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RESEARCH PAPER

Studies on methionine secreting micro-organisms from sago industrial wastes and standardization of growth parameters for maximum methionine secretion

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Abstract : Methionine is a dietary essential amino acid secreted by various microorganisms and can be supplemented in food and feed for enhancing the growth and body functions of the mammals. From a total of 141 microorganisms isolated from various sources such as curd, yogurt, cheese, and sago industrial waste 2 yeast culture and 2 bactreial cultures were confirmed to be elite methionine secreting organisms. The microorganisms through molecular characterization were identified as *Candida tropicalis* ITEM10456, *Kluyveromyces marxianus* CHY1612, *Acetobacter tropicalis* NRIC 0312, and *Lactobacillus paracasei* subsp. *tolerans* JCM 1171. Various parameters such as carbon and nitrogen requirement, optimum pH and temperature for maximum methionine secretion were also investigated. The organisms were found to secrete maximum methionine, *Candida tropicalis* ITEM10456 (1134 µg/ml), *Kluyveromyces marxianus* CHY1612 (1320 µg/ml), *Acetobacter tropicalis* NRIC 0312 (1412 µg/ml), *Lactobacillus paracasei* subsp. *Tolerans* JCM 1171 (1078 µg/ml) after standardization of the growth parameters.

Key Words : Methionine, Micro-organisms, Growth parameters, Standardization

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INTRODUCTION

Methionine $C_5H_{11}SNO_2$ (alpha-L-amino-gamma-methylthio-n-butyric acid) was discovered in the year 1922 by J. Howard Mueller (Howard Mueller, 1922) from casein hydroxlates, the discovery was brought about through microbial research during his study on cultural requirements of *Streptococcus*. Methionine is an α-protein dietary essential amino acid and its deficiency has also been related to childhood rheumatic fever, muscle paralysis, hair loss, depression, schizophrenia and Parkinson's liver deterioration (Rose, 1938). Methionine is frequently a limiting factor in utilization of dietary proteins and according to FAO nutritional report 1973, 22g of methionine should be present in per kg of dietary protein for the successful synthesis of tissue proteins.

The primary protein aminoacids are all manufactured on an industrial scale by three main processes: extraction from protein hydrolysates, fermentation processes (Yamada, 1972) and chemical synthesis (Kaneko *et al.*, 1974). Chemical synthesis and extraction from protein hydrolysates for methionine production are either expensive or undesirable (Kumar and Gomes, 2005). The fermentation production of amino acids using micro-organisms is based on genetic and environmental modifications because the biosynthesis of aminoacids is highly regulated (Rowbury and Woods, 1961; Herrmann and Somerville, 1983). These modifications break the normal regulatory controls, forcing the overproduction and excretion of amino acids (Barrett, 1985). Production of methionine through fermentation process also provide many other aminoacids, hence, a microbial process for commercial production of methionine

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is of more interest (Odunfa *et al*., 2001).

Methionine has been reported to be produced by both bacteria and fungi in appreciable amount, but the wild type strains produce less amount of methionine because of their highly regulated biosynthesis pathway. Ethionine resistant mutant of *Saccharomyces cerevisiae* capable of producing methionine was reported by Heiland and Hill (1993). Lysine and methionine producing *Lactobacillus* and yeasts cultures was isolated from *ogi* a fermented food by Odunfa *et al.* (2001). High methionine producing *Lactobacillus,* yeasts and other micro-organisms are isolated and their production capacity was enhanced by modifying the genes and also by altering their growth requirements (Tokuyama and Hatano, 1996: Mondal *et al.*, 1994). *Lactobacillus plantarum, Lactobacillus* sp., *Leuconostoc* sp., *Corynebacterium* sp. and *Bacillus* sp. capable of secreting methionine was isolated from cassava waste was also reported (Anike and Okafor, 2008). Wild strains of *Acetobacterium woodii* and *Neurospora crassa* capable of accumulationg methionine was also reported (Kageyama *et al.*, 1986: Metzenberg, 1964).

The present study was, therefore, carried out to isolate, screen efficient methionine producing micro-organisms and optimize their growth conditions to enhance the amino acid production.

MATERIAL AND METHODS

The experiments were conducted at Department of Agricultural Microbiology, Agricultural College and Research Institute, Madurai, Tamil Nadu. The curd, yogurt and cheese samples used in the isolation of methionine producing micro-organisms were collected from local stationary shops in Madurai, Tamil Nadu state, India and the sago industrial waste samples were collected from different sago industries of Salem, Tamil Nadu.

Isolation of methionine secreting bacteria and yeasts:

About 1.0 g of each sample was suspended in 100 ml of sterile water and kept in shaker for 30 minutes at 120 rpm, and 1 ml of the suspension was serially diluted ten-fold in sterile double distilled water. Eventually 1 ml of the 10^{-5} dilution was plated into the modified DeMan, Rogosa, Sharpe (MRS) agar devoid of yeast extract as suggested by Kumar and Gomes (2005) (glucose 15.0g, agar 13.5g, peptone 10.0g, beef extract 8.0g, sodium acetate.H_2O 5.0g, K_2HPO_4 2.0g, triammonium citrate 2.0g, $MgSO_4.7H_2O$ 0.2g, $MnSO_4.4H_2O$ 0.05g, tween80 1.0 ml , pH -6.2 ± 0.2 at 25°C) and after inoculation for 2 days at 30°C, the plates were observed for growth. Bacteria and yeast cultures were subcultured and pure cultures of the isolates were streaked on nutrient agar slants and stored at 4°C for further studies.

Screening of the micro-organisms for methionine secretion by paper chromatography method:

A modified paper chromatography method of Khanna and Nag (1973) was followed to screen the micro-organisms for methionine secretion. The seed cultures were grown in 500 ml Erlenmeyer flasks containing 50ml of modified MRS broth at 28°C on rotary shaker at 120 rpm for 2 days. Approximately 1% (v/v) of the seed culture was inoculated into 250 ml Erlenmeyer flask containing 25ml of modified MRS broth at 28°C on rotary shaker at 120 rpm for analysis of methionine secretion. Methionine production was assayed after incubation of the flask for 3 days on a rotary shaker (120 rpm) at 28°C. Uninoculated flasks served as control in all experiments and replications were maintained for each culture.

The 3 days incubated broth cultures were centrifuged at 5000 rpm for 20 min and 5 µl of the supernatant was applied 1.5 cm above one edge of Whatman No.1 filter paper. A 5 ìl standard methionine solution (0.1 mg/ml) was applied alongside with the supernatant as positive control and 5 ìl supernatant of uninoculated modified MRS broth as negative control. A rectangular chromatography tank containing the solvent mixture of n-butanol, acetic acid and water (4: 1: 1) was taken and the chromatographic experiment was allowed to run for 24 hrs. The chromatogram was air-dried at room temperature, sprayed with 0.15% ninhydrin solution dissolved in butanol and dried again at room temperature before heating at 60°C for 5 min in an oven. The Rf value (distance traveled by the aminoacid/ distance traveled by the solvent mixture) of the ninhydrin-positive spot (bluish-violet/purple) of the supernatant that corresponded with the Rf value of the standard methionine solution was taken as an indication for methionine secretion by the micro-organisms.

Methionine assay:

The methionine secretion by the micro-organisms was quantitatively analyzed by following a modified colorimetric method of Greenstein and Wintz (1961). The paper chromatography screened positive microbial cultures were inoculated on 250 ml Erlenmeyer flask containing 25 ml of modified MRS broth and analyzed for methionine secretion every 24 hrs upto 120 hrs. Five ml volume of broth culture was centrifuged at 5000 rpm for 20 minutes and the cell free supernatant was transferred to a 10 ml screw capped test tube for analyses. To each 5 ml volume of the supernatant 1ml of 5N NaOH was added, followed by the addition of 0.1 ml of 10% sodium nitroprusside solution, the mixture was thoroughly mixed by vortexing and kept undisturbed for 10 minutes. After that 2 ml of 3% aqueous glycine was added to the reaction mixture with frequent shaking for a period of 10 minutes and again allowed to stand for 10 minutes. Two ml of concentrated ortho-phosphoric acid was added drop by drop along the sides of the reaction mixture tubes with

continuous shaking. Eventually 5 minutes time interval was maintained for colour development and the intensity of the colour was measured at 540 nm in a spectrometer. A blank was maintained with the uninoculated culture broth and all the analytical reagents as reference, the results were interpolated on a standard curve obtained through varying concentrations (0.1-0.9 mg/ml) of methionine.

Molecular characterization of the micro-organisms:

The DNA from the screened bacterial and yeast isolates were extracted by following the protocol of Insta Gene Matrix (Bio-Rad, USA). Nearly full-length of 16S rRNA gene was amplified from the screened bacterial isolates using universal eubacterial primers, 27F (AGAGTTTGATCMTGG CTCAg) and 1492R (TACGGYTACCTTGTTACGACTT) and the 18S rRNA gene from the screened yeast isolates was amplified using universal fungal primers ITS1 (TCCGTAGGTGAACCTGG) and ITS4 (TCCTCCGCTTATT GATAT). Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems), following the protocols supplied by the manufacturer. The identity of 16S rDNA and the 18S rRNA gene sequence was established by performing a similarity search against the GenBank database (*http://www.ncbi.nih.gov*/BLAST).

Optimization of fermentation conditions for methionine secretion:

Factors like varying glucose concentrations, nitrogen concentrations, pH and temperature affecting the secretion of were optimized by adopting the search technique by varying one factor at a time. The experiments were conducted in 250ml Erlenmeyer flasks containing 25 ml of modified MRS broth inoculated with 1% v/v of the seed cultures . The effect of different glucose concentrations (10, 20, 30, 40 g/l of modified MRS broth) on methionine secretion by the yeast and bacterial isolates were analyzed. Different triammonium citrate concentrations (1.0, 2.0, 4.0, 6.0, 8.0, 10.0 g/l of modified MRS broth) was tried to optimize the nitrogen requirement. The optimization of pH and temperature were done by analyzing the methionine secretion at varying pH (4,5,6,7,8,9) and temperature (24 ºC, 28ºC, 32 ºC, 36 ºC). All the experiments were carried out in four sets with a control and the methionine secretion was analysed every 24hrs unto 120hrs.

Stastistical analysis:

The standard error and critical difference were analyzed from the data obtained. All data from the experiments were analyzed using the ANOVA procedure of SAS (2009) for Completely Randomized Designs.

RESULTS AND DISCUSSION

A total of 96 bacterial and 45 yeast cultures were isolated from 19 different samples such as cheese, curd, yogurt and sago industrial waste. Screening of these micro-organisms by paper chromotagraphy technique resulted in 2 yeast and 2 bacterial cultures isolated from sago industrial waste was proved to be efficient in methionine secretion. Odunfa *et al*. (2001) reported that 25.0% of the *Lactobacillus* and 87.8% of the yeast isolates from *Ogi* produced methionine. Molecular characterization of the screened organisms revealed that the organisms were *Candida tropicalis* ITEM10456, *Kluyveromyces marxianus* CHY1612, *Acetobacter tropicalis* NRIC 0312, and *Lactobacillus paracasei* subsp. *tolerans* JCM 1171. Methionine secreting yeast cultures *Candida boidinni* E500-78 had been reported earlier (Tani *et al*., 1988).

Methionine secretion by the screened bacterial and yeast isolates are shown in Table 1. Amongst all the bacterial and yeast isolates *Acetobacter tropicalis* NRIC 0312 showed maximum methionine yield of 1150 µg/ml and *Kluyveromyces marxianus* CHY1612 showed an appreciable methionine yield of 1150 µg/ml after 96 hrs of incubation. Kitamoto and Nakahara (1994) observed methionine yield of 14.2 mg/g of the substrate by yeast isolate *kluyveromyces lactis* IPU 126. The methionine secretion was abruptly reduced after 96 hrs, the reason may be due to the utilization of methionine as carbon and nitrogen source by the isolates. Li *et al*. (2011) investigated several rumen micro-organisms capable of utilizing methionine and lysine as sole carbon

Table 1 : Methionine secretion by screened yeast and bacterial isolates in modified MRS before optimization of fermentation conditions

	Methionine (µg/ml)			
Time	*Candida tropicalis*	*Kluyveromyces marxianus*	*Acetobacter tropicalis*	*Lactobacillus paracasei* subsp. *tolerans*
24 hrs	163±0.33^{e}	210±2.00^{e}	138±0.19^{e}	192±3.79^{e}
48 hrs	512±12.54^{d}	641±16.14^{d}	601±13.09^{d}	474±11.29^{d}
72 hrs	789±17.72^{b}	874±18.44^{b}	865±4.71^{b}	658±11.19^{c}
96 hrs	930±26.58^{a}	1003±15.70^{a}	1150±17.21^{a}	853±0.58^{a}
120 hrs	658±18.80^{c}	718±22.96^{c}	704±1.92^{c}	673±3.66^{b}
S.E.±	12.37	11.73	7.02	5.29
C.D. (P=0.05)	26.37	25.01	14.97	11.29

and nitrogen source. Lactic acid bacteria (LAB) poorly convert L-methionine to volatile sulfur compounds were mentioned by Dias and Weimer (1998). Confirmed Evidence for distinct L-methionine catabolic pathways in the yeast *Geotrichum candidum* and the bacterium *Brevibacterium linens* was illustrated elaborately by Arfi *et al.* (2005).

Methionine secretion by the elite micro-organisms under varying glucose concentrations (Fig. 1) confirmed that increase in glucose concentration above 20 g resulted in lower methionine secretion except for *Candida tropicalis* ITEM10456. The bacterial isolate *Acetobacter tropicalis* NRIC 0312 showed maximum methionine secretion 1130 µg/ml at 20 g glucose (Fig. 1a), where as yeast isolate *Candida tropicalis* ITEM10456 showed higher methionine secretion (1079 µg/ml) in MRS broth with 30g glucose (Fig 1b). Similar results have been observed by Anike and Okafor (2008) increase in sugar concentrations from 2g to 10g per litre of the medium eventually increased the methionine secretion from 0.25g/l to 1.40g/l in case of *Lactobacillus plantarum*.

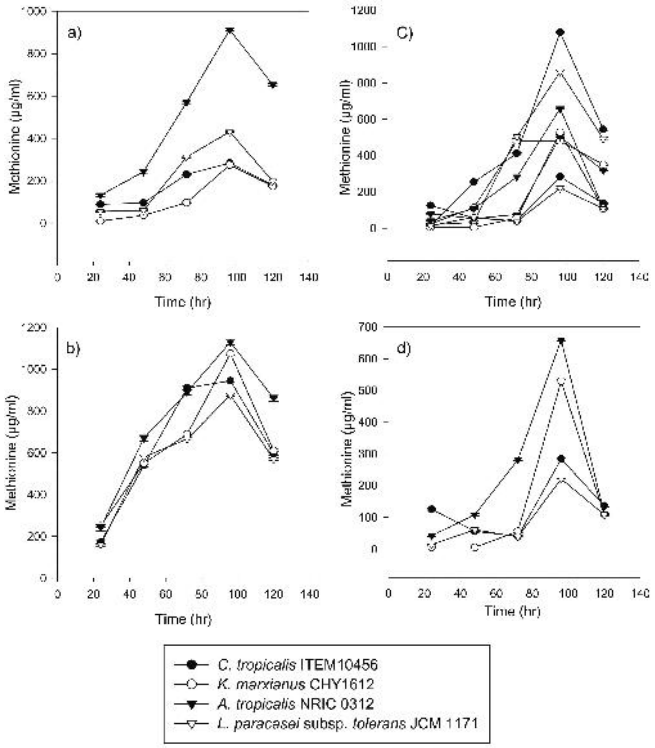

Fig. 1 : Effect of different glucose concentrations, a) 10g/l of broth, b) 20g/l of broth, c) 30g/l of broth,d) 40g/l of broth, on methionine secretion by the screened yeast and bacterial isolates

Influence of variations in nitrogen (triammonium citrate) concentrations were illustrated in Fig. 2. No considerable variation in methionine secretion was seen upto 2g increase in triammonium citrate concentration, increase in concentration to 4g boosted the methionine secretion to maximum, *Candida tropicalis* ITEM10456 (979 µg/ml), *Kluyveromyces marxianus* CHY1612 (1073 µg/ml), *Acetobacter tropicalis* NRIC 0312 (1178 µg/ml), *Lactobacillus paracasei* subsp. *Tolerans* JCM 1171 (917 µg/ml). Further increase in triammonium citrate to the culture broth reduced methionine secretion in all the culture broth. Nwachukwu and Ekwealor (2009) reported a *Streptomyces* strain SP-05 secreted 3.72 mg/ml methionine in the culture broth when 8% (w/v) sucrose and 6% (w/v) ammonium chloride were added. Nitrogen concentration more than 4g and glucose concentration higher than 20g in the culture broth affected the growth and methionine secretion. This may be due to adverse osmotic pressure exerted onto the organism. Pham *et al.* (1992) concluded that a nitrogen concentration of 2%, supplied as $(NH)_2SO_4$ gave the optimum methionine secretion by *Corynebacterium glutamicum ATCC* 21608 and further increase in nitrogen

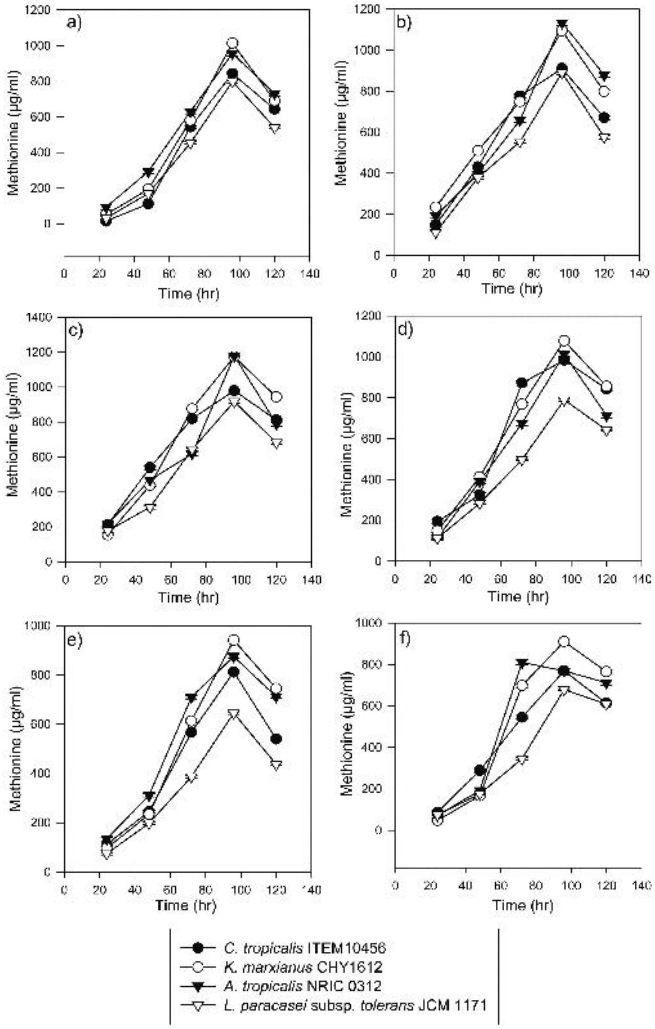

Fig. 2 : Effect of different nitrogen (*Triammonium citrate*) concentrations, a) 1g/l of MRS broth,b) 2g/l of MRS broth,c) 4g/l of MRS broth,d) 8g/l of MRS broth,e) 10g/l of MRS broth, on methionine secretion by the screened yeast and bacterial isolates

concentration having an adverse effect on the organism due to the osmotic pressure exerted.

Fig. 3 shows the methionine secretion by the four isolates under varying pH. At pH4 and 5 all the isolates except for *Candida tropicalis* ITEM10456 did not secrete methionine and *Candida tropicalis* ITEM10456 showed considerable methionine secretion of about 254 µg/ml after 120 hrs of incubation at pH5. The pH 6 (Fig. 3a) was found to most optimum for the methionine secretion by the yeast and bacterial isolates. At pH 8 and 9 only the bacterial isolates showed significant methionine secretion. The effect of varying pH on methionine production by *C.glutamicum* was examined and pH 7 was found to be most siutable for maximum methionine secretion (Venkata Narayana *et al*., 2013).

The final experiment infuence of temperature for maximum methionine secretion, the yeast and bacterial isolates showed significant methionine secretion (Table 2) in all the four varying temperatures (24ºC, 28ºC, 32ºC, 36ºC) and at 32ºC the organisms showed maximum methionine secretion. The methionine secretion was about *Candida tropicalis* ITEM10456 (1134 µg/ml), *Kluyveromyces marxianus* CHY1612 (1320 µg/ml), *Acetobacter tropicalis* NRIC 0312 (1412 µg/ml), *Lactobacillus paracasei* subsp. *tolerans* JCM 1171 (1078 µg/ml) at the optimum fermentation conditions. Variations in methionine secretion by yeast and bacterial isolates before and after optimization of fermentation conditions was shown in Fig 4. Venkata

Table 2 : Influence of temperature on methionine secretion by the screened yeast and bacterial isolates

Temperature	Time	Methionine (µg/ml)			
		Candida tropicalis	*Kluyveromyces marxianus*	*Acetobacter tropicalis*	*Lactobacillus paracasei* subsp. *tolerans*
24 ºC	24 hrs	124±0.76^{e}	145±3.55^{e}	183±3.24^{e}	78±0.69^{e}
	48 hrs	256±1.74^{d}	382±5.46^{d}	269±2.75^{d}	154±1.05^{d}
	72 hrs	435±6.51^{c}	512±16.03^{c}	439±0.90^{c}	286±6.62^{c}
	96 hrs	889±13.91^{a}	719±9.78^{a}	1023±27.15^{a}	765±14.57^{a}
	120 hrs	658±3.58^{b}	695±12.77^{b}	945±16.72^{b}	638±11.29^{b}
	S.E.±	5.02	7.46	10.17	6.20
	C.D. (P=0.05)	10.70	15.92	21.68	13.22
28 ºC	24 hrs	178±1.57^{e}	293±5.78^{e}	201±3.28^{e}	134±2.74^{e}
	48 hrs	613±18.77^{d}	578±8.65^{d}	398±11.92^{d}	435±11.84^{d}
	72 hrs	845±8.62^{b}	867±10.03^{b}	801±17.99^{c}	765±5.21^{b}
	96 hrs	1098±5.98^{a}	1012±12.39^{a}	1223±34.12^{a}	875±29.17^{a}
	120 hrs	761±10.36^{c}	812±24.31^{c}	991±28.32^{b}	619±9.27^{c}
	S.E.±	7.56	9.76	15.62	10.54
	C.D. (P=0.05)	16.12	20.81	33.31	22.47
32 ºC	24 hrs	129±1.14^{e}	83±1.64^{e}	103±2.10^{e}	68±0.88^{e}
	48 hrs	532±9.77^{d}	611±17.05^{d}	438±1.79^{d}	210±3.86^{d}
	72 hrs	845±12.65^{c}	932±9.51^{c}	845±2.87^{c}	740±24.67^{c}
	96 hrs	1134±27.78^{a}	1320±39.52^{a}	1412±11.53^{a}	1078±8.07^{a}
	120 hrs	983±2.01^{b}	1274±12.14^{b}	1198±29.34^{b}	863±8.22^{b}
	S.E.±	10.16	14.46	10.04	8.70
	C.D. (P=0.05)	21.65	30.83	21.42	18.54
36 ºC	24 hrs	145±3.65^{d}	101±0.55^{e}	185^{d}±0.13^{d}	139±0.76^{e}
	48 hrs	553±18.44^{c}	348±1.66^{d}	412^{c}±9.81^{c}	490±2.33^{d}
	72 hrs	890±26.65^{a}	641±19.63^{c}	813^{b}±21.57^{b}	752±13.82^{c}
	96 hrs	893±3.65^{a}	1014±22.08^{a}	1051^{a}±30.75^{a}	893±26.73^{a}
	120 hrs	764±20.79^{b}	803±16.94^{b}	815^{b}±2.22^{b}	793±15.65^{b}
	S.E.±	12.28	10.78	12.29	10.75
	C.D. (P=0.05)	26.18	22.98	26.21	22.92

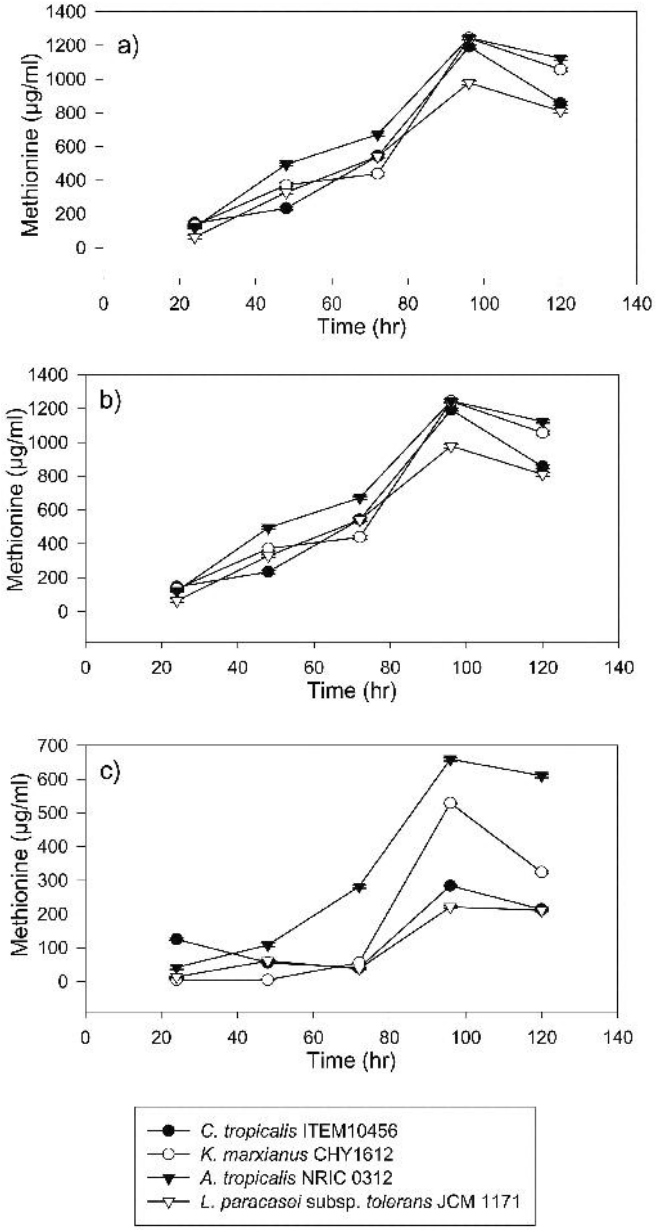

Fig. 3 : Influence of pH ranges a) pH6,b)pH7,c)pH8,on methionine secretion by the screened yeast and bacterial isolates

Narayana *et al.* (2013) studied the effect of temperature on methionine production by *C.glutamicum* confirmed that 30 °C temperature is suitable for maximum methionine secretion also confirmed the experimental results. Hence, the broth composition of the final experiment (glucose 20.0g, peptone 10.0g, beef extract 8.0g, sodium acetate.H_2O 5.0g, K_2HPO_4 2.0g, triammonium citrate 4.0g, $MgSO_4.7H_2O$ 0.2g, $MnSO_4.4H_2O$ 0.05g, tween80 1.0 ml , distilled H_2O 1000 ml, pH - 6.0 ± 0.2) at 32 °C was standardized as the optimum condition for maximum methionine secretion of the elite isolated cultures.

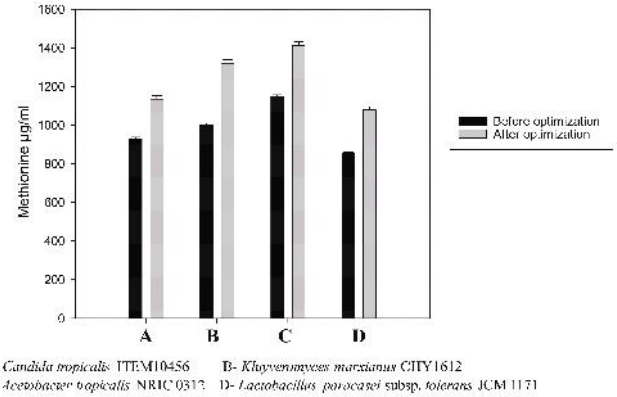

Fig. 4 : Methionine secretion by yeast and bacterial isolates before and after optimization of growth conditions

Among the various micro-organisms isolated from different samples only a very few possessed the capacity to secrete methionine at a considerable amount, which shows the meagre presence of such organisms in the environment. The study clearly proves that modifications in the nutritional and environmental conditions of micro-organisms can enhance the aminoacid particularly methionine secretion under fermentation conditions.

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