JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Nov 2015. Vol. **9**(Spl. Edn. 2), p. 333-338

Resazurin Dye Reduction Test for Determining Microbial Quality of Chevon Meat

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(Received: 08 August 2015; accepted: 09 October 2015)

Growth of spoilage bacteria results in reduced shelf-life of meat and meat products and causing major economic losses to the industry. The number of bacteria in food products is an important index of hygiene during food processing. Most of the available methods enable us to determine the total microbial load but in most cases, they require sophisticated technology / instruments or they are time consuming. A dye based assay which is simple and rapid require simple instrument to perform. Therefore a study was conducted on fresh chevon meat at alternate day interval of 0, 3, 5 and 7 under refrigeration storage using resazurin dye. Results indicated that there was a visible colour change of the dye from initial light green to purple and finally to pink. It can give result within half an hour depending upon the microbial load in meat. This study has more application in current Indian scenario with limited instrumental facility to enumerate bacterial load in meat and meat products.

Key words: Resazurin dye, microbial quality, refrigeration, spoilage, chevon meat, colour change.

Growth of spoilage bacteria in meat and meat products results in reduction of shelf-life and creates great economic losses [1]. The number of such bacteria in food products is an important index of quality and hygiene during various food processing operations. Determination of total bacterial load in meat and meat products by standard plate count method requires around 48 h. Even though colony counting is the method used traditionally [2], it is labour intensive and time consuming. In this context, the content of volatile basic nitrogen (VBN) and trimethylamine (TMA) can be regarded as spoilage indicators for protein-rich foods [3, 4], but these assays need several hours and complex techniques.

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Several new methods have been developed for rapid and indirect determination of bacterial load in various food items such as electrical impedance methods [5, 6, 7], electrochemical methods [8, 9], ultrasonic techniques [10], biosensors [11], flow cytometry methods [12] and the capillary electrophoresis techniques [13]. All these methods enable us to determine the microbial cell density rapidly and accurately. However, in most cases, these techniques require high-technology instruments and high capital investments.

Therefore the interest of food microbiologists and industry has been changed to simple techniques utilising colorimetric and flourimetric dyes/compounds. The Alamar Blue, also known as resazurin, assay incorporates a colorimetric and fluorometric growth indicator that can detect metabolic activity of microbial cells [14]. Moreover resazurin is nontoxic, water-soluble dye which is readily reduced by electron transfer reactions associated with cellular respiration and

producing resorufin, a water-soluble product easily measured by fluorescence or visible light spectrophotometry [15]. As bacterial metabolism proceeds, resazurin changes its color from blue to pink and finally to clear, because oxygen becomes limiting within the medium [16]. The first stage of resazurin reduction is due to the loss of an oxygen atom loosely bound to the nitrogen of the phenoxazine nucleus and this reduction to pink resofurin is not reversible by atmospheric oxygen and that is independent of both reduction potential and oxygen content in the medium. But the second stage of reduction to the colorless state is reversible by atmospheric oxygen [17]. More importantly resazurin is very stable in a culture medium devoid of active cells, but it is rapidly reduced in the presence of living cells in the medium. Several microbial reductases such as *diaphorases* and *NADPH dehydrogenase* can use resazurin as an electron acceptor and that is responsible for the reduction of resazurin into resofurin [17]. Resazurin has been used already to assess bacterial viability and contamination, to evaluate antimicrobial activity and as an indicator of bacterial cell numbers [18, 19]. But the applicability of resazurin dye reduction test to assess the microbial load in meat has not been explored properly.

Therefore the aim of the research was to develop resazurin dye based simple and rapid biochemical test to assess microbial load in fresh chevon meat under refrigerated storage.

MATERIALS AND METHODS

Meat samples and chemicals preparation

Fresh chevon meat from healthy animals was collected within 2 h of slaughter. Aseptically prepared meat devoid of connective tissue were used as day zero samples. The meat was further stored at refrigerated conditions and samples were drawn aseptically on day 3, 5 and 7 respectively to conduct various experiments as described below. Two different concentrations of resazurin dye solution (5mg/100ml and 10mg/100ml distilled water) were prepared. Phosphate Buffer Saline (PBS) was prepared by dissolving one tablet (Bio basic Canada Inc.) in 100ml distilled water.

Resazurin dye reduction test (RDRT)

To 10 g of aseptically drawn meat sample, 90 ml sterile prepared PBS was added and homogenised at 1000rpm for 10 seconds. Then homogenate was filtered through Whatmann filter paper No.1. Four test tubes were taken and numbered serially from 1 to 4. 9ml filtrate was added to test tube 1 and 2 and 9 ml PBS to test tube 3 and 4. In tube number 1 and 3, one ml of 5mg dye solution was added and in tube number 2 and 4, one ml of 10 mg dye solution was added. Both the Tube 3 and 4 acted as blanks for each representative dye concentration. Then all the four test tubes were incubated at 37°C. Time required for colour change from initial colour to final pink colour was noted.

Standard plate count (SPC)

It was determined as per method described by APHA [20]. Preparation of samples and serial dilutions were done near the flame of a horizontal laminar flow apparatus (model: YSI-188, Yarco Sales Pvt. Ltd., New Delhi) which were presterilized by ultraviolet irradiation and keeping all aseptic precautions. Duplicate plates were prepared and the counts were expressed as colony forming units (cfu) per gram. About 10gm sample was aseptically weighed and transferred to sterile beaker. To this 90ml distilled water was added to make 10^{-1} dilution and then homogenised at 1000 rpm for 30 s in an Ultra Turrax tissue homogenizer. 1ml homogenate was transferred to 9ml distilled water to make 10^{-2} dilution and serial dilutions were prepared further. 23.5 g plate count agar obtained from Hi-Media Laboratories Pvt Ltd., Mumbai (Code No.M091) was suspended in 1000 ml distilled water and boiled to dissolve the medium completely and sterilized by autoclaving at 15 lb pressure at 121°C for 15 min. Final pH of the medium was adjusted to 7.0±0.2. Duplicate sets of petri dishes were inoculated aseptically with 1 ml aliquots from appropriate dilutions. About 20 ml of plate count agar, melted and maintained at 44-46°C, was poured gently. The plates were incubated at 37±1°C for 48 h. Plates showing 30-300 colonies were counted. The number of colonies was multiplied with reciprocal of the dilution and expressed as $\log_{10}$ cfu/g.

pH and oxidation-reduction potential

The tissue homogenate was prepared by blending 10g meat sample with 90 ml distilled water using an Ultra Turrax tissue homogenizer (Ultra Turrax IKA, Model T18 Basic, IKA Wares Inc.,Willmington, U.S.A) for one min. The pH was

recorded by immersing combined glass electrode of digital pH meter (Model CP 901, Century Instrument Ltd, Chandigarh) into the meat homogenate. The oxidation-reduction potential was measured by pressing mV button in the same pH meter.

Estimation of myoglobin content

It was estimated by procedure of Warris [21] with suitable modifications. 10gm meat sample was cut into small pieces and connective tissue was separated. To this 50 ml cold phosphate buffer of pH 6.8 was added and homogenized at 1000rpm for 30 seconds. After incubating at1˚C for 1 hour, it was centrifuged at 5600rpm for 30 minutes and filtered through Whatmann filter paper No.1. Absorbance of filtrate was measured using spectrophotometer at 525 nm and 700 nm wavelength.

Concentration of myoglobin was calculated using formula below.

Myoglobin concentration in sample (mg/g)

$$= (A^{525}-A^{700}) \times 2.303 \times 5$$

(5=dilution factor and 2.303=multiplication factor)

RESULTS AND DISCUSSION

Resazurin dye reduction test for fresh meat quality

Initial colour of resazurin dye solution in test tubes added with 5mg and 10mg concentration resazurin were light green and dark green respectively. The colour change in dye in relation to time was given in the Table 1 and Fig 1-5. On day zero, initial light green colour changed to light purple and violet in 30 minute time for each respective dye concentration. The final pink colour and dark purple colour were obtained after 75 min. On day 3, results similar to day zero were obtained. But on day 5, initial colour change was occurred within 15 minutes and a same result was obtained for day 7 of refrigerated storage. The results on 0 and 3rd day were same and took more time for colour change hence meat contained less number of microbes compared to further days of storage.

Physico-chemical parameters

The various physico-chemical parameters like pH, OR potential, myoglobin concentration along with microbiological parameters were given in Table 2.

pH

pH value of chevon meat on day zero was 6.12 and reached to 5.24 on 3rd day of refrigerated storage. Then it started increasing to 6.25 on 5th day and 6.90 on 7th. There was no significant ($P<0.05$) difference between pH on 0 and 5th day. On 0 day, all the parameters were measured 4 hours post slaughter. So the ultimate pH of meat was not attained. No significant ($P<0.05$) increase in pH was recorded up to 3rd day and pH increased significantly ($P<0.05$) thereafter. Similar results were obtained in beef at refrigerated storage [22]. Increase in pH was probably due to bacterial activity that resulted in the production of ammonia, amine and other alkaline substances [23]. The increased pH during the storage may be due to growth of gram-negative bacteria such as *Pseudomonas*, *Moraxella* and *Acinetobacter* etc. [24]. The results also agreed with findings in meat samples in which pH reached to 6.09 on day 9 at refrigeration storage [25]. A highly significant increase in ammonia content during storage period was probably a reason for increase in pH with prolonged storage of goat meat.

Myoglobin concentration

Myoglobin content decreased from 6.01 mg/g of meat on day zero to 3.99 mg/g on day 7 of

Fig. 1. Initial colour of meat extract and blank (Distilled water)

Fig. 2. Immediately after addition of a. 10mg b. 5mg resazurin

storage period. There was significant (P<0.05) difference between myoglobin concentration of meat during refrigerated storage. Red colour of meat depends upon the concentration of myoglobin (Mb) and its derivatives [26]. During storage, the desired red colour of tuna flesh undergoes discolouration and develops an unappealing brown colour, which results from the oxidation of ferrous myoglobin (deoxymyoglobin and oxymyoglobin) derivatives to ferric metmyoglobin [27]. Therefore, colour changes in meat are mainly due to the reaction of myoglobin with other muscle components, especially myofibrillar proteins [28]. During the handling and storage of fish, a number of biochemical, chemical and microbiological changes occur, leading to discoloration [26]. Discoloration of tuna during frozen storage is caused by the formation of metmyoglobin [29]. This phenomenon can be influenced by many factors, such as pH, temperature, ionic strength and oxygen consumption reaction[30]. Metmyoglobin formation is positively correlated with lipid oxidation[31]. The results indicated that there was reduction in myoglobin concentration during storage due to the formation of metmyoglobin.

Oxidation-Reduction (OR) potential

There was no significant (P<0.05) correlation between the redox potential and refrigeration storage of meat. OR potential varies from +100 mV to -100 mV in raw meat[32]. Positive redox potential favours the growth of aerobic microbes on meat surface. The results of this study

Table 1. Color chart of Resazurin dye reduction test with respect to time during the refrigerated storage (4±1C°) of chevon meat

Sr.No.	Day	Time(min.)	Colour at concentration 5mg/100mL	10mg/100mL
1.	0	15	No change	No change
		30	Light purple	Violet
		45	Light pink	Purple
		60	Pink	Purple
		75	Pink	Dark purple
2.	3	15	No change	No change
		30	Light purple	Violet
		45	Light purple	Violet
		60	Light pink	Purple
		75	Pink	Dark purple
3.	5	15	Light purple	Violet
		30	Light pink	Purple
		45	Pink	Dark purple
4.	7	15	Light purple	Violet
		30	Light pink	Purple
		45	Pink	Dark purple

Table 2. Change in physicochemical and microbial quality parameters of chevon meat during refrigerated storage (4±1°C)

Days	0	3	5	7
pH	6.12 ± 0.05^{b}	5.24 ± 0.05^{c}	6.25 ± 0.05^{b}	6.90 ± 0.10^{a}
Oxidation-Reduction potential (mV)	49 ± 1.84^{d}	99 ± 1.00^{a}	72 ± 1.41^{c}	85 ± 1.85^{b}
Myoglobin content (mg/g)	6.01 ± 0.14^{a}	5.38 ± 0.12^{b}	4.22 ± 0.08^{c}	3.99 ± 0.05^{c}
Total plate count (Log_{10} cfu/g)	4.27 ± 0.15^{c}	5.48 ± 0.20^{b}	6.36 ± 0.10^{a}	6.72 ± 0.04^{a}

Means ± SE with different superscripts differ significantly (P<0.05)

Fig. 3. After 15 minutes in meat stored for 7 days in refrigerator

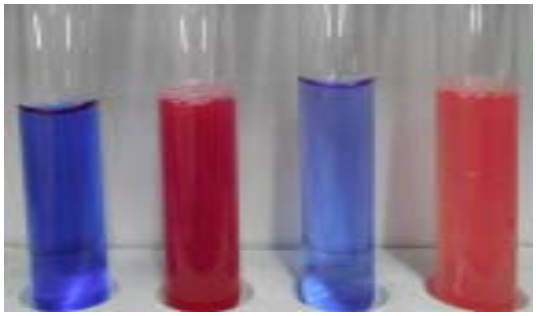

Fig. 5. After 45 minutes in meat stored for 7 days in refrigerator

indicated that there was increase in microbial count with positive redox potential.

Microbiological parameters

SPC started increasing from 4.27 $\log_{10}$ cfu/ g on 0 day to 6.72 $\log_{10}$ cfu/ g on 7th day. SPC of goat meat stored under refrigeration condition increased significantly ($P<0.05$) and coincide with change in colour of dye. A close relation was observed between dye colour change and increase in SPC. Similarly, an increase in SPC of buffalo meat was reported under refrigerated storage[33]. Increased SPC during refrigeration storage was due to multiplication of existing organism under favourable condition of growth using meat as suitable medium. A sharp increase in SPC was also recorded in refrigeration stored minced goat meat by Verma and Sahoo [25] and a significant ($P<0.05$) difference in SPC values was observed between storage days.

CONCLUSION

It is concluded from this research that if the bacterial load is less than 6 $\log_{10}$ cfu/gm, intial colour changed to light purple and violet after 30 minutes. While the colour changed within 30 minutes, if the microbial load was higher than 6 $\log_{10}$ cfu/gm. Hence it is concluded that Resazurin dye reduction test can be used as a rapid and simple test to detect and quantify microbial load in refrigerated stored chevon meat.

Fig. 4. After 30 minutes in meat stored for 7 days in refrigerator

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar for the facilities provided. The work was carried out using the institute fund provided for the project approved by The Director and The Joint Director (Research), Indian veterinary Research Institute.

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