African Journal of Biotechnology Vol. 8 (23), pp. 6716-6720, 1 December, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Ethanologenic potential of the bacterium *Bacillus cereus* NB-19 in media comprising of sugar mill and dairy industrial wastes

Naureen Chaudhary, Ayaz Aslam and Javed Iqbal Qazi*

Department of Zoology, University of the Punjab, Lahore, Pakistan.

Accepted 29 September, 2009

Ethanologenic bacterium was cultivated in a suspension of sugarcane bagasse and processed yogurt whey under the predetermined growth optimized conditions. It was found that blending of processed defatted yogurt whey with 2% sugarcane bagasse (MNCH-9 medium) caused significantly higher growth of the bacterium after 24 h of incubation as compared to the values obtained when the *Bacillus cereus*-NB-19 was cultivated in MNCH-2 (2% bagasse in distilled water) and MNCH-10 (only processed whey). Saccharification potential of the bacterium increased significantly when the bagasse was supplemented with whey. Glucose contents of the cultured MNCH-9 turned out significantly higher as compared to the corresponding values of MNCH-2 at various sampling periods. Provision of whey caused significant increase in xylose content, so that the media MNCH-9 and MNCH-10 attained 3.77 and 4.74 folds of the pentose sugar, respectively, as compared to the value obtained for the MNCH-2. Likewise, much elevated levels of proteins and lipids were found in the culture fluids of MNCH-9 and MNCH-10 as compared to the corresponding figures for the MNCH-2. Cellulase activities of cultivated MNCH-9 and MNCH-10 turned out to be 5.75 folds higher at first sampling period as compared to the value obtained for MNCH-2 culture. At 12th day of the fermentation, MNCH-9 culture fluid showed more than 30% higher ethanol content as compared to the yield obtained in case of MNCH-2. The MNCH-10 expressed ethanol even less than the value found for the MNCH-2. Conclusively, blending of processed whey to sugarcane bagasse is very useful for obtaining yields of the different products including cell mass and ethanol as compared to the cultivation of the *B. cereus* NB-19 in media containing only bagasse or whey. These results dictate the importance of blending agro-industrial wastes of varying nature for their efficient and economical upgradation tied up with the selection of suitable microorganism(s). Such trends are likely to gain more attention of the scientists in related areas.

Key words: Agrowaste, bioethanol, sugarcane bagasse, whey.

INTRODUCTION

At present, many of the agro-industrial wastes are considered first waste and their resource status is taken secondary. This notion becomes important when a given waste material is naturally found deficient in some nutrients which are required for the growth and/or production efficiency of the microorganism(s) being employed for biological/ biotechnological upgradation of the substrate. Sugarcane bagasse is obviously not an exception in this regard. Here blending of two or more appropriate waste substrates may supplement each other in terms of nutrient provision so that the microbial agents' growth and/or production efficiencies are strengthened. In the present study, the bagasse mixed with defatted yogurt whey was employed to compare the growth and ethanologenic efficiency of a bacterial isolate as compared to its potential when only bagasse or defatted whey was used as substrate. Whey is aqueous fraction of milk produced in large amounts as by product following cheese and yogurt manufacturing. Major content of whey is lactose, fluctuating about a few percent, while protein, salts and

*Corresponding author. E-mail: qazi@scientist.com.

vitamins are present in minor amounts. Low concentrations of these components render their recovery uneconomical, thus whey is usually dumped directly to the environment where it becomes detrimental and causes contamination problems (Ben-Hassan and Ghaly, 1994; Roostita and Fleet, 1996; Speer, 1998; Cristiani-Urbina et al., 2000).

The bacterial isolates being reported here appear highly promising for developing simultaneous saccharification and fermentation of the substrate. Better performance of the bacterial isolate in terms of ethanol yield from the cellulosic substrate was supplemented with yogurt whey indicated importance of proper blending of two wastes for the process economics. Results of the present study add further to understanding of bioethanol production from cellulosic materials and help widening the resources for the green fuel generation.

MATERIALS AND METHODS

Identification and source of bacteria

Isolation of the bacterial isolates and preparation of the cellulosic material, the sugarcane bagasse have been described elsewhere (Chaudhary, 2008). The bacterial isolate of this study was identified by sequencing of 16S rRNA gene.

DNA extraction

The bacterial isolates designated as NB-19 was revived in nutrient broth. Then 100 µl of overnight incubated culture was used to harvest the cell and the pellet suspended in 20 µl of lysis solution. The PCR cell lysis solution is comprised of 0.05 M NaOH in 25% sodium dodecyl sulfate. The cell suspension was heated at 95°C for 15 min and cooled rapidly in PCR machine. Then 180 µl of sterile MilliQ water was added. The contents were mixed centrifuged for 5 min and the lysate (supernatant) was saved in fresh eppendrof tubes, which were subsequently stored in freezer till further use.

PCR amplification of 16S rDNA

The extracted DNA was used to amplify 16SrRNA genes by using the universal primers; 27F (5´-AGAGTTGATCMTGGCTCAG-3´) and 1387R (5´GGGCGGGWGTGTACAAGGC-3´) (MGW Biotech, Germany) and Promega Go-Taq® Flexi DNA Polymerase (MGW Biotech, Germany).

For good practicing no source of DNA was allowed to enter the PCR-room at all. For 20 µl PCR reaction the following reactants were mixed together for a given reaction. Then 16S bacterial PCR programme was selected and run. The Touchdown PCR was performed (Don et al., 1991). Denaturation for 30 s at 95°C, anneling for 30 s at 55°C, with the temperature dropping by 1°C each 2 cycles followed by an additional 15 cycles of annealing at 45°C; elongation at 72°C for 90 s and a final 10 min incubation period at 72°C.

The PCR product along with 1 Kb ladder from Gib coBRL were then loaded on 0.8% agarose gel in 0.5X Tris-boric acid buffer, containing 1 µl of 10 mg/ml of ethidium bromide/30 g of the gel. The gel was electrphoresed at 100 V for 20 min. The gel was then observed under U.V. illumination.

Successful amplified 16S rDNA PCR products were purified by using QIA quick PCR purification kit protocol, following manufacturer's instructions.

Sequencing using Backman CEQ8000

Most reagents used were supplied with dye Terminator sequencing (DTCS) kit from Backman. Master mix for a given reaction was prepared by mixing 1 µl primer 27F (5 pmol/µl), 2 µl of DTCS Quick start mix. 0.5µl of 10X sequencing buffer and 5.5µl of MilliQ water. Then to 9 µl of the above master mix, 1 µl of DNA (template) was added, vortexed and briefly centrifuged. The eppendrof tubes were then placed in PCR for 3 h and 25 min at 55 sequencing programme. Sequencing reaction was carried out in PCR machine using the following for 35 cycles: 95°C for 30 s, annealing temperature for 30 s and 60°C for 4 min, and then held at 4°C.

Purification of product for sequencing

For this procedure a master mix for one reaction was prepared by mixing 10 µl of MilliQ water, 2 µl sodium acetate (3M) pH 5.2 (using acetic acid), 2 µl EDTA (100 mM) and 1 µl glycogen (20 mg/ml). To the PCR-product (55 sequencing), 15 µl of the above master mix was added followed by addition of 60 µl of absolute ethanol (-20°C).

The eppendrofs were soon centrifuged at 13000 rpm in a refrigerated centrifuge for 15 min. The supernatant was aspirated and discarded saving the pellet. Then 150 µl of 70% ethanol (4°C) was added and withdrawn with the help of Pasteur-pipette attached aspirator. The contents within the eppendrofs were allowed for air drying at least for 30 min. The precipitate in each eppendrof was then suspended in 30 µl of sample loading solution. The solution was transferred into a well in the sample tray, a 96-well tray for use in the Beckman CEQ8000. Then a drop of mineral oil was added to each cavity (well) having the sample. For obtaining good quality, long reads (800 nt) the program LFR-a was used on the CEQ 8000.

Raw sequencing data from the CEQ 8000 machine was transferred to PC. Text files of the raw sequencing data were made using Chromas software programme. For BLAST, the website www.ncbi.nlm.nih.gov was used. On this website the path; Molecular databases → BLAST →Nucleotide blast → Nucleotide collection (nr/nt) was followed by creating a new window for each datum. The maximum % identity score for an identified bacterium was used to identify the test organism (Lane, 1991; Don et al., 1991; Hallberg et al., 2006).

Experimental procedure

Whey was obtained from a milk/yogurt shop and immediately brought to the laboratory. It is important to note that besides the industrial production of yogurt, majority of the population uses the yogurt made at small scale by milk/yogurt shopkeepers in Pakistan. During this process boiled-cooled milk is routinely inoculated from a previous stock of yogurt. There is no quality control for the process and yogurt and whey vary, in general, in different lots. The whey was treated to separate fats according to a modified method of Rajvaidya and Markandey (2004). In the original method, whey was boiled but in the present study it was autoclaved, routinely. The treatment resulted into separation and floatation of fats and the fluid portion was then filtered through Whatman filter paper No. 1. pH of the whey filtrate was found to be 4.17 at room temperature (22 ± 1°C). The processed whey was dispensed, 5 ml per test tube into 21 tubes. The test tubes were cotton plugged and autoclaved at 15 lb/inch^2 for 15 min. Then a bacterial colony from overnight incubated nutrient agar plates was shifted to each of the test tube. Six bacterial isolates were employed for separate inoculations (Chaudhary, 2008). The colonial inoculations were then dispersed by mixing on

gyro mixer for about 1 min. The inoculated test tubes were incubated at 50°C in orbital shaker at 100 rpm for an overnight period. Growth of the bacterial isolates was then determined at 600 nm against the un-inoculated processed whey (MNCH-9). pH of the culture fluid was also determined.

Based upon best growth, the isolate NB-19 was selected for further experiments employing whey as sole or part of the fermentation substrate(s). The bacterial isolate NB-19 was cultured in media containing 2% sugarcane bagasse in distilled water, in diluted (1:1) processed yogurt and in processed yogurt only. These media were designated as MNCH-2, MNCH-9 and MNCH-10 broth media, respectively, at its pre determined optimum growth conditions (Chaudhary, 2008). The experiments were conducted in plastic containers fitted with plastic pipes for venting the CO_2 .Outer ends of the pipes were immersed in water. The cultures were sampled at 24 h, 6th and 12th days post inoculations. Culture fluids at first sampling period were processed for determination of growth (O.D.), pH, glucose, xylose, protein, lipids, cellulase activity and ash contents. Glucose was determined by O-toluindine method by Hartel et al. (1969), and xylose estimation as described by Raphael (1976). Biuret method of Lowry et al. (1951) was used for the estimation of protein while lipid was estimated by the method of Zollner and Krich (1962). Cellulase activity and ash content were determined as described by Sharma and Sharma (1980) and Ranganna (1986), respectively. Samplings of the 2nd and 3rd periods were processed for the determination of pH, growth (O.D.), glucose, xylose, protein, lipids and ethanol according to the methods as described above and Snell and Snell (1973), respectively.

Statistical comparisons were made by employings single factor analysis of variance and detailed comparisons made according to Campbell (1989).

RESULTS AND DISCUSSION

Six select bacterial isolates were cultivated in the processed yogurt whey in triplicates. After 24 h of incubation, optical densities of the isolates designated as NB-3, NB-5, NB-14, NB-18, NB-19 and NB-22 turned out to be 0.109 ± 0.042, 0.065 ± 0.03, 0.087 ± 0.012, 0.037 ± 0.016, 0.024 ± 0.008 and 0.093 ± 0.025, respectively. pH levels of the filtered whey after autoclaving and following growth of the bacteria did not differ much and was found to be 4.14 and 4.10, respectively. Based on higher ethanol yield as described earlier, the NB-19 was selected for further studies involving media containing the whey. The isolate NB-19 showed 100% similarity regarding its rDNA gene sequence with *Bacillus cereus.* Phenotypic characteristics, that is, being gram +ve, endospore former, motile and positive for lecithinase, catalase and oxidase tests also matched with the described subject bacterium (Holt et al., 2000). Thus the isolate NB-19 was identified as *B. cereus.*

The bacterium showed several folds higher growth in MNCH-9 at first sampling period as compared to its growths in MNCH-2 and MNCH-10. This clearly indicated the nutritional richness of the bagasse in processed whey as compared to the media comprising of only bagasse and whey (Table 1). The shooting growth was also followed by a sharp decline in the next sampling periods, so that O.D. of the cultures in MNCH-9 and MNCH-10 turned out to be significantly less than the values obtained for MNCH-2 at 6th day of incubation. However, at 12th day of fermentation, growth of the bacterium MNC H-9 was found significantly higher than the corresponding values obtained in case of cultures from MNCH-2 and MNCH-10 media.

pH of the processed uninoculated whey remained almost consistent throughout the study period and it ranged from 5.20 ± 0.012 to 5.24 ± 0.014. Bacterial culturing of MNCH-9 and MNCH-10 media did cause drops in pH levels. After 24 h of the bacterial growth, pH of MNCH-9 dropped from a value of 5.24 for the uninoculated medium to 5.15. The corresponding figures for MNCH-10 were found to be 5.18 and 5.05. The parameter dropped to 5.04 at 2nd and 3rd sampling periods for the culture fluids of MNCH-10. At all these described differences in the pH of culture fluids and the uninoculated media turned out statistically significant (Table 1).

Significantly higher glucose levels appeared in the culture fluids of MNCH-9 and MNCH-10 media at 6th and 12th days of the bacterial growth as compared to the values obtained when the NB-19 was cultivated in MNCH-2 medium. Uninoculated MNCH-9 and MNCH-10 also had glucose contents higher than the uninoculated MNCH-2. Vivid differences appeared between the xylose contents of the bacterial cultures in the MNCH-9, MNCH-10 and MNCH-2 media. The parameter showed significant 3.77 and 4.74 folds increases in the culture fluids of the MNCH-9 and MNCH-10 media, respectively as compared to the value obtained for MNCH-2 at firs sampling period. The xylose content of MNCH-10 culture fluid was also found to be significantly higher than the corresponding value of MNCH-9 at 24 hours of incubation. Like glucose, xylose contents of uninoculated MNCH-9 and MNCH-10 media also appeared higher than those found for the uninoculated MNCH-2 medium.

Total protein contents of the cell free fluids of MNCH-9 and MNCH-10 were found significantly higher as compared to the corresponding values of MNCH-2 at all the sampling periods. The parameter also showed significant increases at all sampling periods for MNCH-10 over the corresponding values for MNCH-9 medium. Comparable differences also appeared between the protein contents of uninoculated MNCH-10 and MNCH-9 media. Lipids contents of the culture fluids of MNCH-9 and MNCH-10 were also found significantly higher from the corresponding values of MNCH-2 at all sampling periods.

Cellulase activities of MNCH-9 and MNCH-10 culture fluids at 1st sampling period were found to be 5.75 and 5.97 folds, respectively, higher than the value obtained when the NB-19 was cultivated in MNCH-2 medium (Table 1). Ash content of MNCH-9 and MNCH-10 media were also found several folds higher than the value obtained for MNCH-2 medium.

At 6th day of fermentation ethanol yield in the MNCH-9 medium was found to be significantly higher than the

Table 1. Ethanol yield and other accompanying biochemical changes following cultivation of the bacterial isolate *B. cereus* NB-19 in MNCH-2 (2% sugarcane bagasse), MNCH-9 (2% bagasse in diluted (1:1) processed whey) and (processed whey) MNCH-10 media.

Parameter	Sampling days	Cultivation medium					
		MNCH-2		MNCH-9		MNCH-10	
		Control	NB-19	Control	NB-19	Control	NB-19
O.D. (Growth)	1st	W.I.	0.116 a ± 0.010	W.I.	0.604 b ± 0.029	W.I.	0.130 a ± 0.027
	6th	W.I.	0.108 a ± 0.041	W.I.	0.041 b ± 0.008	W.I.	0.037 b ± 0.011
	12th	W.I.	0.036a ± 0.0866	W.I.	0.089 b ± 0.005	W.I.	0.047 a ± 0.009
pH	1st	4.95 ± 0.017	5.25*** ± 0.011	5.24 ± 0.014	5.15** ± 0.006	5.18 ± 0.005	5.05 *** ± 0.002
	6th	5.12 ± 0.014	5.18 ± 0.049	5.21 ± 0.012	5.17 ± 0.03	5.10 ± 0.017	5.04* ± 0.008
	12th	5.10 ± 0.012	5.17 ± 0.049	5.20 ± 0.012	5.17 ± 0.02	5.09 ± 0.016	5.04* ± 0.008
Glucose (mg/ml)	1st	10.2 ± 0.43	11.52 a ± 0.64	12.5 ± 0.42	15.4 ± 1.02	14.1 ± 0.23	16.3 ± 1.49
	6th	17.2 ± 0.67	11.09**a ± 0.90	17.9 ± 0.60	18.06 b ± 0.17	18.1 ± 0.16	18.2 b ± 0.06
	12th	13.3 ± 0.150	12.03**a ± 1.04	16.9 ± 0.56	18.5 b ± 0.14	16.9 ± 1.25	18.3 b ± 0.166
Xylose (mg/ml)	1st	0.45 ± 0.10	0.43 a ± 0.018	1.03 ± 0.05	1.62**b ± 0.079	1.45 ± 0.06	2.04***c ± 0.020
	6th	0.83 ± 0.031	0.22***a ± 0.049	0.87 ± 0.005	0.611 b ± 0.36	1.07 ± 0.023	1.44**c ± 0.21
	12th	0.59 ± 0.001	0.45** a ± 0.02	0.96 ± 0.009	1.46***b ± 0.03	1.31 ± 0.011	1.84**b ± 0.06
Protein (mg/ml)	1st	11.42 ± 1.33	15.8 a ± 5.00	16.7 ± 2.91	36.04* b ± 5.9	50.4 ± 5.95	65.23 c ± 5.4
	6th	60.5 ± 1.33	36** a ± 4.64	47.14 ± 2.18	72**b ± 2.44	86 ± 6.26	103.7 c ± 4.29
	12th	44 ± 0.46	42.6 a ± 5.2	97.6 ± 0.76	82.9***b ± 1.03	102.2 ± 0.02	118.6**c ±7.2
Lipids (mg/ml)	1st	0.68 ± 0.08	0.94 a ± 0.12	1.00 ±0.138	1.84**b ± 0.08	1.86 ± 0.18	2.36** c ± 0.7
	6th	1.6 ± 0.02	2.56***a ± 0.05	1.9 ± 0.3	3.5* b ± 0.48	1.5 ± 0.05	7.3 c ± 0.36
	12th	1.88 ± 0.002	0.43**a ± 0.29	3.1 ± 0.29	3.8***b ± 0.09	2.5 ± 0.54	3.84* b ± 0.19
Cellulase activity (µmol/ml)	1st	T.N.	6.11***a ± 3.7	T.N.	35.14***b ± 1.58	T.N.	36.5* b ± 1.12
Ash content %	1st	6.6 ± 0.08	4.4 a ± 0.34	16.2 ± 0.05	18.1 b ± 2.13	10.2 ± 0.14	32.9***c ± 0.67
Ethanol % (v/v)	6th	T.N.	0.91 a ± 0.07	T.N.	1.00 b ± 0.179	T.N.	0.62 c ± 0.043
	12th	T.N.	1.65 a ± 0.21	T.N.	2.15 b ± 0.23	N.D	1.24 c ± 0.113

Values represent means ± S.E.M. of quadruplicates.
Values for different cultured media within a row not sharing a common alphabet are significantly different from each other. Single factor of analysis. $P \leq 0.05$. Those with asterisk(s) are significantly different from the corresponding values in the left column. Student's t test.* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.
W.I. = without inoculated; T.N. = Test not performed.

values obtained for the MNCH-2 and MNCH-10 media. This trend became magnificent following further incubation so that after 12th days of fermentation, ethanol contents of the MNCH-9 appeared to be 1.3 and 1.7 folds higher than the corresponding yields in MNCH-2 and MNCH-10, respectively (Table 1).

Agro-industrial wastes, in general, may be deficient in some of the nutrients required for efficient growth, fermentation and/or expression of detoxification potential of a given microorganism. Thus biprocessing of such materials needs their fortification with proper source(s) of nutrients. However, nutritive supplementation should be low-cost or preferably it might be waste from

another source of a different chemical nature, which can enhance the microbial activities including their bioprocess potential when added to a waste substrate aimed to be handled, upgraded or to produce value added commodity. In the present study provision of 50% (v/v) processed defatted yogurt whey to prepare 2% bagasse suspension (MNCH-9) significantly enhanced growth of the ethanologenic bacterium NB-19 as well as yield of the produce (etha-nol). Growth of the bacterium increased upto 420%, while ethanol yield showed 30% elevation in the MNCH-9 medium as compared to the corresponding figures when the bacterium was cultured in MNCH-2 at first and 12th days of the cultivations, respectively. It is interesting to know that bacterial growth and ethanol yields at 1st and 12th days post incubation were found to be comparable in media comprising of only sugarcane bagasse or whey. Infact, many workers have described usage of whey as substrate for ethanol production (Moulin et al., 1980; Siso, 1996; Grba et al., 2002; Kargi and Ozmihci, 2006). In the present study blending of whey with sugarcane bagasse gave better results than using any of the substrate alone. Besides the higher ethanol yield and the rapid increase in bacterial biomass, other parameters viz., glucose, xylose, protein, lipids and cellulase activity were found to be significantly higher as compared to their corresponding levels found in MNCH-2. These results bring support to earlier studies indicating the need of cost-effective nutritive supplementations for microbial production of ethanol form lignocellulosic materials. For example, Lawford and Rousseau (1996) described corn steep liquor (CSL) as cost-effective formulation compatible with production of fuel ethanol in fermentations of lignocellulosic prehydrolysate by a patented recombinant ethanologenic *Escherichia coli* (11303: pL01297). They found that at about 7-8 g/L, the CSL proved to be a com-plete source of nutritional requirements, which supported a fermentation performance that approached the efficiency of the bacterium found in Luria broth, used as fermentation performance standard. These workers estimated economic impact at a cost of CSL of 50/t (dry wt.) as the sole nutritional supplement in a cellulosic ethanol plant to be about 4 cents/gal of ethanol. Above reported informa-tion together with findings of the present study strongly recommend identification of proper blending of different agroindustrial waste for their cost-effective biotechno-logical upgradation.

REFERENCES

Ben-Hassan RM, Ghaley AE (1994). Continuous propagation of *Kluyveromyces fragilis* in cheese whey for pollution potential reduction. Appl. Biochem. Biotechnol. 47: 89-105.

Chaudhary N (2008). Ph.D. Thesis University of the Punjab, Lahore.

Cristiani-Urbina E, Netzahuatl-Munoz AR, Manriquez-Rojas FJ, Juarez-Remirez C, Ruiz-Ordaz N, Galindez-Mayer J (2000). Batch and fed-batch cultures for the treatment of whey with mixed yeast cultures. Proc. Biochem. 35: 649-657.

Don RH, Cox PT, Wainwright BJ, Baker K, Mattick JS (1991). Touchdown PCR to circumvent spurious priming during Gene Amplication. Nucleic Acid Res. 19: 4008-4008.

Grba S, Tomas VS, Stanzer D, Vahcic N, Skrlin A (2002). A selection of yeast strain *Kluyveromyces marxianusi* for alcohol and biomass production on whey. Chem. Biochem. Eng. Q. 16: 13-16.

Hallberg KB, Coupland K, Kimura S, Johnson DB (2006). Microscopic steamer growth in acidic, metal-rich mine waters in north Wales consist of novel and remarkably simple bacterial communities. Appli. Environ. Microbiol. 72: 2022-2030.

Hartel A, Helger R, Lang H (1969). A method for determination of glucose. Z. Klin. Chem. Klin. Biochem. 7: p. 183.

Kargi F, Ozmihci S (2006). Utilization of cheese whey powder (CWP) for ethanol fermentations: Effects of operating parameters. Enzyme Microb. Technol. 38: 711-718.

Lawford HG, Rousseau JD (1996). Studies on nutrient requirements and cost-effective supplements of r ethanol production by recombinant *E. coli*. Appl. Biochem. Biotechnol. 57-58: 307-326.

Lowry OH, Rosebrough NJ, Farr AJ, Randall RJ (1951). Protein measurements with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Moulin G, Guilaume M, Galzy P (1980). Alcohol production by yeast in whey ultrafiltrate. Biotechnol. Bioeng. 22: 1277-1281.

Rajvaidya N, Markandey DK (2004). Ethanologenic potential of the bacterium *Bacillus cereus* NB-19 in media comprising of sugar mill and dairy industrial wastes. Applied Geo Environmental Studies. Appl. Microbiol. A.P.H. Publishers corporation, 2:31.

Ranganna S (1986). Analysis of fruits and vegetable products. Tata McGraw Hill Pub. Comp. Ltd. New Dehli.

Raphael SS (1976). Lynch's Medical Laboratory Technology. W.B. Saunders. Co. Tokyo. Japan.

Roostita R, Fleet GH (1996). Growth of yeasts in milk and associated changes to milk composition. Int. J. Food Microbiol. 31: 215-219.

Sharma AK, Sharma A (1980). Buffer solutions. In: Chromosomes Techniques Theory and Practice, pp. 651-657. Butterworth Co. Publishers Ltd., London.

Siso MIG (1996). The biotechnological utilization of utilization of cheese whey: a review. Bioresour. Technol. 57: 1-11.

Speer E (1998). Milk and dairy product technology. New York: Marcel Dekker.

Snell FD, Snell CT (1973). Colorimetric methods of analysis. Volume III A Robert K, Krieger Publishing Co.

Zollner N, Kirsch K (1962). Micro determination of lipids by the sulphophosphocanillin reaction. Z. Ges. Exp. Med. 135: 545-561.