International Journal of Advance Research in Science and Engineering
Volume No.06, Special Issue No.(01), Nov 2017
www.ijarse.com
IJARSE
ISSN: 2319-8354

Enumeration of Microbial Load in Vegetables Irrigated With Sewage Water

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ABSTRACT

World has seen significant changes in eating habits with the consumption of fresh produce increasingly becoming important in the daily dietary requirement of people. There has been a phenomenal increase in the demand for organically produced foods. Green leafy vegetables are the examples of minimally processed foods, which many times carry high risk of contamination and demands maintenance of hygienic conditions during their production from farm to table.

Contamination of vegetables may take place at all stages of production and processing and originate from sources like soil, excreta, water, ice, animals and harvesting and processing equipment. Soil fertilized with farmyard manure or sewage water poses a higher risk of contamination with pathogenic bacteria. *E. coli*, *Salmonella sp.* and various other microorganisms have been detected in vegetables from farms, wholesale markets, supermarkets and small shops. For fresh cut vegetables that are eaten raw, there is no treatment that can be relied upon for decontamination.

Therefore, in the present investigation pathogenic load was analyzed in randomly collected fresh farm produced samples of ten green leafy vegetables in the areas of village Banur, tehsil Rajpura, district Patiala, Punjab, India. It was found that the microbial load was very high with mean values (MPN/100g) ranging from $353x10^2$ in case of tomato (*Lycopersicon esculentum var. esculentum)* which was lowest to $605x10^5$ in case of Radish *(Raphanus sativus)* which was highest. Effect of single washing and double washing in comparison to control (unwashed samples) was analyzed and it was found that the pathogenic load reduced to a great extent with washing which suggests that double washing could be a good house hold practice to avoid food borne infection before raw consumption. Further, effect of solar irradiation was also checked and it was found that the vegetable samples that were kept in bright sunlight for five hours have less pathogenic load as compared to control (vegetable samples that were kept in shade). The above results indicate that effective management practices if followed can reduce the risk of microbial contamination and reduce the rate of food borne diseases and deaths.

Keywords: leafy vegetables, pathogenic/microbial load, MPN (most probable number).

International Journal of Advance Research in Science and Engineering
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www.ijarse.com
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ISSN: 2319-8354

I. INTRODUCTION

In many parts of world, wastewater used for irrigation is either contaminated with sewage water or completely of sewage origin. When this water is used for irrigation without any treatment the pathogens are applied to the agricultural land. This is a potential health risk to people exposed to it, such as field workers and their families, consumers and handlers of wastewater irrigated vegetables and crops and people living in the neighborhood, passing the fields frequently. Health risks from the use of wastewater can include the spread of infectious diseases by bacteria (typhoid fever, dysentery, and tetanus), virus infection (meningitis, hepatitis, and respiratory diseases), worm infection (roundworm, whipworm, and tapeworm) and other diseases. Infectious diseases due to enteric pathogens are common in many parts of India and untreated wastewater is expected to contain a high concentration of excreted enteric pathogens. Little information is available on the quality of agricultural produce from wastewater-irrigated fields and /or its quality at local markets, although it is evident that the use of wastewater in agriculture is common in many parts of India. The normal practice of harvesting and collecting fresh vegetables does not have any effective microbe elimination step thus, resulting in minor to major contamination with benign or dangerous microorganisms. Presence of moisture on the leaves from precipitation, dew and irrigation favours their survival and multiplication (Beattie and Lindow, 1995, 1999).

Vegetables are an important part of the human diet as they contain carbohydrates, proteins, vitamins, minerals and trace elements and these are essential for normal functioning and health of an individual. In recent years, consumption of raw vegetables as food has increased gradually, particularly among the urban community. This is due to increased awareness on the food value of vegetables, as a result of exposure to other cultures and acquiring proper education (Thompson and Kelly, 2003). Studies have shown that awareness is gradually increasing about the nutritional benefits of green leafy vegetables like palak (*Spinacia oleracea*). While we endeavor to increase the production and consumption of fresh vegetables, it is necessary that the consumer gets safe food.

An increasing association between fresh vegetables and foodborne infection outbreaks has led to concern about contamination of vegetables with pathogenic bacteria in the agricultural environment. Several outbreaks of *E. coli* 0157:H7 infections, *Salmonella* spp. and *Listeria* spp. associated with consumption of raw fruit and vegetable products have been reported (Nguyen-The, 2012; Batz *et al*., 2012). In fact, the foodborne outbreaks caused by *E. coli* and *Salmonella* isolated from fruits and vegetables resulted with 727 cases/6 deaths and 2288 cases/3 deaths, respectively, between the years 2006 and 2010 in the USA (CDC, 2012). In recent years, food borne outbreaks caused by fruits and vegetables have shown an increasing trend. When spoilage and pathogenic microorganisms come in contact with fruit and vegetable production environment, they can rapidly attach and strongly adhere themselves. Some pathogens can also form biofilms on fruit and vegetable surfaces (Sapers and Doyle, 2009; Solomon and Sharma, 2009; Elhariry, 2011). The necessity for effective decontamination process is undeniable as well as being a very critical step and it is cleaning or washing with fresh clean water. The washing methods can reduce the microbial load of the produce. On the other hand, if the washing treatment has not been applied properly, this step can cause cross-contamination (Olaimat and Holley, 2012). Therefore, the

present study is proposed to enumerate the microbial load (total coliform count) in sewage water irrigated vegetables and the use of some of the cheap methods to reduce the pathogenic load of these vegetables.

II. MATERIAL AND METHODS

Study Sites And Sampling:

Sample of vegetables (Spinach, Coriander, Cabbage, Mint, Cauliflower, Onion, Mustard, Radish, Tomato, Fenugreek) were collected from wastewater irrigated fields of village Banur, tehsil Rajpura, district Patiala, Punjab, India (photoplate-1). 10 specimens of each vegetable were collected randomly at a time in sterilized plastic bags and analyzed for the microbial load (APHA, 1995). All the specimen samples were collected carefully by placing the sterilized plastic bag over the plant. The plastic bags were then sealed and immediately transferred to laboratory for preliminary testing and further processing for comparison of microbial load of vegetable samples.

Estimation Of Pathogenic Load Of Selected Vegetable Samples:

The collected vegetable samples were analyzed for the microbial load on preliminary basis and if the results exceeded the limit, then, the microbial load of new specimens of selected unwashed vegetable samples would again be evaluated and the results obtained would be compared with the microbial load obtained after washing the vegetable samples (single and double washing) as well as after solar irradiation of vegetables samples.

Processing Of Samples For Comparison Of Microbial Load:

The collected vegetable samples were processed in different ways and categorized into 4 groups for further evaluation of microbial load (Total Coliform Count) as given below:

1. Unwashed sample (UWS): Samples were neither washed nor irradiated.
2. Single washed sample (SWS): Samples were rinsed once with tap water.
3. Double washed sample (DWS): Samples were rinsed twice with tap water.
4. Solar irradiated sample (SIS): Samples were irradiated with sunlight for 5 hours (not rinsed in tap water).

The mean total coliform count of the unwashed samples was compared with the samples that were washed and also compared with the samples that were solar irradiated. Media utilized and the technique employed for total coliform count is as following:

Preparation Of Media:

All the media viz. lauryl tryptose broth, brilliant green lactose bile broth and EC medium were prepared in distilled water and sterilized in an autoclave at 121°C at 15 lbs pressure for 15 minutes and used for calculation of microbial load (APHA, 1995).

Standard Total Coliform Count Or Most Probable Number (Mpn) Or Multiple-Tube Test: (APHA, 1995)

Presumptive Phase: The lauryl tryptose broth fermentation tubes were used.

Procedure

1. A series of presumptive phase fermentation tubes having inverted vials with appropriate decimal quantitative

(multiple and submultiples of 1ml) of sample were inoculated.

2. The inoculated fermentation tubes were then incubated at 35± 0.5°C. After 24±2 hrs, each tube was shaken gently and examined for gas production and if no gas is formed and trapped in the inverted vial, then it is re-incubated and examined at the end of 48±3hrs.

Confirmed Phase: The brilliant green lactose bile broth fermentation tubes were used.

Procedure

1. All Primary fermentation tubes showing any amount of gas within 24 hrs of incubation to the confirmed phase were submitted. If an additional primary fermentation tube showed gas production or heavy growth at the end of 48hr incubation period, these were also submitted to confirmed phase.

2. The primary fermentation tube showing gas production were shaken gently with a sterile metal loop of 3 mm diameter, one loopful of culture was transferred to a fermentation tube containing brilliant green lactose bile broth. The inoculated brilliant green lactose bile broth tube was incubated for 48±3 hour at 35 ± 0.5°C.

Estimation Of Bacterial Density

The number of positive findings of the coliform group of organisms (presumptive and confirmed) resulting from multiple-portion decimal-dilution plantings as the combination of positive tubes were recorded and computed in terms of Most Probable Number. The values in computing the MPN in portion plantings were used as per APHA, 1995 and are given in table 1.

Table-1: MPN index and 95% confidence limits for various combinations of positive results when 5 tubes are used per dilutions (10 ml, 1.0ml, 0.1ml) as per APHA, 1995.

Combination of positives	MPN index/100ml	95% confidence limits		Combination of positive	MPN index/100 ml	95% confidence limits	
		Lower	Upper			Lower	Upper
0-0-0	<2	-	-	4-2-0	22	9.0	56
0-0-1	2	1.0	10	4-2-1	26	12	65
0-1-0	2	1.0	10	4-3-0	27	12	67
0-2-0	4	1.0	13	4-3-1	33	15	77
1-0-0	2	1.0	11	4-4-0	34	16	80
1-0-1	4	1.0	15	5-0-0	23	90	86

1-1-0	4	1.0	15	5-0-1	30	10	110
1-1-1	6	2.0	18	5-0-2	40	20	140
1-2-0	6	2.0	18	5-1-0	30	10	120
2-0-0	4	1.0	17	5-1-1	50	20	150
2-0-1	7	2.0	20	5-1-2	60	30	180
2-1-0	7	2.0	21	5-2-0	50	20	170
2-1-1	9	3.0	24	5-2-1	70	30	210
2-2-0	9	3.0	25	5-2-2	90	40	250
2-3-0	12	5.0	29	5-3-0	80	30	250
3-0-0	8	3.0	24	5-3-1	110	40	300
3-0-1	11	4.0	25	5-3-2	140	60	360
3-1-0	11	4.0	29	5-3-3	170	80	410
3-1-1	14	6.0	35	5-4-0	130	50	390
3-2-0	14	6.0	35	5-4-1	170	80	480
3-2-1	17	7.0	40	5-4-2	220	100	580
4-0-0	13	5.0	38	5-4-3	280	120	690
4-0-1	17	7.0	45	5-4-4	350	160	820
4-1-0	17	7.0	46	5-5-0	240	100	940
4-1-1	21	9.0	55	5-5-1	300	100	1300
4-1-2	26	12	63	5-5-3	900	300	2900
				5-5-2	500	200	2000

				5-5-4	1600	600	5300
				5-5-5	>1600	-	-

III. RESULTS AND DISCUSSION

Pathogenic Load Of Wastewater Irrigated Samples

Ten specimens of each selected vegetable samples (that is 10 in number) were collected from wastewater fields of village Banur and analyzed for the microbial load. All the sample were processed and mean total coliform count (n=10) was determined (Table 2).

Table 2: Total coliform count of vegetable samples collected from Banur village (n=10)

Vegetables	Scientific Name	Total Coliform (MPN/100 g)	
		Ranges	Mean
Spinach	*Spinacea oleracea*	$60x10^3$-$70x10^5$	$353x10^4$
Coriander	*Coriandrum sativum*	$70x40^4$-$60x10^5$	$335x10^4$
Fenugreek	*Trigonella foenum-graecum*	$80x10^3$-$80x10^5$	$355x10^3$
Cabbage	*Brassica oleracea var. capitata*	$65x10^3$-$75x10^5$	$378x10^4$
Mustard	*Brassica Juncea*	$80x10^3$-$80x10^5$	$355x10^4$
Tomato	*Lycopersicon esculentum var. esculentum*	$80x10^2$-$70x10^3$	$353x10^2$
Mint	*Mentha requienii*	$60x10^3$-$90x10^5$	$457x10^3$
Cauliflower	*Brassica oleracea var. botrytis*	$80x10^3$-$60x10^5$	$304x10^3$
Onion	*Allium cepa*	$110x10^4$-$60x10^6$	$500x10^5$
Radish	*Raphanus sativus*	$110x10^4$-$110x10^6$	$605x10^5$

During preliminary study, it was observed that the vegetable samples of banur village contained elevated levels of microbial load. So, new specimens of selected vegetable samples were again taken to check the effect of washing and solar irradiation.

Effect of Washing On Total Coliform Count:

Firstly, unwashed vegetable samples were analyzed for total coliform count and after that it was followed by washing once and then twice of the same vegetable samples to observe the total coliform counts. All the unwashed specimens showed very high range of coliform count which may be due to wastewater used in irrigation, but the pathogenic contamination varied with the sample. Due to washing, contamination decreased and the decrease in trend was visible in each replica of vegetables. In unwashed samples, maximum mean total coliform count was observed in cabbage and radish ($620x10^4$ MPN/100g). By single

wash and double wash, mean coliform count decreased to $354x10^3$ MPN/100g and $150x10^3$ MPN/100g in case of cabbage whereas in case of radish it reduced to $306x10^3$ MPN/100g and $170x10^2$ MPN/100g respectively. The same decreasing trend in count with increase in number of washings was observed for all the vegetables (Table 3).

Table 3: Effect of washing on total coliform count in vegetables of Banur village (n=10)

Vegetables	Scientific Name	Treatment	Total Coliform (MPN/100 g)	
			Ranges	Mean
Spinach	*Spinacea oleracea*	UWS	$130x10^4$-$90x10^5$	$455x10^4$
		SWS	$80x10^3$-$60x10^4$	$340x10^3$
		DWS	$60x10^2$-$30x10^4$	$153x10^3$
Coriander	*Coriandrum sativum*	UWS	$130x10^3$-$90x10^5$	$456x10^4$
		SWS	$80x10^2$-$60x10^4$	$304x10^3$
		DWS	$40x10^2$-$27x10^4$	$137x10^3$
Fenugreek	*Trigonella foenum-graecum*	UWS	$110x10^4$-$110x10^4$	$60x10^4$
		SWS	$90x10^2$-$80x10^3$	$40x10^3$
		DWS	$30x10^2$-$33x10^3$	$18x10^2$
Cabbage	*Brassica oleracea var. capitata*	UWS	$140x10^4$-$110x10^5$	$620x10^4$
		SWS	$80x10^2$-$70x10^4$	$354x10^3$
		DWS	$30x10^3$-$27x10^4$	$150x10^3$
Mustard	*Brassica Juncea*	UWS	$140x10^4$-$90x10^5$	$520x10^4$
		SWS	$90x10^2$-$80x10^4$	$305x10^3$
		DWS	$50x10^2$-$23x10^3$	$140x10^2$
Tomato	*Lycopersicon esculentum var. esculentum*	UWS	$110x10^4$-$110x10^5$	$60x10^5$
		SWS	$80x10^2$-$60x10^4$	$30x10^3$
		DWS	$40x10^2$-$34x10^3$	$19x10^2$
Mint	*Mentha requienii*	UWS	$13x10^3$-$90x10^4$	$45x10^4$
		SWS	$70x10^2$-$70x10^3$	$38x10^3$
		DWS	$30x10^2$-$30x10^3$	$16x10^2$
Cauliflower	*Brassica oleracea var. botrytis*	UWS	$140x10^3$-$90x10^5$	$457x10^4$
		SWS	$90x10^3$-$70x10^4$	$395x10^3$
		DWS	$50x10^2$-$33x10^4$	$168x10^3$
Onion	*Allium cepa*	UWS	$130x10^3$-$110x10^5$	$556x10^4$
		SWS	$110x10^3$-$60x10^4$	$335x10^3$
		DWS	$40x10^2$-$34x10^4$	$162x10^3$
Radish	*Raphanus sativus*	UWS	$140x10^4$-$110x10^5$	$620x10^4$
		SWS	$110x10^2$-$60x10^4$	$306x10^3$
		DWS	$40x10^2$-$30x10^3$	$170x10^2$

The association of fresh vegetables and food born infections has led to concern about contamination of vegetables with faecal pathogenic bacteria in agricultural environment. Now-a-days in developing cities, due to large population and depleted water reserve, the vegetables are irrigated with urban wastewater and the wastewater contains various pathogenic bacteria including faecal coliform. These pathogenic bacteria contaminate the vegetables as they are present in water due to faecal contamination. There are different ways by which the microorganisms enter into water supply example broken sewer line, congested center and inappropriate treatment. In the hazard identification step, background information on the pathogens in a specific

system is described. It also includes the spectra of human illness and disease associated with the identified microorganisms (Haas *et al.*, 1993).

The transmission of *E. coli* 0157:H7 from manure contaminated soil and irrigation water to lettuce plants was demonstrated using larger scanning convoke microscopy, fluorescence microscopy and recovery of viable cells from inner tissue of plants *E. coli* 0157:H7 migrated to internal locations in plant tissue and was thus protected from the action of sanitizing agents by virtue of its inaccessibility. The pathogen is known to be shed in manure by cattle and survives in animal waste and wastewater (Elder *et al.*, 2000). Experiments demonstrate that *E. coli* 0157:H7 can enter the lettuce plant through root system and migrate throughout the edible portion of the plant (Solomon *et al.*, 2002).

Natvig *et al.* (2002) compared mean total coliform count in the underground and above ground samples of vegetables collected from different villages were studied. These samples were homogenized and processed in the laboratory as single wash (SWS), double wash (DWS) and unwashed samples (UWS). A preliminary experiment showed the presence of coliform in different vegetables. The presence of coliforms may be due to manure application or manure storage (Bovine manure), untreated waste water used for irrigation, broiler litter, cattle grazing, feces shed by cattle in the field here the cattle is the reservoir of *E. coli*. The coliform presence may also be due to rainfall event, which influence the leaching of faecal pathogens, and due to unhygienic food handling. Weather conditions, desiccation, soil type, degree of manure application are likely to effect the survival of pathogen. Washing of vegetables is a general house hold practice before consumption. Double washing indicates that the risk of contamination could be avoided when the coliform count could be reduced to permissible limits.

Effect of Solar Irradiation On Total Coliform Count:

Mean total coliform count of freshly collected samples (without irradiation, at zero hour) were compared with the solar irradiated samples and the results are given in table 4. It was observed that there was decrease in total coliform count in vegetable samples kept in sunlight, as compared to control and it varied within all samples. Maximum reduction in mean total coliform count was observed in fenugreek (from $51x10^4$ to $25x10^2$ MPN/100g) followed by mint (from $404x10^3$ to $40x10^2$ MPN/100g) and least reduction was in case of radish (from $359x10^4$ to $354x10^3$ MPN/100g).

Exposure to sunlight enhances the die-off rate of some pathogenic microorganisms like *Leptospira, Brucella, Mycobacterium, Salmonella* and *E. coli* (Ellis and McCalla, 1976). Survival of the microorganisms is related to temperature variations and it decreases with increase in temperature and vice-versa (Nicholson *et al.*, 2000). Our results are also in line with the findings of Tanook and Smith (1971), as they also reported less reduction of coliform count in vegetables that were placed in shade as compared to that placed in sunlight. Gelderich and Bordner (1971) reported in their work that total coliform count was less in vegetable samples treated with 4 hours of sunlight as compared to 2 hours treatment. Pathogenic load gets reduced below to permissible levels in the produce that is harvested after sun drying in the fields itself, whereas the parts that come in direct contact with waste water was most severely contaminated (Minhas *et al.*, 2006).

Table 4: Effect of solar irradiation on Total Coliform count

Vegetables	Scientific Name	Total Coliform (MPN/100 gm)			
		0 hour irradiation		5 hour irradiation	
		Ranges	Mean	Ranges	Mean
Spinach	*Spinacea oleracea*	$110x10^4$-$60x10^5$	$355x10^4$	$90x10^2$-$50x10^4$	$255x10^3$
Coriander	*Coriandrum sativum*	$170x10^4$-$60x10^5$	$385x10^4$	$90x10^3$-$60x10^4$	$345x10^3$
Fenugreek	*Trigonella foenum-graecum*	$130x10^4$-$90x10^4$	$51x10^4$	$60x10^3$-$5x10^2$	$25x10^2$
Cabbage	*Brassica oleracea var. capitata*	$170x10^3$-$110x10^5$	$555x10^4$	$90x10^2$-$80x10^4$	$404x10^3$
Mustard	*Brassica Juncea*	$140x10^4$-$110x10^5$	$620x10^4$	$90x10^2$-$80x10^4$	$405x10^3$
Tomato	*Lycopersicon esculentum var. esculentum*	$130x10^4$-$90x10^5$	$515x10^4$	$80x10^3$-$60x10^4$	$340x10^3$
Mint	*Mentha requienii*	$90x10^3$-$80x10^4$	$404x10^3$	$80x10^2$-$80x10^3$	$40x10^2$
Cauliflower	*(Brassica oleracea var. botrytis)*	$130\ x10^3$-$90x10^5$	$457x10^4$	$70x10^3$-$50x10^4$	$285x10^3$
Onion	*(Allium cepa)*	$110x10^3$-$110x10^5$	$558x10^4$	$60x10^3$-$50x10^4$	$253x10^3$
Radish	*(Raphanus sativus)*	$170x10^3$-$70x10^5$	$359x10^4$	$90x10^2$-$70x10^4$	$354x10^3$

Sunlight is thought to be the most important factor contributing to the death of these bacteria (Davies *et al.*, 1995). As temperature is considered to be important for the survival of pathogens, sun drying is a cheap practice and it can be utilized in developing countries like India.

IV. CONCLUSION

The present study reveals the potential hazards associated with pathogenic contamination of edible parts of vegetables grown using sewage water. Considering some of the options tested for minimizing the health risks for the consumers, washing and exposure to sunlight seems to be effective low cost strategies and most beneficial for poor people living in villages and remote areas.

ACKNOWLEDGMENT

The authors acknowledge the support of the Dolphin (PG) College of Life Sciences, Chunnikalan, for providing laboratory facilities for this study.

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Photo Plate 1 : Waste water irrigation at Banur Village