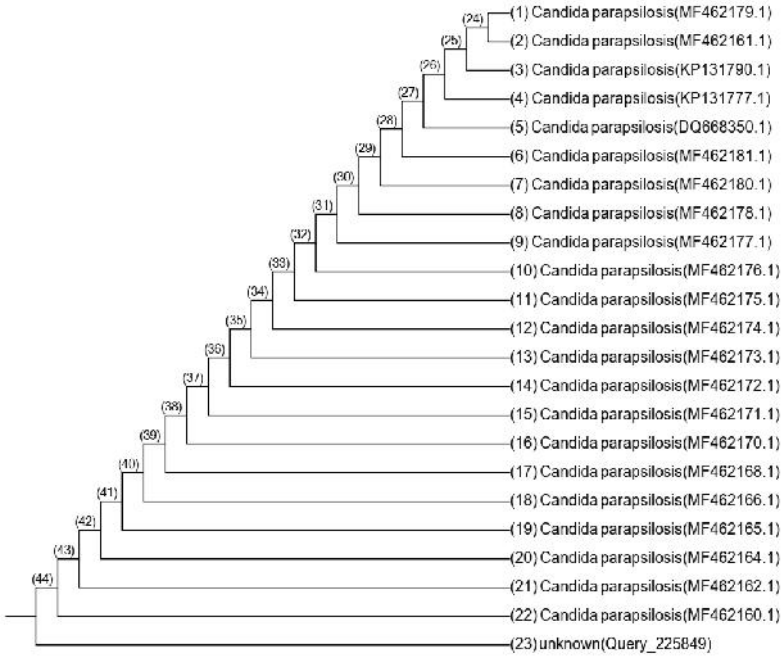


Figure 1. Phylogenetic analysis of 18S rRNA sequences of the fungal isolates (NH3) with the sequences from NCBI. Symbol ■ refers to 18S rRNA gene fragments retrieved from this study. The analysis was conducted with MEGA 6 using neighbor joining method. Bar 0.1 nucleotide substitutions per position

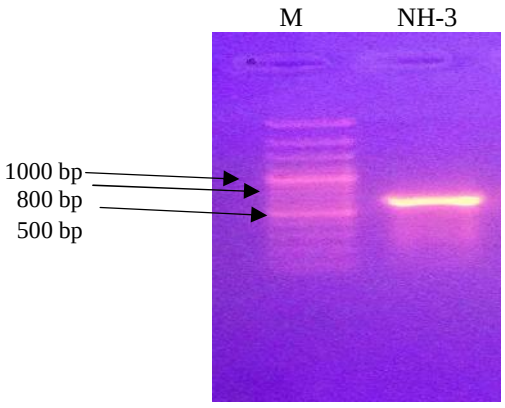


Plate 2. PCR product of DNA gene for the isolates NH-3, where (M) DNA ladder (marker).

3.4. Factors affecting CA production

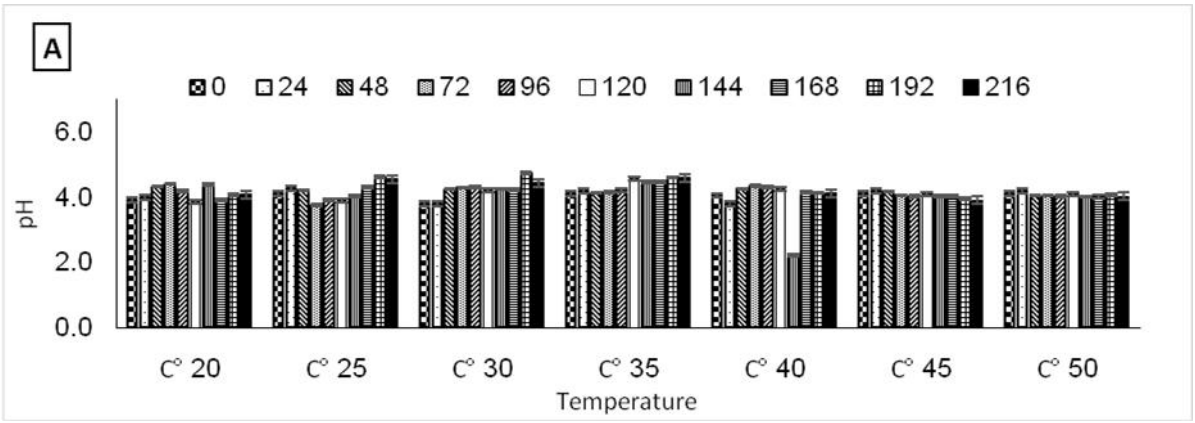
3.4.1. Effect of Temperature

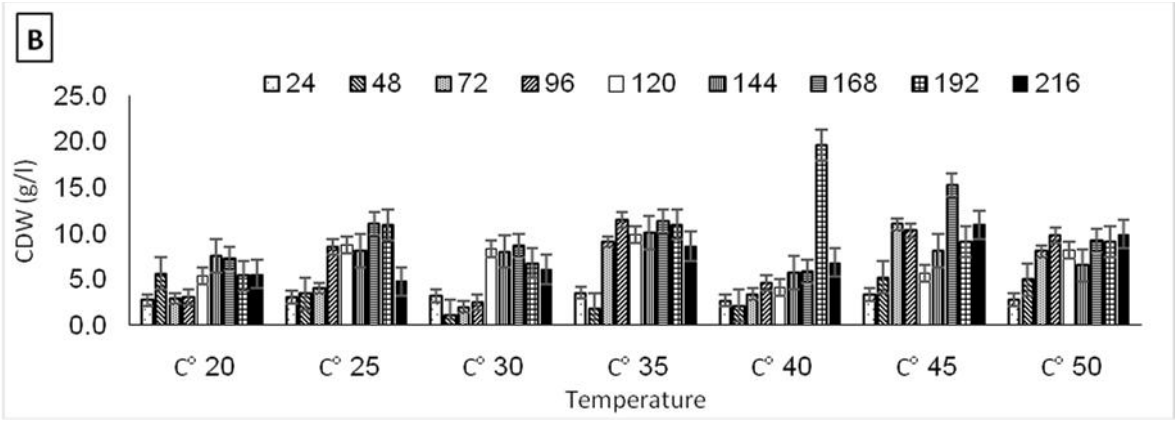
Strain NH-3 was cultivated in MSM medium at different incubation temperatures (20–50 °C). The DCW was increased at higher temperature 40-45°C [ranged, 15.30-19.65g/L] than obtained at lower temperature 20-35°C [ranged, 7.54-9.05 g/L]. However, a variable sugar consumption was achieved ranged 3.75 -28.79 g/L, almost comparable CA was obtained at all tested incubated temperatures that ranged 2.06-3.55 g/L (Figure2.D) that greatly affected the CA yields (ranged 0.08-0.95 g/g-consumed sugar) (Figure 2.E). The consumed sugar of 3.75 g/L for the highest CA production of 3.55g/L with CA yield of 0.20 g/g and the biomass yield was 19.65 g/g-based on consumed sugar obtained at incubation temperature 40°C after 192 h that resulted in low productivity at 0.02g/L/h (Table 3). This shows that the isolate achieved the best kinetic parameters for CA production at elevated temperature. From the above results, temperature 40°C was considered as the best temperature for CA production by strain NH-3.

Table 3: Effect of different temperature on citric acid production by *Yeast* NH-3 with the maximum values over a span of 216 hours

Temperature (°C)	pH	CDW (g/L)	CS (g/L)	$Y_{(CDW/CS)}$ [a] (g/g)	CA (g/L)	$Y_{(CA/CS)}$ [b] (g/g)	Productivity (g/L/h) [C]
20 °C	4.08 ± 0.03	7.54 ± 16.69	5.88 ± 1.18	1.28	3.26 ± 0.19 (216 h)	0.55	0.02
25 °C	4.02 ± 0.12	9.05 ± 13.08	28.79 ± 0.87	0.31	2.43 ± 0.02 (216 h)	0.08	0.01
30 °C	4.29 ± 0.25	8.63 ± 11.81	19.09 ± 1.11	4.52	2.06 ± 0.23 (216 h)	0.11	0.01
35 °C	4.59 ± 0.20	12.61 ± 54.45	23.95 ± 4.04	0.53	2.35 ± 0.04 (216 h)	0.10	0.01
40 °C	4.12 ± 0.02	19.65 ± 67.25	3.75 ± 1.09	5.24	3.55 ± 0.02 (192 h)	0.95	0.02
45 °C	3.91 ± 0.01	15.30 ± 18.88	23.06± 2.30	0.66	2.41± 0.06 (216 h)	0.10	0.01
50 °C	4.02 ± 0.09	9.90 ± 13.01	12.93 ± 0.00	0.77	2.57 ± 0.00 (216 h)	0.20	0.01

[a]Yield of biomass based on substrate consumed; [b] Yield of CA based on substrate consumed; [c]Productivity of CA





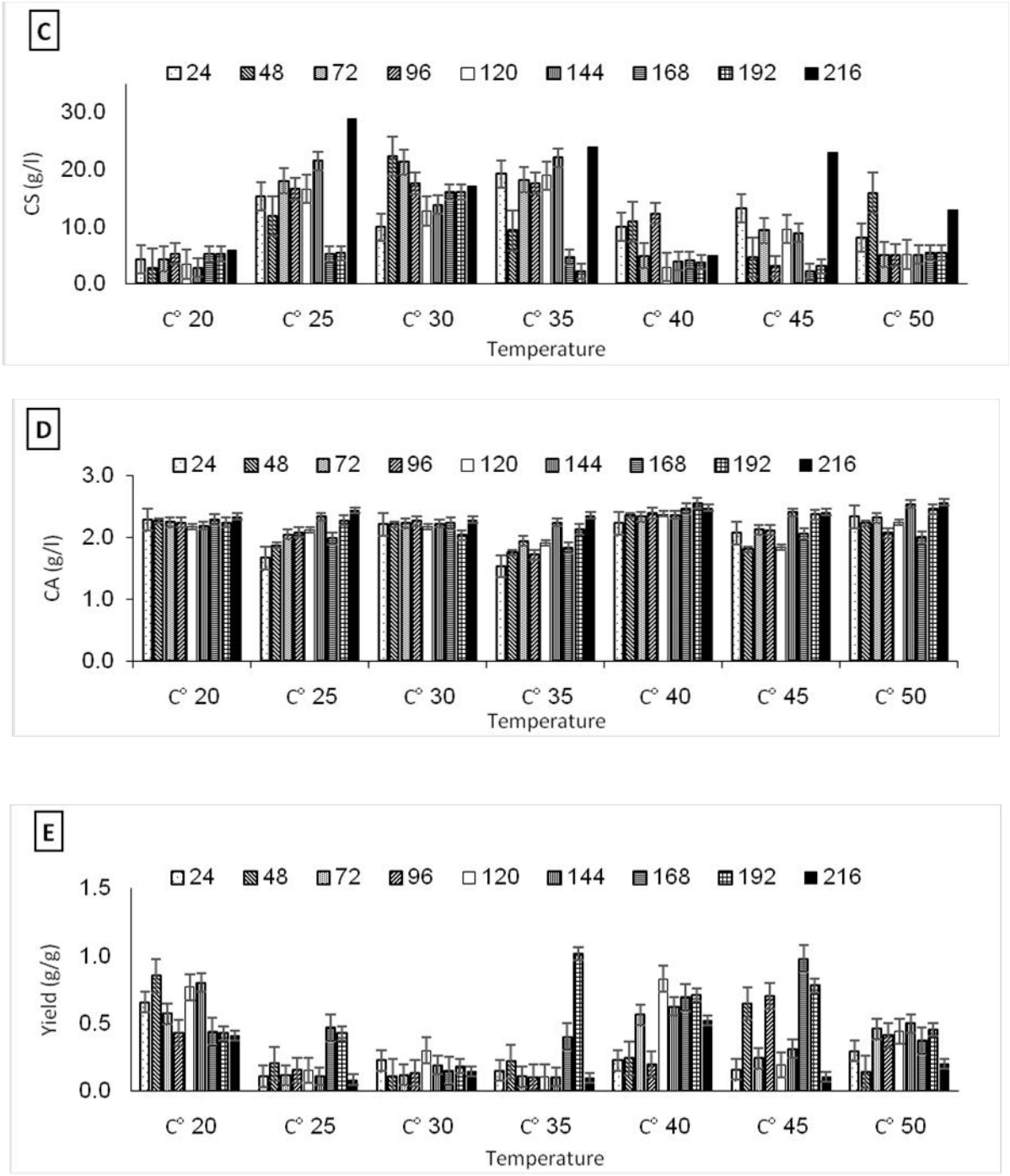


Figure 2. Effect of incubation temperature on CA production from Sugar Cane molasses by *C.parapsilosis* NH-3 (A) pH, (B) CDW, g/l, (C) CS, g/l, (D) CA production, g/l, and (E) CA yield. The standard deviation is less than that corresponding to the size of the symbol if no error bars are seen.

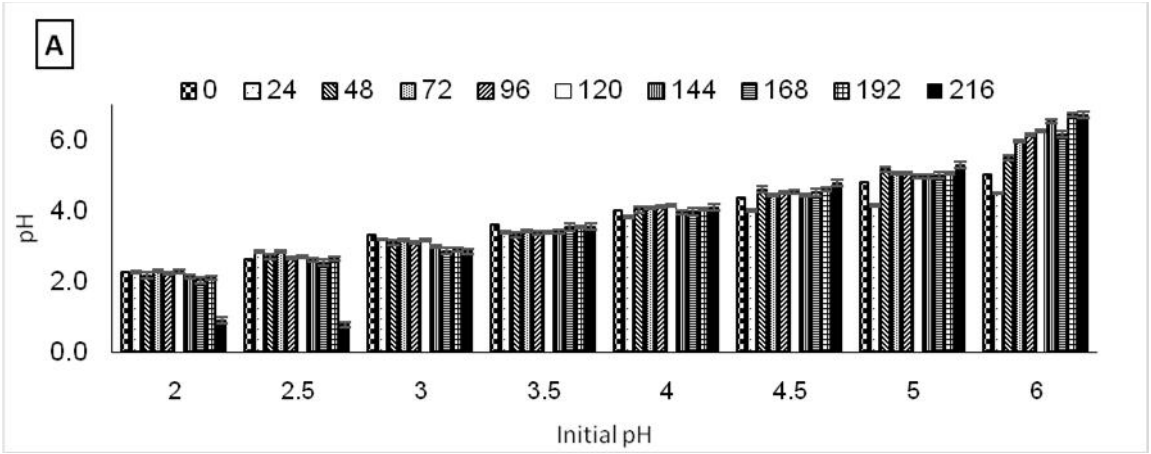
3.4.2. Optimization of pH

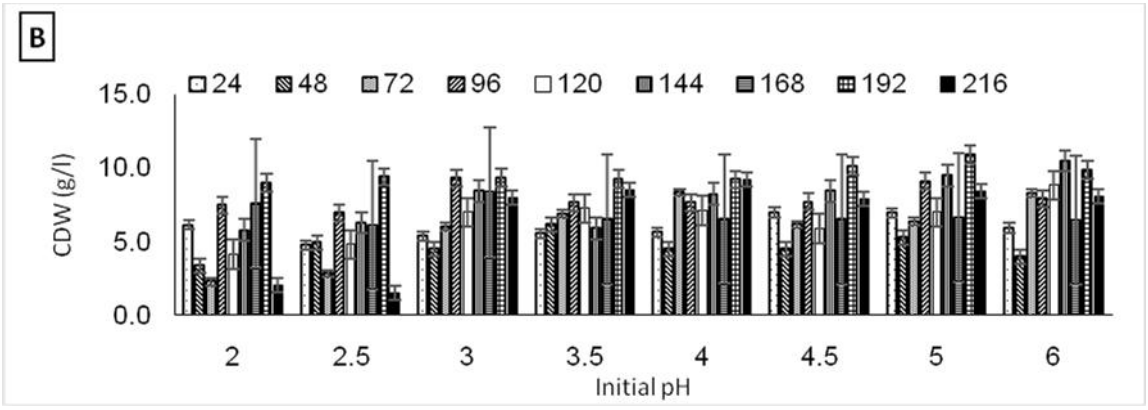
Different pH values were investigated as shown in Table 4 and Figure 3. High DCW was obtained at high pH (4.5-6.0) as compare to higher pH value (2.0-4.0) with production of comparable CA. This results in higher specific production of CA at higher pH values (data not shown). pH 6.0, pH 2.5 and pH 2.0 attained high CA yield at 0.81, 0.58, and 0.54 g/L, respectively. The consumed sugar of 16.08 g/L with highest CA production of 2.38 g/L were obtained at pH 5.0 after 48 h (Figure 3.D). With pH 5.0, the biomass yields 0.68g/g, CA yield of 0.15 g/g, and productivity of 0.05 g/L/h was achieved. On the other hand, pH values3.0 showed CA lower than 2.29 g/L by strain NH-3.

Table 4: Effect of different pH values on citric acid production by *C. parapsilosis*NH-3 with the maximum values over a span of 216 hours at 45 °C.

Initial pH	pH	CDW (g/L)	CS (g/L)	$Y_{(CDW/CS)}$ [a] (g/g)	CA (g/L)	$Y_{(CA/CS)}$ [b] (g/g)	Productivity (g/L/h) [C]
2.00	2.33 ±0.10	8.97 ± 0.68	4.32 ± 0.66	2.08	2.34 ± 0.14 (24 h)	0.54	0.10
2.50	2.43 ± 0.01	9.39± 0.20	4.18 ± 1.13	2.25	2.33 ± 0.02 (48 h)	0.58	0.05
3.00	3.14 ± 0.04	9.34 ± 0.25	13.94 ± 1.91	0.67	2.29 ± 0.08 (24 h)	0.16	0.10
3.50	3.69± 0.10	9.27 ± 0.45	27.42 ± 0.03	0.34	2.33± 0.05 (144 h)	0.08	0.02
4.00	3.535 ± 0.01	9.20 ± 0.11	4.81 ± 3.76	1.91	2.32 ± 0.04 (48 h)	0.48	0.05
4.50	4.23 ± 0.18	10.15 ±0.03	2.85 ± 0.16	3.56	2.31 ± 0.10 (24 h)	0.81	0.10
5.00	4.30 ± 0.17	10.91 ± 0.30	16.08 ± 0.35	0.68	2.38 ± 0.14 (48 h)	0.15	0.05
6.00	4.60 ± 0.00	10.47 ± 0.26	2.85 ± 0.57	3.67	2.37 ± 0.07 (24 h)	0.83	0.10

[a] Yield of biomass based on substrate consumed; [b] Yield of CA based on substrate consumed; [C]Productivity of CA





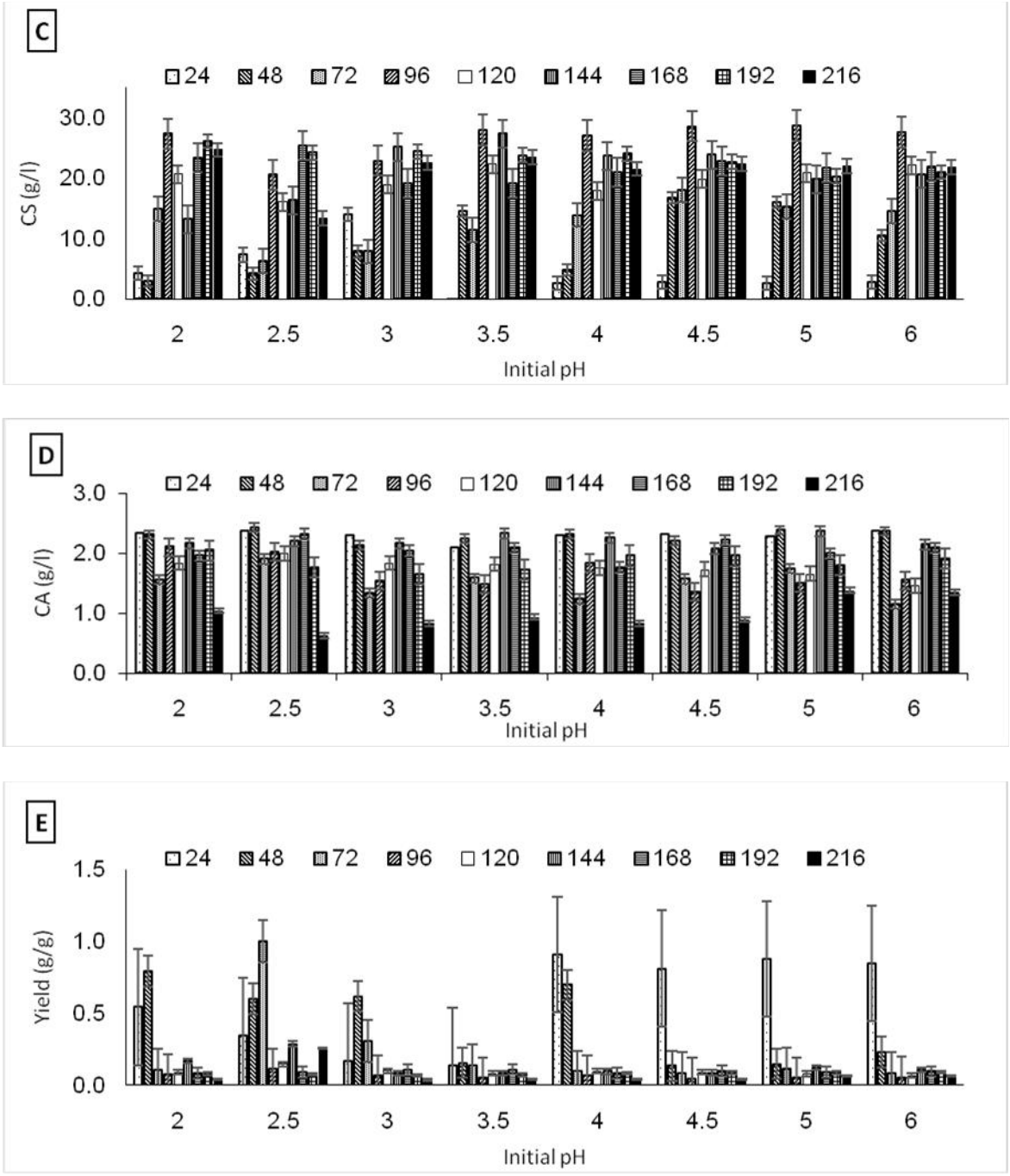


Figure 3. Effect of pH value on CA production from Sugar Cane molasses by *C. parapsilosis* NH-3incubated at 40 °C (A) pH, (B) CDW, g/l, (C) CS, g/l, (D) CA production, g/l and (E) CA yield. The standard deviation is less than that corresponding to the size of the symbol if no error bars are seen.

3.4.3. Effect of SCM concentrations

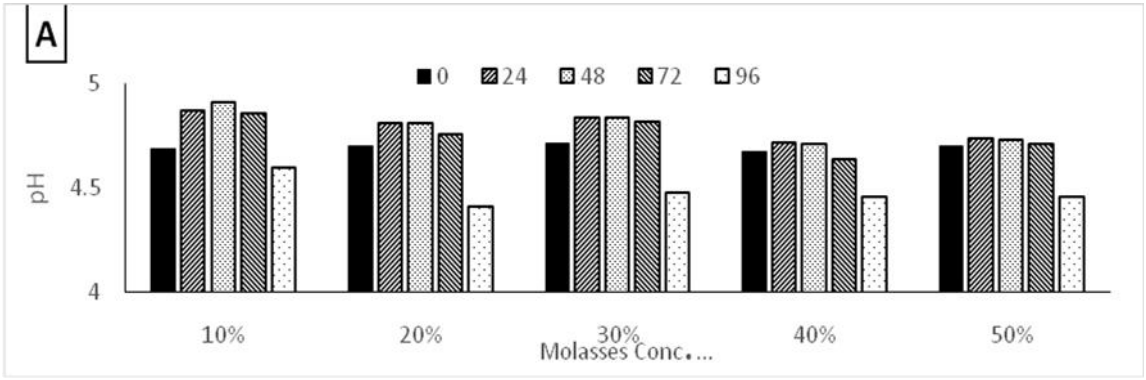
C. parapsilosis NH-3 was cultivated in MSM medium containing different sugarcane molasses concentrations (10–50 %) with and without filtration through activated carbon (Table 4). The DCW and consumed sugar were increased with the increment of sugar concentration. However, comparable CA concentrations were obtained [3.30-4.30 g/l]. The yield of biomass based on consumed sugar ($Y_{DCW/CS}$) was higher without using charcoal filtered molasses than using molasses with charcoal filtration (Table 4).

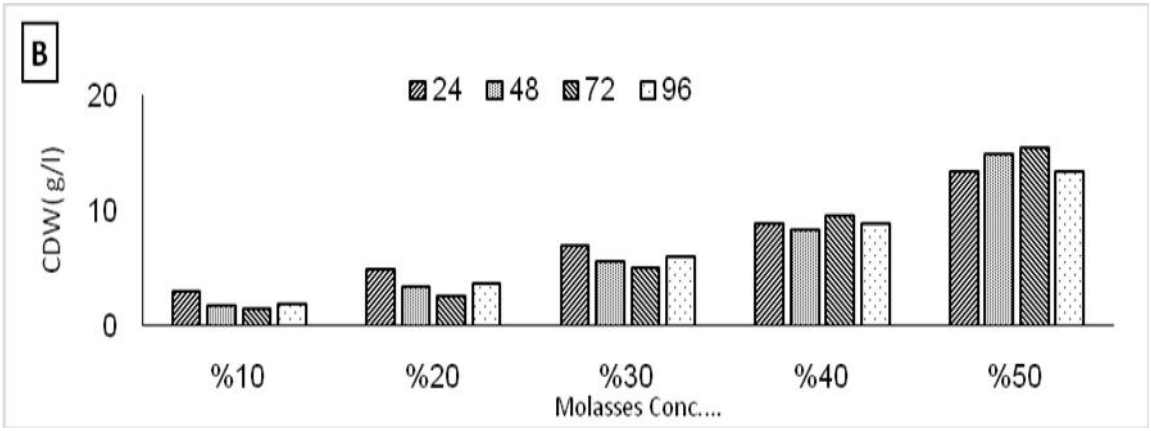
The consumed sugar of 49.64 g/l with the highest CA production of 4.30 g/L at yield of 0.09 g/g was obtained using 30 % SCM without filtration after 48 h (Figure 4.E) that resulted in productivity of 0.09 g/L/h. Beyond this concentration (> 30%), decline in CA production was obtained. From the above results, concentration 30 % of SCM without filtration was considered as the best concentration for production CA by strain NH-3.

Table 4: Effect of different molasses concentration filtrated with/without activated charcoal filtration on citric acid production by *C. parapsilosis* NH-3 with the maximum values over a span of 96 hours.

SCM conc. (%)	C-Filtration	pH	CDW (g/L)	CS (g/L)	$Y_{(CDW/CS)}$ [a] (g/g)	CA (g/L)	$Y_{(CA/CS)}$ [b] (g/g)	Productivity (g/L/h) [C]
10	Without	4.87	2.16	4.93	0.44	4.27 (24 h)	0.87	0.18
	C-Fil	4.87	2.16	4.53	0.48	3.98 (24 h)	0.88	0.17
20	Without	4.81	2.59	31.77	0.08	4.3 (48 h)	0.14	0.09
	C-Fil	4.81	2.59	44.85	0.06	4.3 (24 h)	0.10	0.18
30	Without	4.84	5.09	49.64	0.10	4.3 (48 h)	0.09	0.09
	C-Fil	4.84	5.09	94.90	0.05	4.3 (48 h)	0.05	0.09
40	Without	4.71	8.43	133.83	0.06	4.3 (48 h)	0.03	0.09
	C-Fil	4.71	8.43	11.15	0.76	4.3 (24 h)	0.04	0.18
50	Without	4.74	11.8	191.90	0.06	4.3 (24 h)	0.02	0.18
	C-Fil	4.74	11.8	192.15	0.06	4.3 (72 h)	0.02	0.06

[a]Yield of biomass based on substrate consumed; [b] Yield of CA based on substrate consumed; [c] Productivity of CA





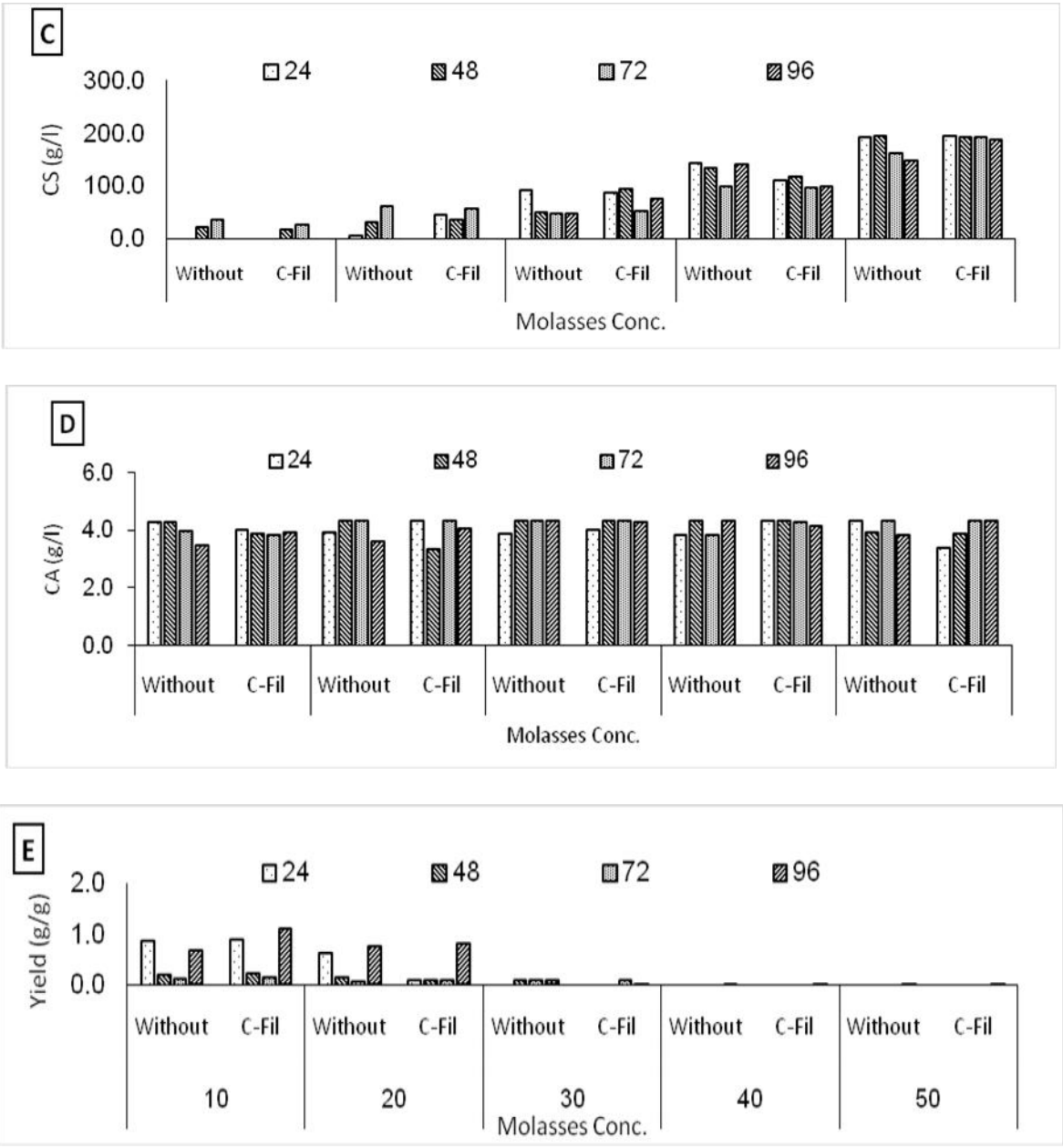


Figure 4. Effect of different Sugar Cane molasses concentration on CA production by *C.parapsilosis* NH-3(A) pH, (B) CDW, g/l, (C) CS, g/l, (D) CA production, g/l and (E) CA yield.

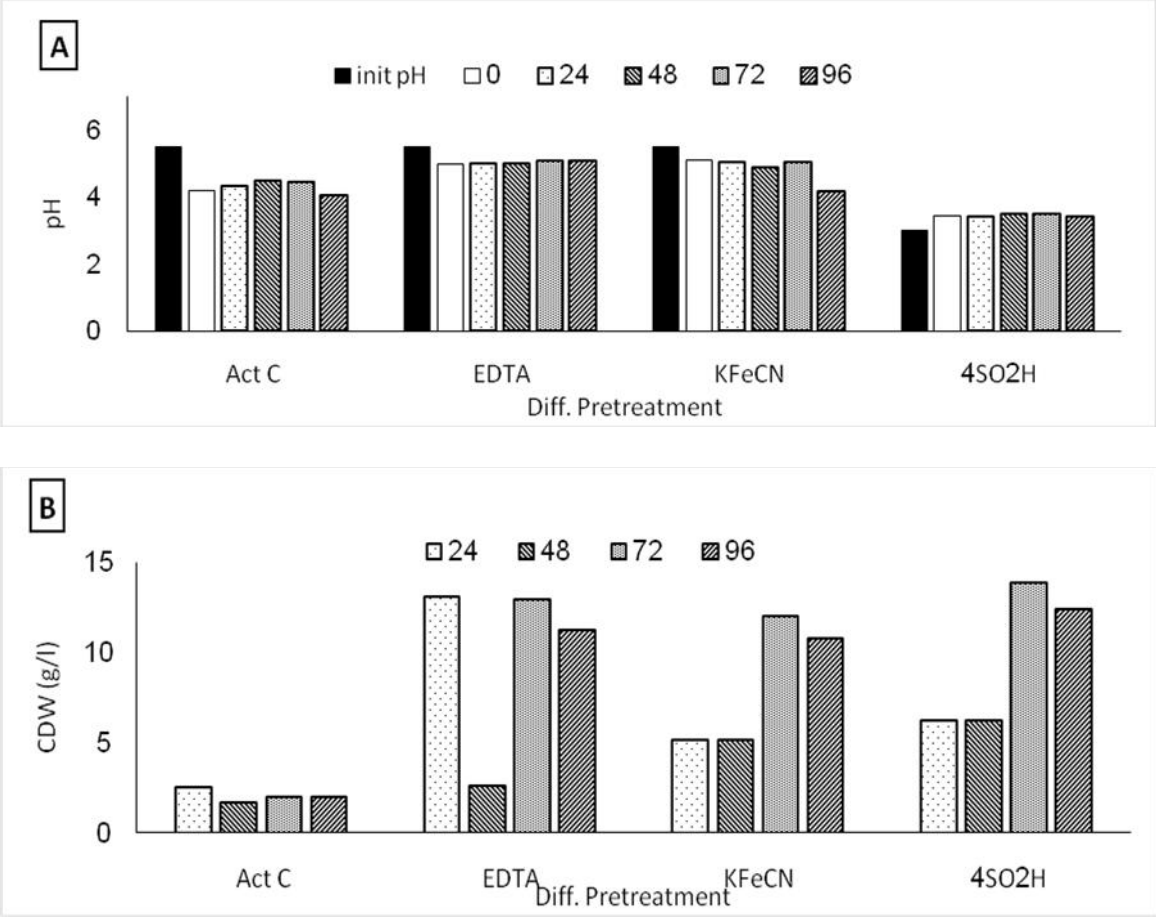
3.4.4. Effect of SCM pre-treatment

The influence of different SCM pre-treatment ($K_4Fe(CN)_6$, EDTA and Sulfuric acid) on CA production by strain NH-3 were investigated as shown in Figure (5.A-E). A comparable result in term of DCW, CS, and CA production were obtained using the tested treatment methods. It was found that, there is a positive relation between method of pre-treatment and CS /CA production by strain NH-3. Using EDTA treatment method, CS was increased achieving maximum value of 4.30g/l with CA production 4.11 g/L- giving yield reached to 0.96 g/g and productivity 0.18 g/L/h after 24 h. Therefore, EDTA treatment method was considered as the optimal method for SCM pretreatment for CA production byNH-3.

Table 5: Effect of different molasses pretreatment on citric acid production by *C.parapsilosis* NH-3 with the maximum values over a span of 96 hours.

Pre-treatment method	pH	CDW (g/L)	CS (g/L)	$Y_{(CDW/CS)}$ [a] (g/g)	CA (g/L)	$Y_{(CA/CS)}$ [b] (g/g)	Productivity (g/L/h) [C]
ACT-C	4.05	2.57	8.35 ±0.00	0.31	0.74 ±0.00 (96 h)	0.09	0.01
EDTA	5.00	13.03	4.30 ±0.31	3.03	4.11 ±0.00 (24 h)	0.96	0.18
$K_4Fe(CN)_6$	4.17	12.07	7.27 ±0.07	1.66	3.77 ± 0.00 (96 h)	0.52	0.04
H_2SO_4	3.43	13.95	6.66 ±0.00	2.09	4.00 ± 0.60 (96 h)	0.60	0.04

[a] Yield of biomass based on substrate consumed; [b] Yield of CA based on substrate consumed; [c]Productivity of CA



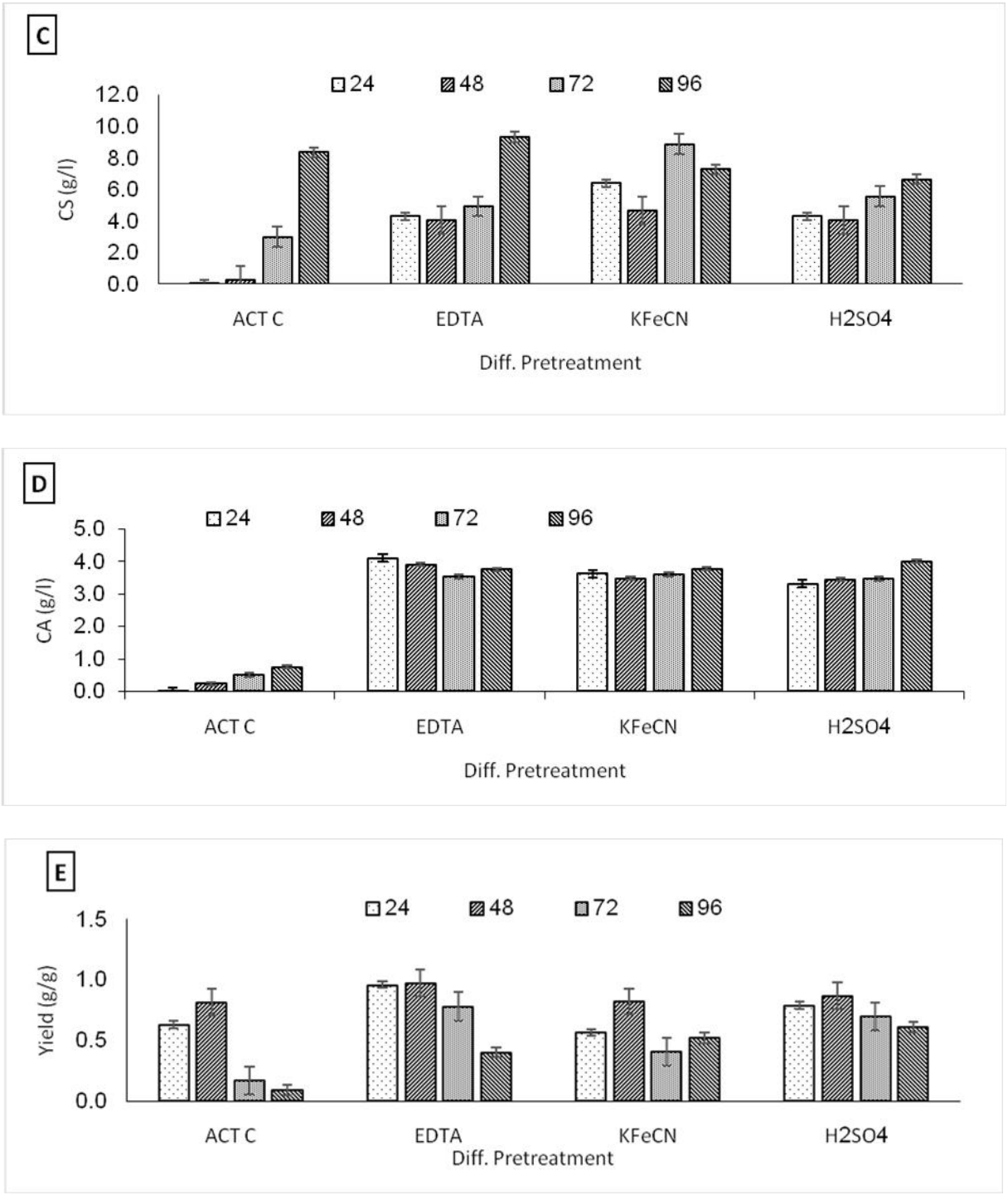


Figure 5. Effect of different pretreatment of Sugar Cane molasses on CA production by *C. parapsilosis* NH-3 (A) pH, (B) CDW, g/l, (C) CS, g/l, (D) CA production, g/l and (E) CA yield. *Initial pH* : the adjusted pH before autoclaving, *Zero pH* : the pH after autoclaving

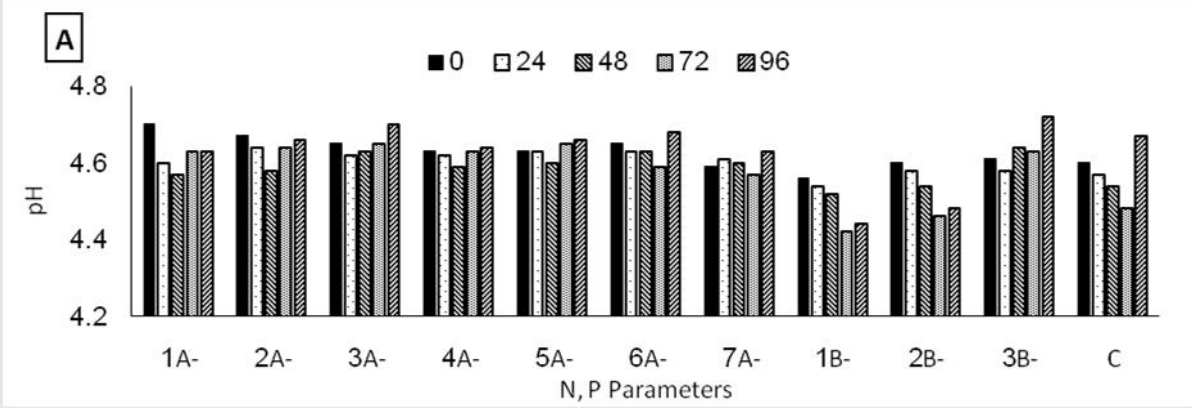
3.4.5. Optimization of Nitrogen and phosphorus limitation

Different organic and inorganic nitrogen and phosphorus sources were used in MSM for CA production which need to limitation for reducing the production cost as shown in Table 6. Nitrogen limitation resulted in increased consumed sugar compared to control. On the other hand, the fungal strain produced comparable CA using all treatment tested. CA production of 4.30 g/l- with the highest CA yield at 0.09 g/g (Figure E) and productivity 0.18 g/L/h was obtained with all MSM components except KH_2PO_4 (B-1) after 24 h compared to other limitations tested (Figure 6). In addition, the decreased DCW obtained using this medium compared to others have resulted in increased specific production of CA. Therefore, B-1 was considered as the optimal limitation for CA production by strain NH-3.

Table 6: Effect of nitrogen and phosphorus limitation on citric acid production by *C.parapsilosis* NH-3 with the maximum values over a span of 96 hours at 40 °C, pH 5.0, molasses conc 20% and Pretreated with EDTA.

N,P- limitation	pH	CDW (g/L)	CS (g/L)	$Y_{(CDW/CS)}$ [a] (g/g)	CA (g/L)	$Y_{(CA/CS)}$ [b] (g/g)	Productivity (g/L/h) [C]
A-1	4.64	17.78	52.59	0.34	4.30 (24 h)	0.08	0.18
A-2	4.60	15.83	53.08	0.30	4.30 (24 h)	0.08	0.18
A-3	4.60	32.19	76.26	0.42	4.30 (24 h)	0.06	0.18
A-4	4.58	16.77	65.19	0.26	4.30 (48 h)	0.07	0.09
A-5	4.53	20.17	73.25	0.28	4.30 (24 h)	0.06	0.18
A-6	4.58	20.53	52.59	0.39	4.30 (48 h)	0.08	0.09
A-7	4.58	22.02	72.57	0.30	4.30 (24 h)	0.06	0.18
B-1	4.61	24.97	49.07	0.51	4.30 (24 h)	0.09	0.18
B-2	4.57	24.59	52.05	0.47	4.30 (24 h)	0.08	0.18
B-3	4.57	30.47	52.74	0.58	4.30 (48 h)	0.08	0.09
C	4.57	10.11	51.85	0.19	4.30 (24 h)	0.08	0.18

[A-1]MSM without $Ca(NO_3)_2$; **[A-2]**MSM without Yeast Ext; **[A-3]**MSM without $(NH_4)_2SO_4$; **[A-4]** MSM without $Ca(NO_3)_2$, Yeast Ext; **[A-5]**MSM without $Ca(NO_3)_2$, $(NH_4)_2SO_4$; **[A-6]**MSM without Yeast Ext,$(NH_4)_2SO_4$; **[A-7]** MSM without $Ca(NO_3)_2$, Yeast Ext, $(NH_4)_2SO_4$; **[B-1]**MSM withoutKH_2PO_4; **[B-2]**MSM without K_2HPO_4; **[B-3]**MSM without KH_2PO_4, K_2HPO_4; **[C]**MSM without $Ca(NO_3)_2$, $(NH_4)_2SO_4$, Yeast Ext, KH_2PO_4, K_2HPO_4.
[a] Yield of biomass based on substrate consumed; [b] Yield of CA based on substrate consumed; [c]Productivity of CA



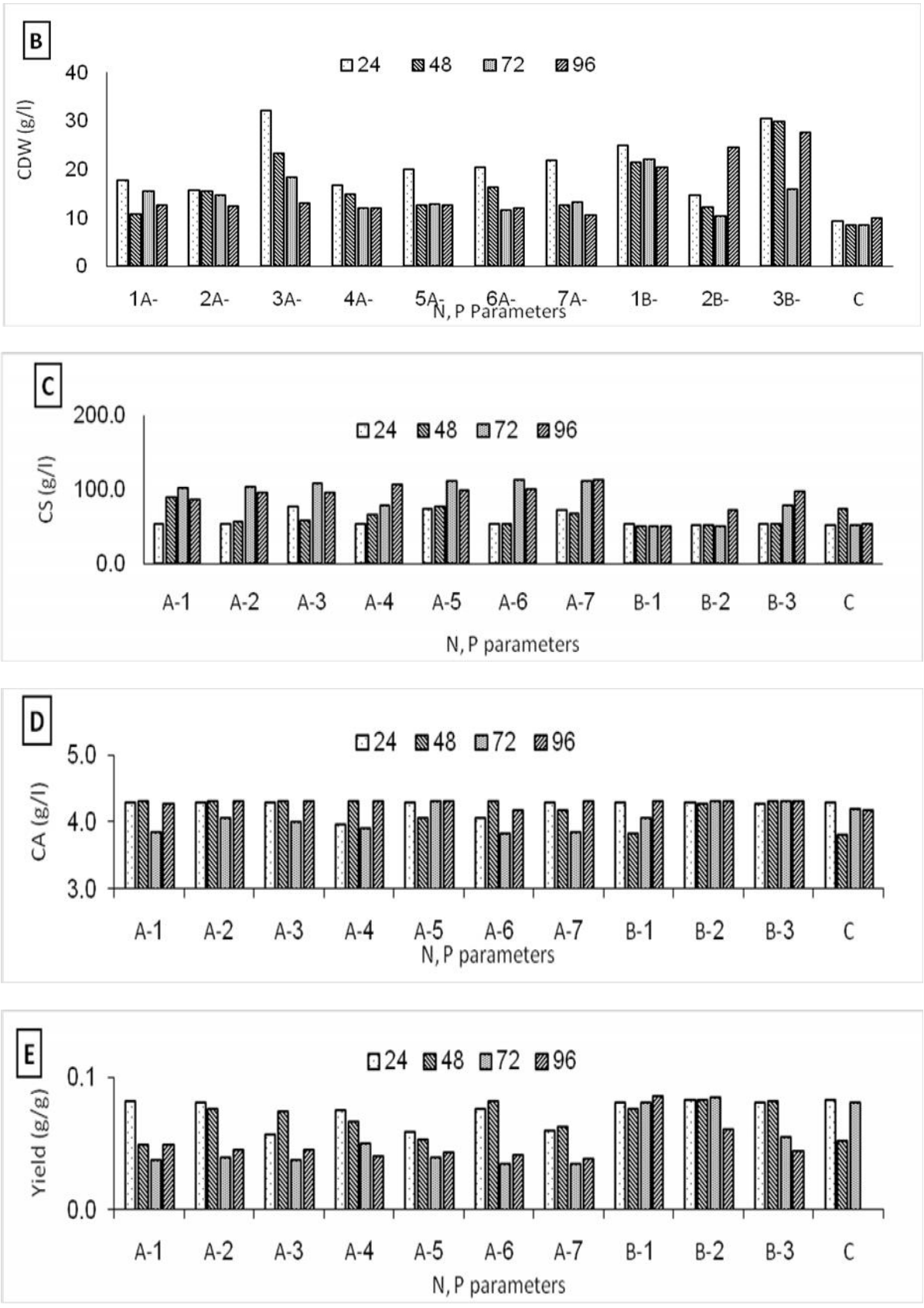


Figure 6. Effect of Nitrogen and Phosphorus limitation on CA production from Sugar Cane molasses by *C. parapsilosis* NH-3 (A) pH, (B) CDW, g/l, (C) CS, g/l, (D) CA production, g/l and (E) CA yield.

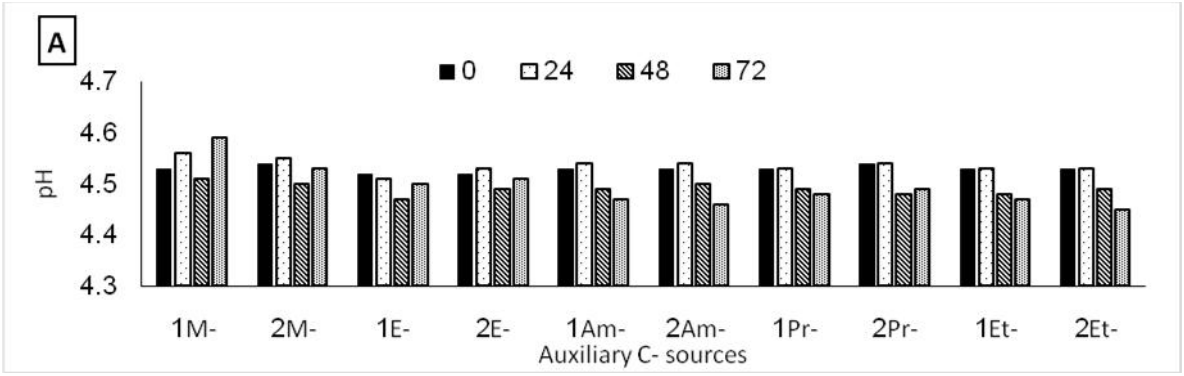
3.4.6. Effect of auxiliary carbon sources (co-substrates)

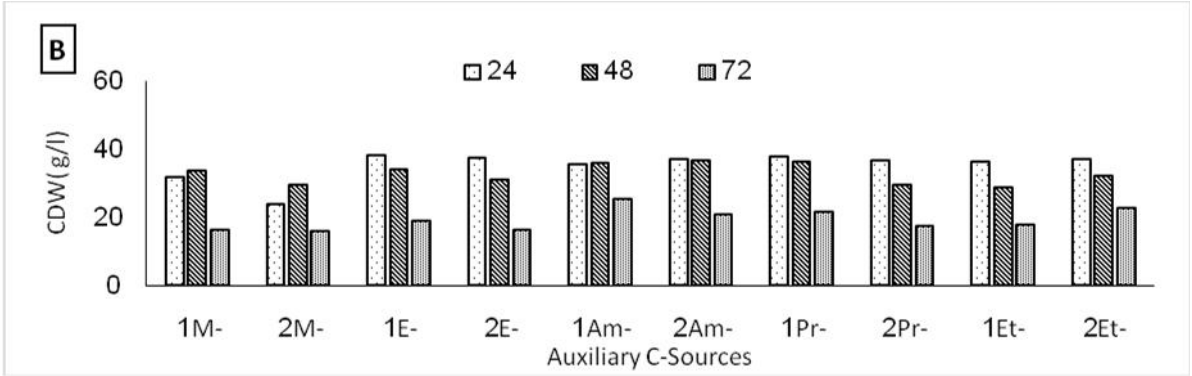
Different additional carbon sources were supplemented separately (Methanol, Ethanol, Amyl alcohol, Propanol, and Ethyl Acetate) at 1 and 2% to optimized MSM media containing 30% SCM pretreated with EDTA method at 50°C and pH 5.0. As shown in Table 7, Figure 7.A-E, almost no differences between auxiliary carbon source supplementation. Amongst all tested materials, methanol resulted in consumed sugar of 55.68 g/L with CA production at 4.30 g/L with CA yield 0.08g/g after 24h. Other tested co-substrates resulted in lower yield. Thus Methanol 1% was considered as the best auxiliary carbon sources for CA production using SCM as a carbon source.

Table 7: Effect of different auxiliary carbon sources on CA production by *C. parapsilosis* NH-3 with the maximum values over a span of 72 hours (at 40 °C, pH 5.0, molasses conc 20% and Pretreated with EDTA without KH_2PO_4).

Auxillary C-Sources	Conc.	pH	CDW (g/L)	CS (g/L)	$Y_{(CDW/CS)}$ [a] (g/g)	CA (g/L)	$Y_{(CA/CS)}$ [b] (g/g)	Productivity (g/L/h) [c]
Methanol	1 %	4.5	33.8	55.68	0.61	4.30 (24 h)	0.08	0.18
	2 %	4.54	29.9	63.91	0.47	4.32 (24 h)	0.07	0.18
Ethanol	1 %	4.53	38.59	63.42	0.61	4.32 (24 h)	0.07	0.18
	2 %	4.5	37.47	63.71	0.59	4.32 (24 h)	0.07	0.18
Amyl Alcohol	1 %	4.5	35.75	63.42	0.56	4.32 (24 h)	0.07	0.18
	2 %	4.5	33.08	63.32	0.52	4.32 (24 h)	0.07	0.18
Propanol	1 %	4.5	38.22	63.61	0.60	4.32 (24 h)	0.07	0.18
	2 %	4.5	36.92	63.51	0.58	4.32 (24 h)	0.07	0.18
Ethyl Acetate	1 %	4.5	36.49	63.42	0.58	4.32 (24 hr)	0.07	0.18
	2 %	4.54	37.28	65.38	0.57	4.32 (24 h)	0.07	0.18

[a] Yield of biomass based on substrate consumed; [b] Yield of CA based on substrate consumed; [c]Productivity of CA





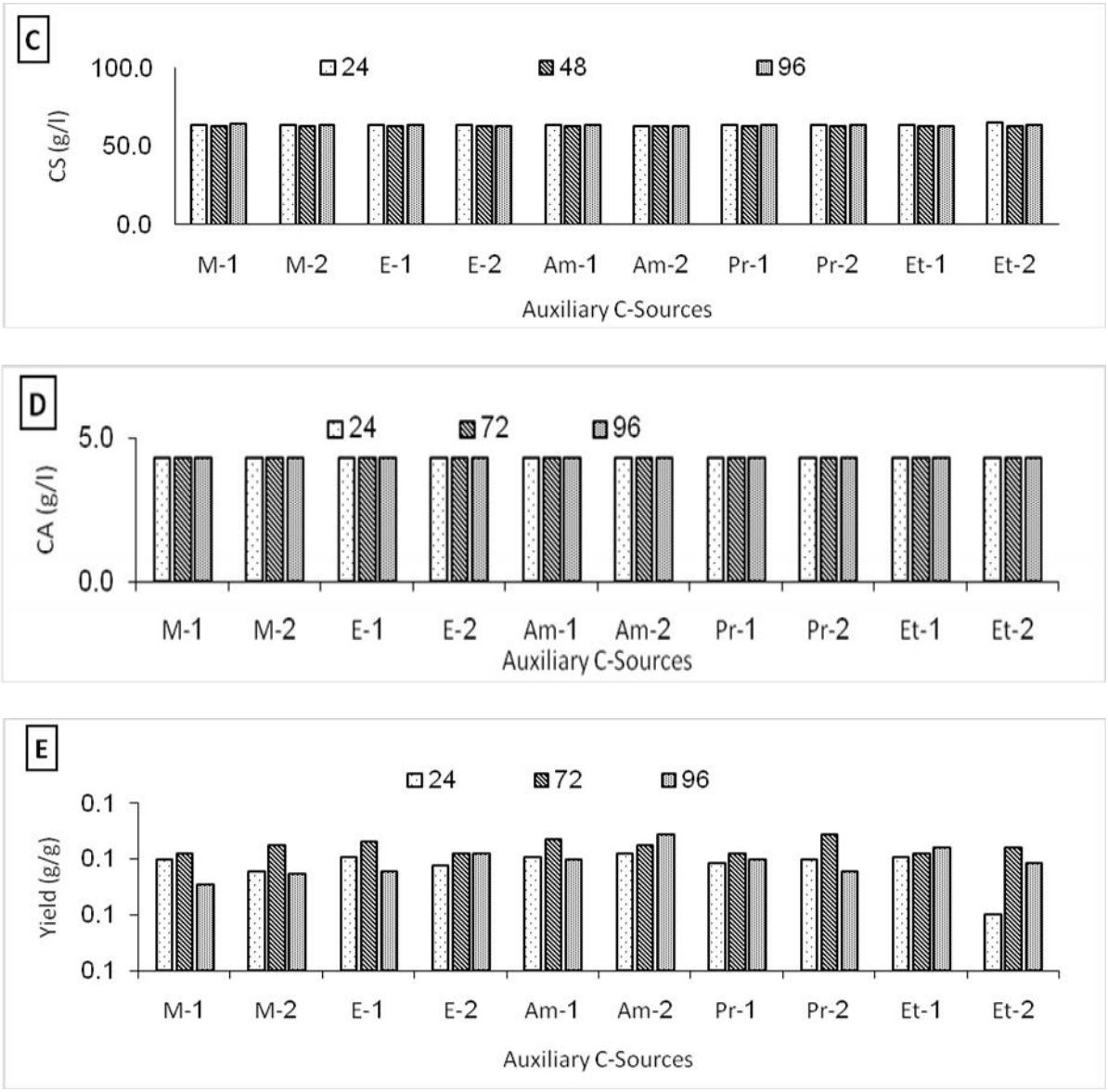


Figure 7. Effect of Auxillary C-Sources on CA production from Sugar Cane molasses by *C. parapsilosis* NH-3 (A) pH, (B) CDW, g/l, (C) CS, g/l, (D) CA production, g/l and (E) CA yield.

3.4.7. Plackett-Burman design (PBD)

In the present study, *C. parapsilosis* NH-3 produced high yield of citric acid (Table 9) and the experimental analysis (Table 8) depending on the evaluated consumed sugar. Incubation temperature (A), initial pH (B), SCM pre-treatment (C), Nitrogen and phosphorus limitation (D), auxiliary carbon source (E), and SCM concentration (F) were studied with low and high level after incubation period 72 hrs. Two variables C and Four of six variables (Table 8) implying that these variables influenced the production process significantly. While, A, B, D, and E were insignificant (Figure 8.A, B).

Table 8: The actual values of the parameters tested in PBD with p - values

Variables		Temp	pH	Pre-Treat	N-P Limit	C-Addition	Molasses conc.
Coded		A	B	C	D	E	F
Low level (-)		30	3.5	$K_4Fe(CN)_6$	A4	None	20
High level (+)		45	5	EDTA	B1	M1	30
p-value	CS	0.068	0.305	0	0.808	0.184	0.001
	Yield	0.477	0.141	0.003	0.783	0.104	0.176
Significant sign				*			*

; **[A-4]**All component except $Ca(NO_3)_2$, Yeast Ext,**[B-1]** All component except KH_2PO_4.

Table 9: Plackett-Burman design matrix for six parameters

Run	A	B	C	D	E	F	CA (g/L)	CS (g/L)	Yield (g/g)	pH
1	-	-	-	-	-	-	4.27	4.69	0.91	3.9
2	-	-	-	+	-	+	4.29	5.24	0.82	3.8
3	+	-	+	+	+	+	4.28	144.83	0.03	4.02
4	+	+	-	-	+	+	4.29	71.04	0.06	4.94
5	+	+	-	-	-	-	4.29	7.70	0.56	4.86
6	-	-	+	+	-	+	4.27	112.98	0.04	4.01
7	+	+	+	+	-	-	4.29	96.50	0.04	4.76
8	-	+	-	+	+	+	4.29	64.52	0.07	4.78
9	+	-	+	-	+	+	4.29	137.94	0.03	4.22
10	-	-	-	-	+	-	4.29	22.83	0.19	3.86
11	-	+	+	+	+	-	4.29	79.28	0.05	4.76
12	-	+	-	+	-	+	4.29	33.77	0.13	4.84
13	-	+	+	-	+	+	4.23	148.89	0.03	4.88
14	+	-	+	+	-	-	4.29	113.59	0.04	3.82
15	+	+	-	+	+	-	4.29	12.00	0.36	4.68
16	+	+	+	-	-	+	4.23	145.69	0.03	5.13
17	+	-	-	-	-	+	4.29	18.52	0.23	3.86
18	+	-	-	+	+	-	4.29	10.65	0.40	4.3
19	-	-	+	-	+	-	4.29	37.83	0.11	3.94
20	-	+	+	-	-	-	4.29	54.06	0.08	4.73

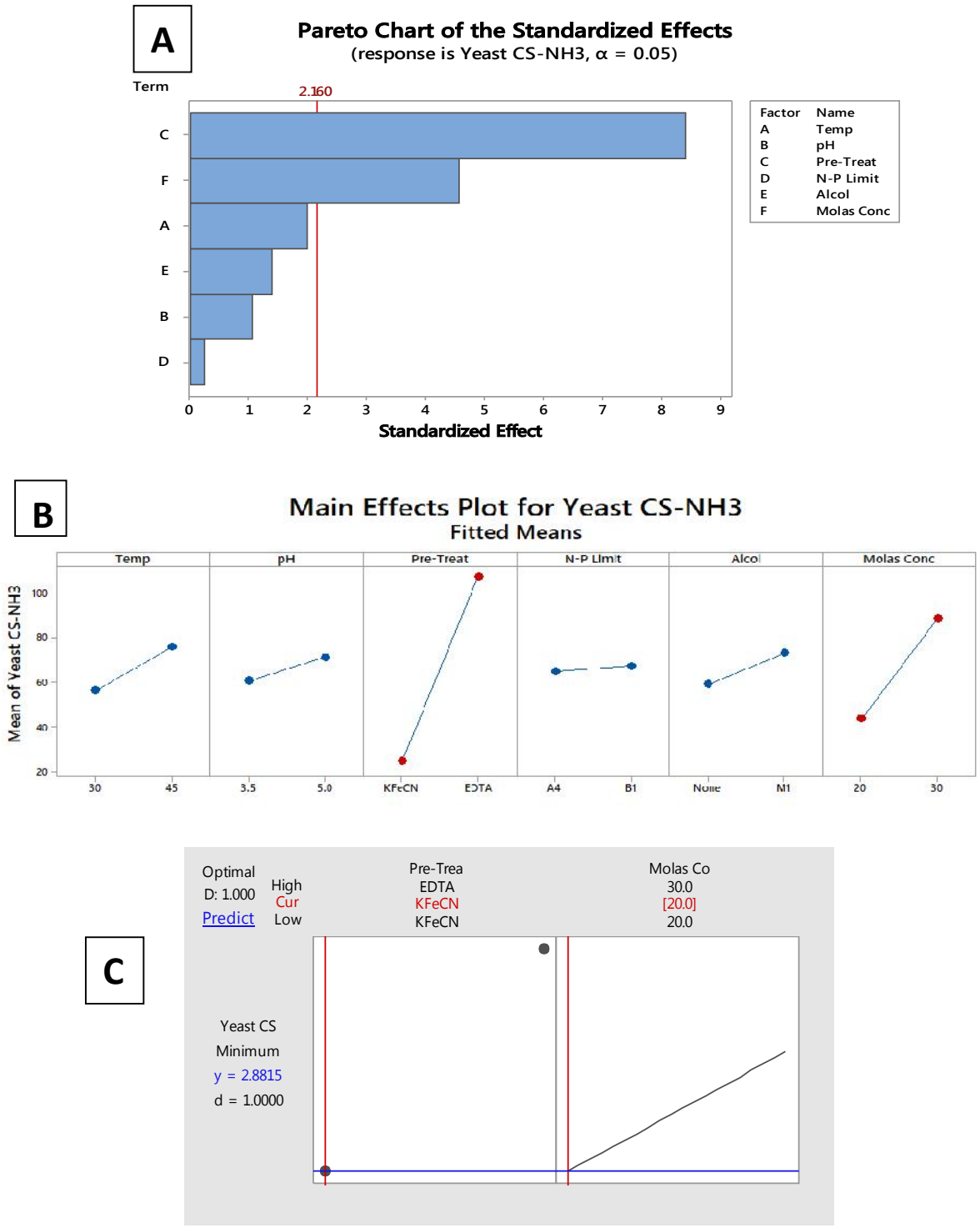


Figure 8. Plackett-Bermann design effect on CA production from Sugar Cane molasses by *C.parapsilosis* NH-3 depending on consumed sugar results (A) Standardized Effect, (B) main effects plot, (C) theoretical prediction.

The highest yield was recorded in run 1 and 2. In run 1, the incubation temperature30 °C, initial pH 3.5, $K_4Fe(CN)_6$ for SCM pre-treatment, MSM without $Ca(NO_3)_2$ and Yeast Ext, no methanol addition, and 20% molasses concentration recorded consumed sugar 4.69 g/L, citric acid production 4.27 g/l giving CA yield 0.91 g/g. While in run 2, the incubation temperature 30°C, initial pH 3.5, $K_4Fe(CN)_6$ for SCM pre-treatment, MSM without KH_2PO_4, without methanol addition, and 30% molasses concentration recorded consumed sugar 5.24 g/L, Citric acid production 4.29 g/l giving CA yield 0.82 g/g.

Consequently, Plackett-Burman design predicted for achieving the highest citric acid yield (Figure 8.C)by *C. parapsilosis* NH-3 will occur by using initial pH (3.5), $K_4Fe(CN)_6$ for SCM pretreatment, MSM without $Ca(NO_3)_2$ and 20 % of sugar cane molasses concentration, which produce 2.8815 g/L consumed sugar and 0.6285 g CA/g CS yield with 100% confidence (d=1).

4. Discussion

Citric acid production process cost need to reduce and the way for that, using cheap carbon source. One of the cheapest carbon sources is sugar cane molasses is a desirable raw material for because of its availability and relatively low price **[Angumeenal and Venkappayya, 2013]**. The major objective of this study was to select potential microbial CA producers using low cost substrates and optimize the production conditions in different fermentation modes. To achieve this aim, 219 microbial isolates were isolated from different localities in Egypt. microbial cultures grown on bromo-cresol green containing medium were analyzed to detect the medium pH **[Sabnis, 2008]**. One hundred and three isolates showed yellow color and used for quantitative analysis of CA produced from SCM as an inexpensive carbon source. Isolate NH-3 could produce the highest amount of CA at 1.15 g/L compared to other obtained isolates, therefore was selected and characterized using biochemical (Plate 1) and genetics 18S rRNA analysis. In the phylogenetic tree based on the neighbor-joining algorithm (Figure 1), Strain NH-3 clustered with the members of the genus *Candida*, showing the highest 18S rDNA gene sequence similarity of 99% with *Candida parapsilosis* and was identified as *Candida parapsilosis* NH-3. The genus *Candida* is well known for being able to synthesis CA using different carbon sources **[Omar and Rehm, 1980; Roehr *et al.*, 1993; Pazouki *et al.*, 2000]**, but to the authors knowledge, there is no report on CA production using sugar cane molasses by *C. parapsilosis*.

When the temperature of medium was increased above 25°C, the biosynthesis of citric acid increased recording the highest production at 40 °C (3.55 g/L) after 192 hrs. This is advantageous for using this strain as the high temperature greatly decrease or avoid the contamination risk during fermentation. Temperature affects the growth of microorganisms, and this effect is variable in media having different compositions. Higher incubation temperatures can cause denaturation of citrate synthase, accumulation of other by-products such as oxalic acid, and can enhance catabolite repression **[Panda *et al.*, 1984]**. Ali *et al.*, **[2002; Miall and Parker, 1974; Crolla and Kennedy, 2001]** reported maximum production of citric acid at 35°C.

pH is one of the important factors affecting cell growth, enzymatic activities and product formation. pH 5.0 was found to be optimum for CA production (2.38 g/L) by strain NH-3 after 48h. Most studies reported neutral to slightly acidic or slightly alkaline pH (pH 5.0-7.0) is the optimal for CA production by several microbial **[Laffey and Butler, 2005; Pazouki *et al.*, 2000]**. The low pH value during the production phase reduces the risk of contamination by other microorganisms and inhibits the production of unwanted organic acids (gluconic and oxalicacids), which makes the product recovery easier **[Max *et al.*, 2010; Abdelrahman *et al.*, 2017]**.

Molasses concentration is one of the critical factors affecting fermentation due to the osmotic stress, enzymatic activities and/or substrate inhibition limitation. 20% of SCM achieved the best CA production by the selected isolate NH-3, beyond this concentration CA production was reduced. This might be attributed to substrate inhibition that cause suppression of α-ketoglutarate dehydrogenase in highly concentrations levels **[Hossain *et al.*, 1984]**, inhibiting the citric acid catabolism via theKrebs cycle, leading to its accumulation **[Rohr and Kubiecek, 1981]**. On the other hand, at low levels the size of the pseudo mycelium is affected **[Laffey and Butler, 2005]** and affect cellular metabolism that decreased enzymatic activities and CA production. 60 g/L crude glycerol was reported as the optimal concentration for CA production by *C. parapsilosis* at 30 °C after 168 h [**West, 2013**], while 3.5% SCM was reported as the optimal concentration for CA production by *C. lipolytica* **[Pazouki *et al.*, 2000]**, whereas 20%sugar beet molasses was the optimal for mutant *Yarrowia lipolytica* **[Żarowska *et al.*, 2001]**.

The production of Citric acid from pretreated Sugar Cane Molasses using different chemical methods to precipitate the heavy metals is shown in Table 5. EDTA treated molasses gave a marked increase in CA concentration (4.11 g/L). CA production from other methods were low in comparison to EDTA treated medium. This means that SCM contained undesirable substances which affected CA production. The superiority of EDTA treatment over other methods may be due to the significant removal of heavy metals from molasses and EDTA was used in order to entrap metal ions from the solution **[Calik *et al.*, 2001]**.

Sugar cane molasses are rich in nitrogen and rarely need to be supplemented with a nitrogen source. The mineral salt medium used in CA production are usually supplemented with calcium nitrate, ammonium sulfate and yeast extract. While phosphate sources in the medium are dipotassium hydrogen phosphate and potassium dihydrogen phosphate, the results of CA production raised (4.30 g/L) when excluding potassium dihydrogen phosphate from MSM media. The reports have conflicting data been cited regarding the effects of phosphate limitation. According to Shu and Johnson **[1948]**, phosphate needs not be limiting for citric acid production; however, when metal levels are not limiting, additional phosphate leads to secondary reactions and promotes biomass growth **[Papagianni *et al.*, 1999A;B,C, Desouky *et al.*, 2017]**. However, Finogenova *et al.* **[2005]** demonstrated that citric acid accumulated when phosphate was limiting, even when nitrogen was not. Instead, Kristiansen and Sinclair **[1979]**, stated that the limitation by nitrogen is essential for theproduction of citric acid.

Addition of lower alcohols enhances citric acid production from Sugar Cane Molasses as reported by several authors **[Hamissa, 1978; Manonmani and Sreekantiah, 1987; Georgieva *et al.*, 1992; Dasgupta *et al.*, 1994; Żarowska *et al.*, 2001]**. So that, in this study we have investigated the effect of methanol, ethanol, amyl alcohol, propanol, and ethyl acetate at1 and 2 %. The optimal concentration of 1% methanol was selected for CA production by strain NH-3depending on yield. Alcohols have been shown to principally act on membrane permeability in micro-organisms by affecting phospholipid composition on the cytoplasmatic membrane **[Orthofer *et al.*, 1979]**. However, Meixner et al. **[1985]** argued against a role of membrane permeability in citric acid accumulation. Ingram and Buttke **[1984]** found that alcohols stimulate citric acid production by affecting growth and sporulation through the action not only on the cell permeability but also the spatial organization of the membrane, or changes in lipid composition of the cell wall.

Plackett-Burman design showed that the pre-treatment of molasses and molasses concentration were seemed to have an important role in the fermentation of citric acid. PBD was used for the screening of the parameters that had significant implications. The P-value was considered as a tool for evaluating the significant nutrients. Parameters with confidence levels greater than 100% were considered to significantly influence the response. The pretreatment (EDTA/ $K_4Fe(CN)_6$) and molasses concentration showed a significant influence in the both out of the six parameters. Pre-treatment was observed to affect the citric acid yield through the effect of the citric acid concentration, where the P-value was 0.000. The maximum yield obtained from this set of experiments was 0.91 g citric acid/g sugar cane molasses (experiment no. 1).

The determination coefficient (R^2) provides an indicator for the variability of the predicted response with the experimental results. The closer R^2 is to 1, the better able the model is to predict the response **[Wang 2006; Aghaie *et al.*, 2009;Imandi *et al.*, 2013]**. The R^2 of the following model was 1.000, indicating that 100% of the variability in the experimental results could be explained by the estimated model, which confirmed that the model was highly significant. Consequently, the media with 20% SCM treated by $K_4Fe(CN)_6$, without $Ca(NO_3)_2$, Yeast Ext, and alcohol addition, which adjusted at pH 3.5 were selected by PBD as significant parameters for citric acid production from SCM.

5. Conclusion

In the present study, CA production and optimization using SCM as substrate was investigated by *Candida parapsilosis* NH-3 strain. Incubation temperature at 40°C; pH 5.0; SCM at 30%; EDTA pretreatment; exclude from MSM KH_2PO_4; methanol 1 % were the optimal fermentation conditions for CA production by NH-3 strain. Under these conditions, maximum CA production of 4.30 g/L with CA yield of 0.09g/g were achieved after 24 h. Plackett-Burman design based on optimized parameters the best result was recorded with 20% SCM treated by $K_4Fe(CN)_6$, without $Ca(NO_3)_2$, Yeast Ext, and alcohol addition, and pH 3.5 as significant parameters for citric acid production from SCM.

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