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by

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Isolation and Screening of Indurially important microorganism

One of the most fundamental tasks of a microbiologist is to isolate an organism with particular properties in a pure state, free of contamination, i.e. the selection of one type of organism from numerous others with which it is normally associated in nature. The ultimate sources for fresh isolates of microorganisms when contemplating a new process for industrial use are water, soil, plant material, sewage, spoiled foods and other natural habitats where they exist in complex mixtures. The detection and isolation of micro organisms form the natural sources is called screening which requires a selective method for isolation of a pure culture from natural environments. The desired organism may be less rapidly growing on the ordinary culture medium than other organisms. Selective methods favor the growth of desired species while discouraging the other organisms. There are three main types of selective methods: Chemical methods, Physical methods and Biological methods. Chemical methods of selection: These involve the use of some chemical compounds in the culture media which may favour the growth of the desirable organisms or may inhibition the undesirable ones. One approach used in chemical methods of selection involves the incorporation of a single carbon or nitrogen source that can be used only by the species to be isolated. This particular kind of selection is also known as “enrichment culture technique” (Figure 1) which is a valuable tool in many screening programmes meant for isolating industrially important strains. This involves the alterations in medium so as to favour the growth of the desired micro-organisms. On the other hand, unwanted micro-organisms are eliminated or develop poorly since they do not find suitable growth conditions in the newly created environment. The second approach involves the use of “dilute media” for the isolation of certain aquatic bacteria which are capable of growing with very low levels of carbon or nitrogen sources. This involves the use of a medium with low levels of the nutrients at

which only the desired species are able to grow well while others are not able to grow well. The third approach involves the supplementation of the culture media with low levels of certain chemicals including dyes, antibiotics, bile salts, salts of heavy metals which are inhibitory or toxic to the undesirable organisms so that the only the desirable species, which are not inhibited by these chemicals are able to grow. Many gram -ve bacteria can grow in presence of low concentrations of various dyes that inhibit gram +ve bacteria. Many intestinal bacteria can grow in presence of bile salts whereas non‐intestinal bacteria are inhibited. A medium containing crystal violet and sodium deoxycholate will allow gram negative intestinal bacteria to grow but will inhibit most other kinds of bacteria.

Physical methods of selection: One approach in this involve the use of a heat treatment at which the endospore producing bacteria can be selected by heating a mixed culture to 80oC for 10 min. The second approach involves the use of incubation temperature according to the temperature range of the organisms which are to be screened : low temperature is used for psychrophiles while high temperature for thermophiles. The third approach involves the use of a culture media with specific pH according to the pH range of the organisms to be screened: low pH for acidophiles, high pH for alkalophiles. The fourth approach makes use of small cell diameter or the motility of the culture to be screened. A membrane filter having a pore size of 0.15 μm is placed on the surface of an agar plate and the mixed microbial population is applied over the filter surface. The cells with a diameter less than 0.15 μm penetrates the pores of the filter to reach the underlying agar and grow on the agar while the organisms which are too large to penetrate the membrane filter are unable to grow on the agar. Those which are smaller in size and also have the ability to swim will penetrate through the pores and migrate away from the point thus forming hazy zone within the agar. Biological methods of selection: These involve the exploitation of negative interactions of the organisms towards the other organisms. One approach involves the selection of disease-producing species by taking advantage of pathogenic properties. If a mixed sample is injected in to a mouse the pathogens will multiply extensively while non-pathogens will be inhibited or killed by defense mechanism of the animal. The crowded plate technique is the other approach involving the detection and isolation of antibiotic producers. It consists of preparing a series of dilutions of the soil or other source material followed by spreading of dilutions on the nutrient agar plates. the agar plates having 300 to 400 or more colonies per plate are considered. The ability of a colony to exhibit antibiotic activity is indicated by the presence of a zone of growth inhibition surrounding the colony. If one is interested

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to isolate ISOLATION OF PURE CULTURES: Once the desires microorganisms are enriched and screened by the selective methods, the same are purified, sub cultured and the strains of the same are preserved for future use. A variety of the techniques have been developed for isolation into pure cultures. One of the methods, "Streak-plate technique", involve the streaking of the culture on agar plate across the surface and colony developing from one cell will be sufficently apart from another. A single streak plate does not automatically assure purity – slime or chain producing bacteria may adsorb some contaminants. It is advisable to streak a culture several times in succession, preferably on a non-selective medium. A modification of streaking method, “Roll-tube technique” has been developed for the isolation of strict anaerobes. This involves the use of stoppered anaerobic culture tubes whose inner walls have been coated with a prereduced agar medium . The tube containing an atmosphere of O2-free CO2 is rotated by a motor and inoculated with a transfer loop held against the agar surface starting at the bottom and drawing the loop gradually upward . After inoculation the tube is re-stoppered and incubated . In the other two methods, "Pour plate" and "spread plate techniques", the mixed culture is first diluted (only few cells/ml) before used to inoculate media. In pour plate, mixed culture is diluted directly in tubes of cooled, liquid agar medium and then dispensed into Petridishs, allowed to solidify and then incubated. In spread plate, the culture is not diluted in medium but in sterile saline. Small amount of sample (diluted culture) is placed on to the surface of agar plate and spread evenly with the help of bent, sterile glass rod. Here, only surface colonies develop. Another method for obtaining pure culture involves the use of a device called the micromanipulator in conjuction with a microscope to pick a single bacterial cell from a mixed culture. Schematic illustration of a single bacterium from a mixture of cells is shown in Fig 3. The microscopic field (1) shows the point of the microprobe touching a bacterial cell and causing the organism to float in a small amount of water. The microscopic stage is then moved to the left (2) while the microscope remains fixed. This causes the bacterium to follow the probe and becomes separated from the other cells.

ISOLATION AND ENUMERATION OF BACTERIA: Lactic acid bacteria have been isolated by either of three procedures: (i) enrichment of the sample in broth culture followed by plating of this culture onto agar medium; (ii) direct inoculation of the sample onto an agar medium, and (iii) membrane filtration of the sample followed by incubation of the membrane onto an agar medium. The most useful and preferred procedure is direct inoculation (spread plating) onto an agar medium because the colonies which develop can be enumerated, differentiated by their appearance and isolated for identification. Numerous media have been used for the isolation and enumeration of lactic acid bacteria from various food samples and these include de Man, Rogosa Sharpe (MRS) agar, Tomato

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juice (TJ) agar, Irrmann agar, tomato juice wine agar, yeast extract agar and various modifications of MRS agar to incorporate either grape juice, tomato juice, cysteine, malic acid and various sugars. Nakagawa agar and sucrose agar, developed for the isolation of brewery lactic acid bacteria could be useful for wine lactic acid bacteria. The addition of cycloheximide (100 mg/l) or pimaricin (50 mg/l) to the plating medium is necessary to inhibit the growth of yeasts and moulds. Incubation of plates under an atmosphere enriched in carbon dioxide or nitrogen can encourage faster growth of the colonies of lactic acid bacteria. Acetic acid bacteria are isolated by direct (spread) inoculation of samples over the surface of an appropriate agar medium. A range of media have been used to isolate acetic acid bacteria. Glucose-yeast extract-calcium carbonate-agar has been used most effectively to survey the presence of Acetic acid bacteria. The calcium carbonate gives the medium milky white appearance and serves to neutralise the acid produced by colonies of acetic acid bacteria. These colonies are recognised as they are surrounded by clear zones due to the solubilization of calcium carbonate. It is necessary to incorporate either cycloheximide or pimaricin into the medium to inhibit the growth of yeasts. Wine lactic acid bacteria appear not to grow very well on this medium and consequently donot interfere with the isolation of acetic acid bacteria. If their growth becomes a problem then it may be controlled by the addition of streptomycin (25 mg/l) which does not affect the growth of acetic acid bacteria. Alternatively it may be necessary to use a medium such as yeast extract-ethanol agar which will selectively support the growth of acetic acid bacteria but not the lactic acid bacteria. The use of an agar medium comprising nutrients, glucose, acetic acid and ethanol for selectively isolating acetic acid bacteria has also been reported by certain workers. Bacillus species can be isolated by plating the samples onto a nutrient agar medium (plate count agar) to which cycloheximide has been added to prevent yeast growth. The plates are incubated aerobically at 25-30oC for 24-48 h. Colonies that are comprised of Gram positive rods, are catalase positive and produce endospores are considered as the species of Bacillus. ISOLATION AND ENUMERATION OF YEASTS Both pour and spread plating techniques are used as the standard procedures for isolating and enumerating yeasts from various sources. However, there is increasing evidence that spread plating gives more accurate data. Other useful methods for enumerating yeasts are membrane filtration and microscopic examination. A variety of plating media have been used for the isolation of yeasts from various sources sources and these include grape juice agar, malt extract agar, Yeast-malt (YM) agar and other nutrient agars. These are non selective media that allow the growth of all yeast species associated with the fermentation. A limitation in their use, however, occurs with samples containing mixtures of yeast species of significantly different populations. In these circumstances colonies of the most prevalent species dominate on the plate preventing observation of those

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species that are present in the sample at lesser populations. Lysine agar is used most effectively to selectively isolate and enumerate the populations of non-Saccharomyces species including Kloeckera apuculata, Candida stellata during wine fermentations. Predominating Saccharomyces cerevisiae does not interfere with the growth of these yeasts on this medium because it is unable to use lysine as a nitrogen source and consequently does not grow. The successful use of lysine agar to specifically monitor the growth of non-Saccharomyces yeasts during wine fermentation has now been demonstrated by many researchers. Nutrient medium containing ethanol (12%) and sodium metabisulphite (0.015%) is also for the selective enumeration of Saccharomyces species during wine fermentation, because of their lesser tolerance to ethanol and sulphur dioxide. Species of Kloeckera and Hanseniaspora in particular and other non-Saccharomyces yeasts have difficulty growing in this medium therefore making it useful to monitor the growth of S. cerevisae during the early stages of fermentation. Use of a medium containing elevated concentrations of sorbate or benzoate for the selective isolation of Zygosaccharomyces bacilii, a species mostly associated with wine spoilage has also been reported. Biggy agar can be used to monitor the presence of hydrogen sulphide (H2S) producing yeasts. This medium contains bismuth sulphite which reacts with H2S-producing colonies causing them to appear brown-black. Bacterial growth on yeast isolation plates may be a problem when examining samples of grapes or musts. Such growth can be controlled without detriment to yeast development, by incorporating oxytetracycline (100 mg/l) or other bacterial antibiotic into the medium composition. Fungal contamination of yeast isolation plates can also be a problem with some grape samples. Some authors have added sodium propionate (0.1-0.2%) or biphenyl (0.02%) to the medium to control this interference. The detection of killer toxin producing yeast is done by the seeded agar plate technique using malt extract agar buffered at pH 4.5 with 0.5M phosphate citrate buffer. Approximately 105 cells/ml of the senstitive strain are suspended in 15 ml of pre-sterilized, molten (45oC) medium and then the mixture is poured into the petridish and allowed to solidify. Strains of the killer yeast are streak inoculated onto the surface of the seeded agar which is then incubated at 20oC for 48 h. Killer activity is recognised by inhibition of growth (clear zones) of the seeded strain in the region surrounding growth of the killer strain. Although enumeration of yeasts by agar plating is suitable for ecological studies, it is too slow to meet the requirements of modern quality assurance programs. Generally, it is necessary to incubate agar plates for upto four days before reliable data on counts is obtained. More rapid, automated methods are being developed for the enumeration of yeast populations and these include the application of impedance, ATP-bioluminescence and fluorescence microscopy techniques. In some cases these techniques have been applied to wine yeasts but further research and development of these applications is required.

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ISOLATION AND ENUMERATION OF MOULDS Procedures for the isolation and enumeration of fungi from foods and beverages have undergone extensive revision in recent years and are discussed in detail by many workers.. Direct plating of sample suspensions onto dichloran-rose bengal-chloramphenicol (DRBC) agar is now widely accepted as the preferred method for isolation of fungi from foods and beverages. The plates are incubated aerobically at 20-25oC for 72-96 h. The advantage of this medium over others that have been used in the past (acidified potato dextrose agar) lies in the combined use of dichloran and rose bengal to limit the spreading-lateral growth of fungal colonies without inhibiting their initial development. MAINTENANCE AND PRESERVATION OF INDUSTRIALLY IMPORTANT MICROORGANISMS: Several methods are available for the preservations of the microorganisms but there are two criteria for selecting a method of preservation for a given culture. They are: i) period of preservation desired and ii) nature of the culture to be preserved. Cultures are preserved for several reasons including demonstration of specific properties, research, assay, comparison studies, type strains, production of fermentation products and starter cultures. It is most desirable to preserve the cultures in such a way that they can be handled and transported with ease, and remain physiologically active as near to initial performance as possible without loss of genetic unity or of biochemical characteristics. In general each preservation method can be assigned to one of the following groups: Metabolically active methods: The cultures remain metabolically active by these methods and need to be maintained at low temperatures, in the refrigerators during the period of the preservation. There include periodic transfer on agar or in liquid medium or keeping agar cultures under mineral oil.

Periodic transfer to fresh media: The temperature and the type of medium chosen should support a slow rate of growth. Overlaying cultures with mineral oil: Oil must cover the slant completely - It should be about ½ inch above the tip of the slanted surface. Maintenance of strains by metabolically active methods are only for short-term preservation and should be used only in case a strain cannot be preserved by one of the metabolically inactive methods, or in addition to one of these methods Metabolically inactive preservation techniques: The cultures are made metabolically inactive by these methods either by cryopreservation or by drying. Freezing and low temperature storage in or above liquid nitrogen: Dense suspension containing some cryoprotective agent – glycerol, DMSO (dimethyl sulfoxide) is frozen to -150oC and then stored in liquid nitrogen refrigerator either by immersing in liquid nitrogen (-196oC) or in gas phase above liquid nitrogen (-150oC). Freezing and low temperature storage below -70°C: Dense suspension is generally made in 10% glycerol and frozen in the deep freezers. Freeze drying (Lyophilization): The advantages of freeze-drying are obvious. It is a convenient method for the preservation and long-term storage of a wide variety of

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microorganisms. However, special precautions are needed for the preservation of microorganisms sensitive to desication, light, oxygen, osmotic pressure, surface tension and other factors. Some effective protective agents for example skim milk and meso-inositol or honey or glutamate or raffinose, are used to suspend cells to be freeze-dried inorder to protect these against known freezing and drying injuries. Several anaerobic bacteria which are sensitive to aerobic freeze-drying, can successfuly be preserved using activated charcoal (5% w/v) in the suspending media along with the above protective agents. Freeze-drying involves the removal of water from frozen cell suspension by sublimation under reduced pressure. Most bacteria die when the cultures become dry although spore formers remain viable for many years. Suspension is frozen at -60 to -70oC and then connected to vacuum. Sublimation occurs causing dehydration of bacteria with minimum damage to the cell. Small cotton-plugged vials containing frozen suspensions of the microorganisms are placed in a flask, attached to a condenser which is connected to a vacuum pump. The microbial cells become dessicated as the ice in the frozen suspension sublimes directly to water vapours which are trapped in the condenser thus preventing it from entering the vacuum pump. After dessication the vials are removed and hermetically sealed under a vacuum. Liquid drying: Liquid-drying (L-Drying) involves vacuum-drying of samples from the liquid state without freezing. It is one of the sophisticated techniques used for the long-term preservation of microorganisms. Several microorganisms, which are sensitive to freezing or freeze-drying, can successfully be preserved by liquid drying. Liquid-drying has several advantages over freeze-drying and has been effectively used for preserving large collections of fragile microorganisms in various culture collections. However, specialized equipment is required for L-drying. Recently a simple and effective liquid-drying method has been described for the successful preservation of sensitive microorganisms (including various anerobes) which fail to survive freezing or freeze□drying. Since micro-organisms can survive for years in a completely dry state, freeze drying (lyophilization), as well as soil tube storage are most effective and require the least maintenance for long-term storage. Cultures lyophilized in tubes sealed and stored in liquid nitrogen, at 4oC in a cold room, or in a refrigerator have been known to survive storage for 30 or more years. Together with this, efficient record keeping and a precise inventory system asists in maintaining reliable records and eliminating costly mix-up. With proper care for nutrition, temperature, and inoculum development, the lyophilized cultures are easily rehydrated and restored to vegetative growth. The verification of the culture after retrieval is usually checked microscopically for the presence of contamination, morphological variation and tested in shake flasks for authentication of the expected physiology. SUBSTRATES USED IN MEDIA FORMULATIONS IN FERMENTATION TECHNOLOGY Media used for the cultivation of microorganisms must contain all

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elements in the form suitable for synthesis of cell substances and for the production of metabolic products. In laboratory research with microorganisms, pure defined chemicals may be used for the production of culture media but in industrial fermentations, complex, almost indefinable substrates are frequently used foreconomic reasons. Depending upon the process 25-70% of the total cost of the fermentationmay be due to the carbohydratesource. In many cases the media components are the byproducts of other industries and are extremely varied in composition. An optimally balanced culturemedium is mandatory for maximal production. Ingredients used In bioprocesses must be readily available, cheap and responsible for high product yield. Substratesused as Carbon sources: Carbohydrates are traditional energy sources in the media. For economic reasons, pure glucose or sucrose are not generally used as the sole carbon sources except in some cases which demand exact fermentation control. Molasses – one of the cheapestsources of carbohydrates. In addition to sugar, molasses contains nitrogenous substances, vitamins and trace elements. Composition is variable depending upon the raw material used for sugar production. Quality of molasses varies depending upon the location, climatic conditions and production process of the sugar factory. In addition to conventional molasses, a residue from starch saccharification which accumulates after the crystallization of glucose is also used as a fermentation substrate. “Hydrol” molasses a byproduct of glucose production from corn Malt extract: an aqueous extract of malted barley is an excellent substrate for many fungi and actinomycetes. Dry malt extract contains 90-92% carbohydrates and is composed of hexoses (glucose, fructose), disaccharides (maltose, sucrose), trisaccharides (maltotriose) and dextrins. Nitrogenous substances include proteins peptides, amino acids, purines, pyrimidines and vitamins. Amino acid composition varies according to grain used but proline always makes up about 50% of total amino acids. Media containing malt extract must be carefully sterilized. Overheating in presence of low pH and high proportion of reducing sugars, Maillard reaction occurs in which amino groups of amines, amino acids or proteins react with carbonyl groups of reducing sugars, aldehydes or ketones which lead to the formation of brown condensationproducts. Starch and dextrins: These can be directly metabolized by amylase producing organisms. It is commonly used for the preparation of glucose syrups, fructose syrups, maltose syrups, ethanol etc. Sulfite waste liquors: Sugar-containing waste products of paper industry with a dry weight of 9-13%. Primarily used for the cultivation of yeasts. Sulfite liquor from coniferous trees have a total sugar content of 2-3% and 80% of the sugars are hexoses (glucose, mannose, galactose) and others being pentoses (xylose, arabinose). Sulfite liquor from deciduous trees contain mainly pentose sugars. Cellulose: Wide availability. Occurs as a waste in the form of straw, corn cobs, wood wastes, bagasse and waste paper. It is not usually possible to use cellulose directly as a carbon source so it must first be hydrolysed

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chemically or enzymatically. The sugar syrup formed from cellulose hydrolysis is used for ethanol production and fermentative production of butanol, acetone and isopropanol. Whey: a byproduct of dairy industry. Contains 1.6% lactose and 0.27% casein. Used for the production of ethanol, single cell protein, xanthan gum, vitamin B12 , 2-3, butanediol, lactic acid and gibberellic acid. Animal and plant oils: Not generally used as sole fermentable C sources but are supplemented substrates. In antibiotic fermentation,soy, palm and olive oils are supplemented. Methanol: Cheapest fermentation substrate but metabolized by only a few bacteria and yeasts. Generally used as a substrate for single cell protein, glutamic acid, serine and vitamin B12 production. Ethanol: can be metabolized by many microorganisms as sole carbon source. Acetic acid is presently made by oxidation of ethanol. Alkanes: with a chain of C12 to C18 are readily metabolized by many microorganisms.

Substratesused as Nitrogen sources: • Ammoniumsalts • Urea • Gaseous NH3 • Corn steep liquor – a concentrated extract formed during starch production/extraction from corn. 4% nitrogen – contains various amino acids including alanine, arginine, glutamic acid, isoleucine, threonine, valine, phenyl alanine, methionine, cystine. Sugar present in corn steep liquor gets largely converted into lactic acid by lactic acid bacteria. Yeast Extract – a concentrated extract produced from autolysed Baker's yeast. Lysis is carried out at 50- 60C or through plasmolysis in the presence of high concentration of NaCl – contains amino acids, peptides, water soluble vitamins and carbohydrates. Composition of yeast extract is variable depending upon the substrates used for yeast cultivation. Peptones: protein hydrolysates. Composition variable depending upon their origin. Sources of peptones include meat, casein, gelatin, keratin, peanut seeds, soy meal, cotton seeds and sunflower seeds. Peptone from gelatin is rich in praline and hydroxyproline but has no sulphur containing amino acids. Peptone from keratin has a large proportion of praline and cystine but lacks lysine. Peptones from plant origin (soy peptone, cotton seed peptone) have large proportions of carbohydrates. The composition is also influenced by the type of hydrolysis, whether acid or enzymatic especially in regard to its tryptophan content. Soy/peanut/ground nut/cotton seed meal: The residue from soyabeans/ peanut/ ground nut/ cotton seed after the extraction of oil. Protein content - 50%o, carbohydrate content 30%o (sucrose, stachypse, raffinose, arabinan, arabinoglucan etc), 1%o residualfat

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Qualitative screening of enzyme producing microorganisms from natural biodiversity: The samples of soils containing decaying kitchen garbage are collected from dumping ground of Chandigarh city. The samples are explored for fungal biodiversity capable of producing multiple depolymerizing enzymes. The organisms are enriched in sterile Yeast Nitrogen Base (HI-Media, India) supplemented with 2% w/v fresh kitchen waste rich in cellulose, hemicellulose, pectin, starch, protein and lipids. After inoculation with the soil samples, the flasks are incubated on a rotary shaker at 30°C and 150 rpm for 96 h. The enriched fungal biodiversity is screened on yeast extract-Malt extract agar plates containing either of carboxymethyl cellulose, xylan, guar gum, starch, inulin, pectin, skimmed milk powder, tributyrin and incubated at 30° C for 96 h. The plates showing fungal growth are visualized for hydrolytic capabilities of the strains on various polymers to know about their potential to produce various hydrolytic enzymes. The degradation of starch is evaluated by flooding starch containing media plate with 0.2 % iodine solution, where the cleared zone is seen around amylase producing colony against the blue background. The clearing zone around the colony in inulin and pectin containing medium after the addition of Logule's iodine solution (1% (w/v) I2 in 1.5% solution of KI) is taken as a criteria for determining the inulinolytic activity of the fungal strains. For assessing the cellulolytic, xylanolytic and mannanolytic activities, media plates containing CMC, xylan and guar gum, are flooded with an aqueous solution of 0.1 % Congo red for 15 min and then wash with 1N NaCl. The degradation of skimmed milk powder is indicated by the appearance of a clear zone around the colony capable of producing protease. The ability to degrade lipid by production of lipase is indicated by the appearance of clear zone around the colony is trubutyrin containing plates. Further screening of the selected isolates is carried out by assessing their ability to co-produce multiple hydrolytic enzymes including cellulases, hemicellulases, pectinases, amylases, inulinases, proteases and lipases by solid state fermentation by taking 5 g of shredded kitchen waste in 250 ml Erlenmeyer flasks. The flasks are autoclaved, cooled to room temperature and then inoculated with 2.5 ml of spore suspension of the screened isolates. The flasks are incubated at 30oC for 4 days under stationary conditions. The enzyme(s) are extracted by adding 100 ml of distilled water to each flask along with 0.1% tween 80 and the flasks are kept on rotary shaker at 150 rpm for 30 min at room temperature. Churning of the contents of the flasks is carried out in a laboratory grinder followed by filteration through a metallic sieve. The solid residue left is pressed to release maximum liquid possible. The filtrate is centrifuged at 10,000 rpm and 4oC for 10 min. The supernatant obtained is used as enzyme preparation supernatants obtained from solid state cultures are assayed at 50oC, pH 4.0 for cellulases (exo-β-1,4-glucanase, endo-β-1,4-glucanase, and β-1,4-glucosidases), hemicellulases (xylanase, xylosidase, mannanase, mannosidase), pectinase (pectin lyase,

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polygalactouronase), inulinase, amylases (α-amylase, glucoamylase, pullulanase, α-glucosidase). protrease, lipase and alginate lyase. Production of various hydrolytic enzymes by Solid, surface and submerged state fermentation of agro□industrial residues The agro-industrial residues including malt spent grain, rice spent grain, wheat bran, rice bran, mixture of malt spent grain and rice spent grain, mixture of wheat bran and malt spent grain, mixture of wheat bran and rice spent grain are evaluated for supporting the growth of fungal strain and inducing the production of enzymes viz. α-amylase, glucoamylase, pullulanase, inulinase, endo-β-1,4-glucanase, exo-β-1,4-glucanase, β-1,4-glucosidase, endo-β-1,4-xylanase and endo-β-1,4-mannanase under liquid and solid state fermentation. The selected fungal strain SS-25 is further analysed for enzyme productivities under solid as well as liquid state fermentation conditions using malt spent grain, rice spent grain, wheat bran, rice bran, mixture of malt spent grain and rice spent grain, mixture of wheat bran and malt spent grain, mixture of wheat bran and rice spent grain as medium. Liquid culture fermentation is studied both as submerged (shaking) as well as surface (static) state fermentation. For this, either 5 g of the wheat bran is used as media for both surface and submerged culture fermentations in 250 ml Erlenmeyer flasks containing 100 ml distilled water. The selected fungal isolates are assessed for their ability to grow under solid state conditions on wheat bran. Five grams of wheat bran is taken as basal medium for SSF in 250 ml Erlenmeyer flasks (in duplicate), moistened with 7.5 ml of distilled water, autoclaved, cooled and inoculated with five discs (7mm) cut from the periphery of actively growing colonies on PDA plates followed by incubation at 30oC for 96 h under stationary conditions. After 96 h of incubation, the enzyme(s) is extracted by adding 100 ml distilled water and 0.1% Tween 80, shaking the flask on rotary shaker (New Brunswick, USA) at 150 rpm for 30 min at room temperature. The contents of the flask are then churned in a laboratory grinder and then filtered through a metallic sieve, the solid residue is pressed to release left over liquid. Supernatant thus obtained is centrifuged at 10,000 rpm at 4ºC for 10 min and the clear supernatant thus obtained is used as enzyme preparation. Quantitative estimation of enzymes The supernatants obtained from solid state cultures are assayed at 50oC, pH 4.5 for various enzymes including cellulases, hemicellulases, pectinase, amylases, inulinases, proteases, lipases and alginate lyase. The yields are expressed as IU/ml Cellulases Cellulase complex is measured in terms of endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase activities using carboxymethyl cellulose (CMC), Whatmann filter paper No. 1 strips (1× 6 cm), salicin, cellobiose and 4- Nitrophenyl β-D-glucopyranoside) as the substrates and expressed in terms of CMCase, FPase, salicinase, cellobiase and β-glucosidase respectively by determining the µmoles of glucose liberates/min using dinitrosalicylic acid reagent for CMCase, FPase and Salicinase. while glucose is liberated in case of cellobiase is assayed by Glucose-Peroxidase

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method. The β-glucosidase is quantified in terms of µmoles of p-nitro phenol (PNP) liberates/min. CMCase (Endo-β-1,4-glucanase) 0.5 ml of the appropriately diluted enzyme and 0.5 ml of 0.5% CMC solution prepare in 0.1 M acetate buffer (4.5) are added in a test tube to make the reaction mixture. The tubes are incubated at 50oC for 30 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. The absorbance is read at 560 nm. One unit of CMCase is expressed as the amount of enzyme require to release one µmole of glucose from carboxymethylcellulose in one min under assay conditions (50oC; pH 4.5). FPase (Exo-β-1,4- glucanase) 0.5 ml of the appropriately diluted enzyme and Whatman filter paper No. 1 (1 x 6 cm strip) are added in a test tube to make the reaction mixture and the final volume of 1 ml is made by adding 0.1 M acetate buffer (4.5). The mixture is incubated at 50oC for 60 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. The absorbance is read at 560 nm. One unit of FPase is expressed as the amount of enzyme require to release one µmole of glucose from filter paper in one min under assay conditions. (50oC; pH 4.5) β-glucosidase 0.5 ml of the appropriately diluted enzyme and 0.5 ml of 1mM β-D-glucopyranoside (pNPG) solution prepare in 0.1 M acetate buffer (4.5) are added in a test tube to make the reaction mixture. The tubes are incubated at 50oC for 30 min after which the reaction is stopped by boiling for 5 min The p-nitro phenol (pNP) liberate as a result of enzyme reaction is determined by adding 4 ml of 1.8 N NaOH and measuring the absorbance at 410 nm. One unit of β-glucosidase is expressed as the amount of enzyme require to release one µmole of pNP from pNPG in one min under standard assay conditions. Salicinase (β-glucosidase) 0.5 ml of the appropriately diluted enzyme and 0.5 ml of 0.5% salicin solution prepare in 0.1 M acetate buffer (4.5) are added in a test tube to make the reaction mixture. The tubes are incubated at 50oC for 30 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. The absorbance is read at 560 nm. One unit of Salicinase is expressed as the amount of enzyme required to release one µmole of glucose from carboxymethylcellulose in one min under assay conditions (50oC; pH 4.5). Cellobiase (β-glucosidase) /0.5 ml of the appropriately diluted enzyme and 0.5 ml of 0.5% cellobiose solution prepare in 0.1 M acetate buffer (4.5) are added in a test tube to make the reaction mixture. The tubes are incubated at 50oC for 30 min after which the reaction is stopped by boiling for 5 min The glucose liberate as a result of enzyme reaction is determined by taking 0.1 ml of the sample in another test tube. To this 1.0 ml of Glucose oxidase, peroxidase, 4– aminoantipyrine reagent is added followed by the addition of 1.0 ml distilled water and incubation at room temperature for 30 min.

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The absorbance is read at 500 nm. One unit of cellobiase is expressed as the amount of enzyme require to release one µmole of glucose from cellobiose in one min under assay conditions (50oC; pH 4.5)

Hemicellulases Hemicellulases are determined in terms of endo-β-1,4-xylanase and endo-β-1,4-mannanase activities using xylan and guar gum, respectively, as the substrates and determining the µmoles of xylose and mannose liberates/min, respectively using dinitrosalicylic acid reagent. Xylanase (Endo-1,4-β-xylanase) 0.5 ml of the appropriately diluted enzyme and 0.5 ml of 0.5% xylan solution prepare in 0.1 M acetate buffer (pH 4.5) is added in a test tube of 30 ml capacity to make the reaction mixture. In enzyme blank, xylan solution is replaced by buffer and in substrate blank, enzyme is replaced by buffer in the above described composition of reaction mixture. The tubes are incubated at 50oC for 15 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. OD is read at 560 nm. One unit of xylanase is expressed as the amount of enzyme require to release one µmole of xylose from xylan in one min under standard assay conditions. β-Xylosidase 0.5 ml of the appropriately diluted enzyme and 0.5 ml of 1mM Methyl β-D-xylopyranoside solution prepare in 0.1 M acetate buffer (4.5) are added in a test tube to make the reaction mixture. The tubes are incubated at 50oC for 30 min after which the reaction is stopped by boiling for 5 min The xylose liberates as a result of enzyme reaction is determined by adding 4 ml of 1.8 N NaOH and measuring the absorbance at 410 nm. One unit of Xylosidase is expressed as the amount of enzyme required to release one µmole of xylose from Methyl β-D□xylopyranoside in one min under standard assay conditions. Mannanase (Endo-1,4-β-mannanase) The crude enzyme preparation is appropriately diluted in 0.1 M acetate buffer (pH 4.5). 0.5 ml of the diluted enzyme and 0.5 ml of guar gum solution is added in a test tube of 30 ml capacity to make the reaction mixture. In enzyme blank, guar gum solution is replaced by buffer and in substrate blank, enzyme is replaced by buffer in the above described composition of reaction mixture. The tubes are incubated at 50 oC for 15 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. OD is read at 560 nm. One unit of mannanase is expressed as the amount of enzyme require to release one µmole of mannose from guar gum in one min under standard assay conditions. β-Mannosidase 0.5 ml of the appropriately diluted enzyme and 0.5 ml of 1mM 4-nitrophenyl β-D mannopyranose solution prepare in 0.1 M acetate buffer (4.5) are added in a test tube to make the reaction mixture. The tubes are incubated at 50oC for 15 min after which the reaction is stopped by boiling for 5 min The xylose liberates as a result of enzyme reaction is determined by adding 4 ml of 1.8 N NaOH and measuring

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the absorbance at 410 nm. One unit of mannosidase is expressed as the amount of enzyme required to release one µmole of mannose from 4- nitrophenyl β-D mannopyranose in one min under standard assay conditions. Pectinases Pectinases are determined in terms of endo-pectin lyase and endo polygalacturonase using pectin and polygalacturonic acid as the substrates. Pectin lyase The enzyme is estimated by using pectin as the substrate and determining the µmoles of galactouronic acid liberates/min, using dinitrosalicylic acid reagent (Miller, 1959). The crude enzyme preparation is appropriately diluted in 0.1 M acetate buffer (pH 4.5). 0.5 ml of the diluted enzyme and 0.5 ml of pectin solution is added in a test tube of 30 ml capacity to make the reaction mixture. In enzyme blank, pectin solution is replaced by buffer and in substrate blank, enzyme is replaced by buffer in the above described composition of reaction mixture. The tubes are incubated at 50oC for 15 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. OD is read at 560 nm. One unit of pectinase is expressed as the amount of enzyme require to release one µmole of α-D galacturonic acid from pectin in one min under standard assay conditions. Polygalactouronase The enzyme is estimated by using polygalactouronic acid as the substrate and determining the µmoles of galactouronic acid liberates/min, using dinitrosalicylic acid reagent. The crude enzyme preparation is appropriately diluted in 0.1 M acetate buffer (pH 4.5). 0.5 ml of the diluted enzyme and 0.5 ml of pectin solution is added in a test tube of 30 ml capacity to make the reaction mixture. In enzyme blank, pectin solution is replaced by buffer and in substrate blank, enzyme is replaced by buffer in the above described composition of reaction mixture. The tubes are incubated at 50oC for 15 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. OD is read at 560 nm. One unit of pectinase is expressed as the amount of enzyme required to release one µmole of α-D-galacturonic acid from pectin in one min under standard assay conditions. Amylases Amylases are determined in terms of α-amylase, glucoamylase, pullulanase and α-glucosidase activities using soluble starch, pullulan and maltose as the substrates. α-amylase (1,4-α-D-glucan glucanohydrolase) The crude enzyme preparation is appropriately diluted in 0.1 M acetate buffer (pH 4.5). 0.25 ml of the diluted enzyme and 0.25 ml of starch solution (0.2 %) is added in a test tube of 30 ml capacity to make the reaction mixture. In substrate blank, enzyme is replaced by 0.1 M acetate buffer in the above described composition of reaction mixture. The tubes are incubated at 50oC for 10 min after which the reaction is stopped by adding 0.25 ml of 1N HCl followed by the addition of 0.2% iodine solution to develop the color. 4.0 ml of distilled water is added to all the tubes and OD is read at 660 nm. The percentage reduction is expressed as the amount of enzyme require to reduce the color of

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starch-iodine complex by 10 % in 10 min. Glucoamylase (exo-1,4- α-glucosidase) The crude enzyme preparation is appropriately diluted in 0.1 M acetate buffer (pH 4.5). 0.5 ml of the diluted enzyme and 0.5 ml of starch solution (0.5%) is added in a test tube of 30 ml capacity to make the reaction mixture. In enzyme blank, starch solution is replaced by 0.1 M acetate buffer and in substrate blank, enzyme is replaced by 0.1 M acetate buffer in the above described composition of reaction mixture. The tubes are incubated at 50oC for 15 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. OD is read at 560 nm. One unit of glucoamylase is expressed as the amount of enzyme require to release one µmole of glucose from soluble starch in one min under standard assay conditions. Pullulanase The crude enzyme preparation is appropriately diluted in 0.1 M acetate buffer (pH 4.5). 0.5 ml of the diluted enzyme and 0.5 ml of pullulan solution (0.5%) is added in a test tube of 30 ml capacity to make the reaction mixture. In enzyme blank, pullulan solution is replaced by 0.1 M acetate buffer and in substrate blank, enzyme is replaced by 0.1 M acetate buffer in the above described composition of reaction mixture. The tubes are incubated at 50oC for 15 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. OD is read at 560 nm. One unit of Pullulanase is expressed as the amount of enzyme require to release one µmole of maltodextrin from soluble pullulan in one min under standard assay conditions. α-glucosidase 0.5 ml of the appropriately diluted enzyme and 0.5 ml of 0.5% cellobiose solution prepare in 0.1 M acetate buffer (4.5) are added in a test tube to make the reaction mixture. The tubes are incubated at 50oC for 30 min after which the reaction is stopped by boiling for 5 min The glucose liberates as a result of enzyme reaction is determined by taking 0.1 ml of the sample in another test tube. To this 1.0 ml of Glucose oxidase, peroxidase, 4– aminoantipyrine reagent is added followed by the addition of 1.0 ml distilled water and incubation at room temperature for 30 min. The absorbance is read at 500 nm. One unit of cellobiase is expressed as the amount of enzyme require to release one µmole of glucose from cellobiose in one min under assay conditions (50 oC; pH 4.5.) Inulinase The crude enzyme preparation is appropriately diluted in 0.1 M acetate buffer (pH 4.5). 0.5 ml of the diluted enzyme and 0.5 ml of inulin solution (0.5%) is added in a test tube of 30 ml capacity to make the reaction mixture. In enzyme blank, inulin solution is replaced by buffer and in substrate blank, enzyme is replaced by buffer in the above described composition of reaction mixture. The tubes are incubated at 50oC for 15 min after which the reaction is stopped by adding 1.5 ml of DNSA reagent. This mixture is boiled for 15 min to develop the color followed by the addition of 4 ml of distilled water. OD is read at 560 nm. One unit of Pullulanase is expressed as the amount of enzyme require to

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release one µmole of fructose from soluble inulin in one min under standard assay conditions. Protease Protease activity is assayed by using azocasein as substrate. The reaction mixture contains 20 µl azocasein (5% v/v) and 430 µl of 100 mM Tris HCl buffer (pH 9.0) is preincubated at 50◦C for 5 minutes. The reaction mixture is initiated by addition of 50 µl of enzyme. This is terminated by addition of 500 µl of TCA. These samples are kept in ice for 15 minutes .The precipitate is settled by centrifugation at 10,000 rpm for 5 minutes. 800 µl of supernatant is then mixed with 200 µl of 1.8 N NaOH and absorbance is measured at 420 nm. One unit of protease activity is defined as the enzyme that increase the absorbance by 0.01 caused by enzyme per hour under the assay condition. Lipase Lipase is estimated by using p-nitrophenyl butyrate (pNPB) as substrate. 1.5 ml of Tris-HCl is added to 0.9 ml of working solution (1ml of solution I which is prepared by dissolving 90 mg of p-nitrophenyl butyrate in 30 ml of propane-2-ol in solution II which is prepared by dissolving 2 g of Triton X-100 and 0.5 g of Gum Arabic dissolved in 450 ml of 30 mM Tris HCl of pH 8.0) in 30 ml test tube. 0.5 ml of 75 mM CaCl2.2H2O is added and the mixture is prewarmed at 50◦C for 5 minutes. 0.1 ml of the enzyme supernatant is added and the mixture is mixed properly. The reaction mixture is then incubated at 50◦C for 10 min in the water bath. The reaction is stopped by immersing the tubes in ice. The substrate blank contains 0.1 ml Tris HCl buffer (pH 8.0) instead of enzyme whereas enzyme blank has 0.9 ml of Tris HCl buffer (8.0) instead of substrate. Absorbance is read at 410 nm and the amount of p-nitrophenol liberates is calculated from t stock solution of p-nitrophenol of 1 mg/ml in propane-2-ol. One unit of enzyme activity is defined as equivalent to one microgram of p-nitrophenol liberates perminute. Alginate lyase In the case of alginate lyase, the reaction mixture contains 0.5 ml enzyme and 0.5 ml sodium alginate (0.5 %) as substrate (prepared in acetate buffer (4.5)). Separate enzyme blank and substrate blank are made by replacing substrate (in case of enzyme blank) and enzyme (in case of substrate blank) respectively with 0.1 M acetate buffer (4.5) in the above reaction mixture. After an incubation of 10 min the OD is read at 235 nm. One unit has been defined as an increase in optical density (235nm) of 0.010 per min.

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