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⑤④ Process for denitrification of tobacco.

⑤⑦ A fed-batch fermentation process for reducing nitrate and nitrite content of aqueous tobacco extract is disclosed. Tobacco extract is rapidly and efficiently denitrified in accordance with the process by feeding the extract into a fermentor containing appropriate denitrifying microorganisms while providing necessary additives and maintaining conditions under which the nitrate and nitrite are reduced via a dissimilatory pathway. The denitrified extract may be applied to tobacco from which soluble solids have been extracted to form reconstituted tobacco for use in smoking products.

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PROCESS FOR
DENITRIFICATION OF TOBACCO

The present invention relates to a fed-batch fermentation method for denitrating aqueous tobacco extracts via dissimilatory denitrification.

It is generally recognised that reduced delivery of oxides of nitrogen in smoke of tobacco products is desirable. Therefore, a number of methods have been developed to reduce levels of nitrogen oxide precursors, such as nitrates and nitrites, in smoking products. For example, methods involving microbial treatment of tobacco to accomplish nitrate and/or nitrite reduction have been proposed.

It has now been discovered that relatively concentrated tobacco extracts may be denitrified rapidly and efficiently by means of a fed-batch fermentation. Fed-batch fermentation processes are known in production of yeast, glycerol, organic acids, antibiotics and the like. See e.g. S.J. Pirt, "The Theory of Fed Batch Culture with Reference to the Penicillin Fermentation", J. Appl. Chem. Biotechnol., 24, pp. 415-24 (1974); A. Whitaker, "Fed-Batch Culture", Process Biochem. pp. 10-15

(May 1980). See also Rober Keller et al., "Fed-batch Microbial Culture: Models, Errors and Applications", J. Appl. Chem. Biotechnol., 28, pp. 508-14 (1978). Such processes, however, are not known to have been previously taught or suggested for denitrification of tobacco. Moreover, the known art contains no suggestion that tobacco extract denitrification can be effected on extracts containing relatively high nitrate and soluble solids content, that more efficient nutrient utilization would result, or that denitrification can be accomplished more rapidly by means of a fed batch process.

This invention provides a method for denitrifying tobacco extract employing a fed-batch fermentation process. In accordance with the method a stirred sterile deaerated aqueous tobacco extract containing up to about 21 weight percent soluble solids and up to about 4000 ppm nitrate-nitrogen is fed into a fermentor having therein a stirred culture solution containing an inoculum having a volume equal to 15-30% of the total volume of extract to be denitrified and having at least 10^6 - 10^8 cells/ml of conditioned denitrifying microorganisms at a rate such that the nitrate-nitrogen content in the fermentor does not exceed about 1000 ppm, while providing additives necessary for and maintaining conditions under which nitrate is reduced to nitrogen gas via a dissimilatory pathway. After terminating the feed, the incubation of the extract is continued for about two hours to insure complete denitrification.

The invention also provides a method for denitrifying tobacco wherein an aqueous tobacco extract is formed and denitrified as above described and is then reapplied to the tobacco web resulting from the extraction to form reconstituted tobacco

suitable for use in smoking products.

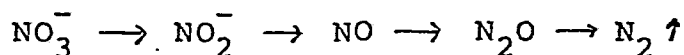
The present invention provides an improved method for reducing nitrate and/or nitrite in aqueous tobacco extract by means of microbial denitrification. The method of the invention permits more rapid and efficient reduction of nitrate in tobacco materials when microorganisms which are capable of dissimilatory denitrification are utilized. Further, more concentrated extracts can be denitrified by means of the invention. This more rapid and efficient reduction is effected by employing a fed-batch culture technique. Smoking articles prepared from tobacco reconstituted with the denitrified extract deliver significantly lowered amounts of oxides of nitrogen on smoking.

In tobacco product manufacture, the time required for microbial denitration can be the rate limiting step. Therefore provision of a shorter processing time for nitrate reduction, as with the present invention, is a significant economic advantage, particularly when operating on a commercial scale. Moreover the present process, provides more efficient utilization of nutrients, thereby reducing manufacturing costs. Finally since extracts having higher soluble solids content can be treated in accordance with the invention, the size of the fermentors, as well as, the time and costs associated with concentration of extract for use in reconstitution processes are reduced.

Broadly stated the process of the invention comprises feeding tobacco extract into a fermentor containing conditioned denitrifying microorganisms at a rate such that the ionic strength of the solution in the fermentor does not rise to a level inhibitory to the microorganisms. The conditions of the system are those which are conducive to efficient reduction of

1 nitrate to nitrogen gas. Further the invention comprises
reducing the nitrate level of tobacco by denitrifying tobacco
extract in accordance with such process and forming reconstituted
tobacco employing the denitrified extract.

5 In the practice of the present invention, micro-
organisms which reduce nitrate or nitrite to elemental nitrogen
via a series of metabolic steps commonly known as dissimilatory
denitrification are used. Nitrate reduction via this pathway is
effected by a series of enzymatic reactions shown schematically
10 below.



On the other hand, during assimilatory nitrate reduction
microorganisms convert nitrate to ammonia and protein.

For the purpose of the present invention, dissim-
15 ilatory reduction is selected since elemental nitrogen is the
end product of nitrate reduction and can be completely removed
from the tobacco materials. Moreover, no other nitrogen-
containing metabolic intermediate products that could poten-
tially affect the subjective characteristics of the denitrated
20 tobacco material or influence the further formation of oxides
of nitrogen in the generated smoke are produced via the
dissimilatory denitrification mechanism. Further, because the
extract stops cell growth, biomass build-up and attendant problem
of waste disposal can be avoided with this process. In addition,
25 since the process is not highly exothermic the need for elaborate
cooling steps can be avoided.

Microorganisms which are effective in dissimilatory
reduction of nitrates include Micrococcus (Paracoccus)
denitrificans, specifically Paracoccus denitrificans, ATCC
30 19367; ATCC 17741 and ATCC 13543 and Micrococcus denitrificans

Beijerinck, NC1B8944. Other dissimilatory denitrifiers are selected species of the genera Pseudomonas, Alcaligenes, Bacillus and Propionibacterium. Microorganisms effective only in the reduction of nitrate to nitrite in tobacco materials are not considered suitable for use in the present process in that the level of oxides of nitrogen is not significantly reduced in smoke from tobacco materials treated with such microorganisms.

The Paracoccus denitrificans strain ATCC 19367 has been utilized in the present process and has been found to be highly effective. This strain was obtained from the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852. Its morphology is set forth in Table 1.

Table 1

Morphological and Biochemical Characteristics of
Paracoccus (Micrococcus) denitrificans ATCC 19367

A. MORPHOLOGY

Cells are coccoid, averaging about 1 μ in diameter. They are gram negative and occur in pairs, singly and aggregates.

B. PHYSIOLOGY

Optimum growth: 25-30°C, Range: 5-37°C.

Nitrates and nitrites are electron acceptors in dissimilation being reduced to nitrous oxide and nitrogen. Anaerobic growth with the production of gas in the presence of nitrate. Indole not produced.

Urease activity-negative.

Catalase-positive.

Aerobic growth on salicin, dulcitol, d-xylose, adonitol, lactose, mannitol, sucrose, sorbitol, maltose, dextrose, mannose, raffinose and rhamnose but with the production of neither acid or gas.

Aerobic growth fructose, and arabinose with the production of trace amounts of acid.

No growth on aesculin or arginine.

Starch is not hydrolyzed.

Gelatin is not hydrolyzed.

Where microorganisms are capable of a number of metabolic processes it is important to subject the microorganisms to an inductive treatment whereby they are acclimated or conditioned to the dissimilatory reduction of nitrates before using

them in accordance with the process of the present invention. Thus, it may be necessary to subject the microorganisms to an induction process during which a build-up of microorganisms with enzyme systems adapted to dissimilatory denitrification is obtained. Reference herein to conditioned microorganisms are intended to mean microorganisms which have such enzyme systems and are acclimated to dissimilatory denitrification. The induction process can be effected by growth and maintenance of the microorganisms under controlled conditions. For example, a broth containing nitrate-nitrogen, possibly derived from aqueous tobacco extracts, may be inoculated with the denitrifying microorganism. Normally the broth should have a nitrate-nitrogen content of at least 10 ppm and preferably about 1400 ppm to support and achieve the desired amount of inoculum build-up. Concentrations of nitrate-nitrogen greater than about 4000 ppm may have adverse effects on the microorganisms.

During inoculum buildup additives necessary for growth of the microorganisms are required. Generally, such additives will include a carbon source, nitrate, phosphate, ammonium salts and metal salts, as sources of metallic ions, such as iron and magnesium. In the buildup of Paracoccus denitrificans, glucose, as the carbon source, potassium nitrate, potassium phosphate salts, ammonium chloride, magnesium sulfate and ferric chloride have been found to result in a suitable medium. Specifically, a medium containing 10 g glucose, 10 g KNO_3 , 10.7 g K_2HPO_4 , 5.24 g KH_2PO_4 , 1 g NH_4Cl , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.002 g FeCl_3 per liter of deionized water has been found adequate for buildup of Paracoccus denitrificans.

It will be recognized by those skilled in the art that the amount of culture added to the nitrate broth is a matter of

judgment. However, addition of inoculum to culture broth to give an initial optical density of about 0.3 to 0.4 at 660 m μ as determined using an Hitachi-Perkin Elmer Spectrophotometer, Model 120, will give an acceptable inoculum build-up within a period of about 8 to 24 hours. Optimally the broth and additives are sterilized prior to inoculation.

The inoculated broth is incubated under conditions such that nitrate is reduced to nitrogen gas via a dissimilatory metabolic pathway. Generally this is most efficiently accomplished if the dissolved oxygen content in the broth during incubation is as close to 0 ppm as possible. The dissolved oxygen may be measured and monitored by employing a dissolved oxygen electrode. The desired low dissolved oxygen levels may be effectively achieved by initially sparging the fermentation vessel being employed for the incubation with an inert gas such as nitrogen or helium at a rate of 0.5 to 1.0 volume/volume/minute until the desired dissolved oxygen level is achieved, and thereafter eliminating the air access to the vessel. Although the dissolved oxygen content may be well above 0 initially, where air access is restricted the dissolved oxygen will be reduced to approximately zero generally within 15 to 30 minutes after commencing fermentation.

The optimum incubation conditions will vary to some degree depending upon the specific microorganism employed.

Where Paracoccus denitrificans is selected, the initial pH of the broth should be between about 7 to 8 and preferably about 7.0 to 7.5. The broth is maintained at a temperature between about 20°C and 40°C, temperatures between about 30°C and 35°C being preferred. During incubation, agitation is generally required and may be achieved by low to medium speed rotary

stirring at about 60 to 300 rpm. Adjustments in these conditions necessary to optimize fermentation with different microorganisms will be apparent to one skilled in the art. The incubation period is generally about 8 to 24 hours to permit maximum build-up but will vary depending upon the initial relative amounts of nitrate and inoculum and the specific incubation conditions. It is to be understood that the inoculum build-up incubation step can be expedited by means of a vacuum in the same manner as is more fully described hereinbelow in connection with the tobacco denitrification process.

For maximum inoculum build-up and adaptation of the microorganisms, it is desirable to transfer the microorganisms resulting from the above induction process to a second nitrate broth and subject them to a further induction incubation as described above. The induction process is preferably carried out serially in this manner at least three times and optimally a total of five times, in each case employing the culture resulting from the previous induction stage to inoculate the nitrate broth. Following the desired number of induction stages the resulting microorganisms may be transferred to a fermentor for use in the present process.

In the practice of the present process, the built-up inoculum is transferred under aseptic and anaerobic conditions to a sterile fermentor large enough to accomodate the inoculum, necessary additives and tobacco extract to be denitrated. The inoculum, at the start of operation of the process of the invention, contains 10^{6-8} cells/ml and has a volume about 15-30% of that of the tobacco extract to be denitrated.

As with the induction process, the optimum fermentation conditions will depend on the specific microorganisms

employed. With the Paracoccus denitrificans strain herein employed effective fermentation is achieved when the temperature is maintained at about 25°C to 40°C, preferably at about 30°C to 35°C and optimally about 35°C. Although lower temperatures may be employed they may slow the fermentation process to a degree which is not commercially practical. The pH of the culture at the commencement of the process is in the range of about 7.0 to 8.0 and optimally at 7.3. Overall conditions of the system should be such that during denitration the pH is maintained between about 7.0 to 9.5.

The fermentation media should be agitated during incubation. A minimum agitation of about 60 rpm is necessary to maintain the microorganisms in suspension and keep them in contact with their environment. Moreover, agitation facilitates diffusion of the nitrogen gas end product from the media. Agitation of about 60-150 rpm is typically employed. Since the volume in the fermentation vessel changes during operation of the process, stirring means effective for such changing volumes are desirable. For example, a multiple impeller arrangement or a single impeller and draft tube might be employed to provide agitation.

During incubation the dissolved oxygen content of the fermentation media should be low enough for dissimilatory reduction of nitrate to nitrogen to occur. Typically dissolved oxygen levels below 0.5 ppm are adequate. Optimally levels as close to zero as possible are desirable in order to expedite dissimilatory denitrification. Although the initial oxygen content of the fermentation media may be above zero, if oxygen access is restricted the content will rapidly be reduced such that desirable low levels are achieved within the early part of

the incubation stage. Typically such oxygen content reduction will be complete within 30 minutes after fermentation commences. By continuing to restrict oxygen access during operation of the process, low, near zero levels can be maintained. Sparging with an inert gas, as nitrogen or helium for 10 minutes at a flow rate equal to the volume being deaerated is generally effective to reach about 0 ppm dissolved oxygen. Sparging, though useful initially, is not required and is generally not employed during operation of the process.

0 The denitrification process of the invention requires presence of certain additives in the medium. Specifically a carbon source is required. Such a carbon source is preferably glucose in an amount equal to 2.5 g/1000 ppm $\text{NO}_3\text{-N}$ for 10% soluble solids increasing to 10 g/1000 ppm $\text{NO}_3\text{-N}$ for 21% soluble
5 solids extract to be denitrified. Other carbon sources such as sucrose, fructose, molasses or the like may also be employed. Preferably the carbon source is all present in the inoculum upon commencement of denitrification since the microorganisms will be more active initially when optimal, non-inhibitory concentrations
0 are present.

 In addition to a carbon source, a phosphate source, certain metallic ions and ammonia are utilized in the present process. References to additives herein are intended to signify materials such as these, which along with nitrate or nitrite,
5 are necessary to the denitrification process. Specifically potassium phosphate, phosphoric acid or other phosphate material in which the phosphate is available to the microorganisms may be employed. Metallic ions, preferably in the form of FeCl_3 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ or other sources of such ions, are employed. Each of
0 these materials may be present in the tobacco extract or the

inoculum. It is preferable, however, that the FeCl_3 be present in the inoculum. Further, when the additives are present in the extract care should be taken to ensure that sufficient amounts of these additives remain available to the microorganisms, that is, unreacted with the tobacco extract components. The actual amounts of these materials required in the process is small. Generally no more than 0.1 gram of each material per liter of extract is required. The additives should be deaerated and sterilized prior to commencing denitrification. These procedures may be effected on the additives per se or following their addition to the extract.

In the practice of the present invention the nitrate content of an aqueous tobacco extract is reduced. Such an extract may be formed employing conventional techniques, as by contacting a tobacco material with an aqueous solution to extract the soluble components, including nitrate salts. The time of contact will depend on the water to tobacco ratio and the temperature of the aqueous solution. The aqueous extract produced by contact with the aqueous solution is separated from the insoluble fibrous tobacco residue, employing conventional solid-liquid separation techniques. For example, squeezing, centrifugation and filtration techniques may be employed.

If necessary the separated tobacco extract is treated to adjust the soluble solids and/or nitrate content. Generally extracts containing up to about 21% soluble solids and up to about 4000 ppm nitrate-nitrogen may be treated in accordance with this invention. Sterilization of the tobacco material prior to commencing the process of the invention is generally preferable to avoid any conflicting microbial process. Such sterilization may be accomplished by conventional means. Auto-

claving or heating may be employed for this purpose. Alternatively sterilization may be accomplished chemically by elevating or reducing the pH of the system to above 11 or below 2.4 for at least about 60 minutes using strong acids or bases which are acceptable in the system. For example, potassium hydroxide or phosphoric acid may be used for this purpose. Deaeration of the extract prior to commencing the process may be accomplished in the manner employed to deaerate the additives and culture broth. The pH of the extract should generally be between about 6-8 and optimally about 7.3 at the start of the process. Addition of KOH or H_3PO_4 to the extract may be employed to achieve the desired pH level.

Application of a vacuum during fermentation involving dissimilatory denitrification causes the denitrification to proceed toward completion at an increased rate. This is believed to be due, at least in part, to the more rapid diffusion of the nitrogen gas and carbon dioxide gas end products and their removal from the system as a result of application of the vacuum. Therefore, during practice of the process a vacuum may be maintained in the fermentation vessel. Any conventional means for producing a vacuum may be employed. The degree of vacuum utilized during fermentation depends in part on the growth kinetics of the microorganisms involved and the organisms' ability to produce the sequential enzyme systems required for the denitrification process under reduced pressure. At sufficiently high vacuum levels microbial functions may be affected. The exact level at which this occurs for a given microorganism can be experimentally determined. In addition, the viscosity of the tobacco material being denitrated and the potential fluid "boil over" effect that may occur at higher vacuums also limit

the degree of vacuum which can be applied to the system. Generally, a vacuum ranging to 500mmHg (below atmospheric) has been found to facilitate denitrification without adversely affecting the microorganisms. With a solution of low viscosity, the pressure reduction should generally be maintained in the range of about 50 mm Hg to about 200 mm Hg, whereas solutions of higher viscosity, for example about 500 centipoises or greater, will require a vacuum in the range of about 150 mm Hg to about 500 mm Hg.

The equipment employed in the practice of the invention may be sterilized prior to commencing the process. This equipment generally comprises a fermentor capable of maintaining the required oxygen exclusion and provided with an inlet port for the extract, a pump such as a peristaltic or piston pump to regulate feed of the extract, and a holding tank for the extract, said tank also being capable of maintaining oxygen exclusion and being provided with an outlet port. The tank is connected to the pump which is in turn connected to the fermentor by means of tubing. All connections should be such as to maintain anaerobic and aseptic conditions in the system.

The extract is fed, preferably at a regular rate, into the fermentor. The rate should be such that the nitrate-nitrogen in the fermentor remains below 1000 ppm, preferably between 500-800 ppm and optimally not above 700 ppm. Upon completion of the feed, the conditions in the fermentor should be maintained for at least two hours to ensure maximum denitrification of the extract. By means of the process essentially complete denitrification can be accomplished in less than 20 hours.

Following denitrification, the extract may be combined with insoluble tobacco materials which have been made into a sheet using conventional reconstitution methods. Prior to such

reconstitution the extract may be concentrated if necessary or desired. Of course, since more concentrated denitrated extracts may be produced in the practice of the present invention the degree of concentration required can be relatively reduced. The resulting reconstituted tobacco may be employed in any smoking product desired. Any such smoking tobacco product will exhibit reduced delivery of nitrogen oxides during combustion.

It is to be understood that the process of the invention may be employed to denitrate extracts from whole tobacco leaf, cut or chopped tobacco, tobacco filler, reconstituted tobacco, tobacco stems and the like. As used herein, references to tobacco and tobacco materials are to be understood to include all such forms of tobacco in various stages of curing. Further it is to be understood that reconstituted tobacco denitrated in accordance with the invention exhibits reduced nitrogen oxide delivery in any tobacco product which is consumed by combustion and that references to smoking tobacco products include, cigars, cigarettes, cigarillos and the like.

The following examples are illustrative of the invention:

Example 1

Inoculum Buildup

Paracoccus denitrificans ATCC #19367 was subjected to an induction process. In this process 25 ml of inoculum containing 10^7 cells/ml was incubated for 12 hours in a sparger flask in 250 ml of culture medium. The sparger flask was provided with a sparger reaching the bottom of the flask and an exhaust port for the removal of metabolic gases or for deaeration during sparging. The culture medium comprised 235 ml of a first sterile solution containing K_2HPO_4 , KH_2PO_4 , KNO_3 , $MgSO_4 \cdot 7H_2O$ and NH_4Cl and 15 ml of

a second sterile solution containing glucose and FeCl_3 . The overall composition of the culture medium was 10.7 g/l K_2HPO_4 , 5.24 g/l KH_2PO_4 , 10.0 g/l KNO_3 , 0.2g/l $\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g/l NH_4Cl , 10.0 g/l glucose and 0.002 g/l FeCl_3 in deionized water. The culture medium was deaerated with sterile nitrogen for 10 minutes and cooled to below 33°C before inoculation. During incubation a temperature of 33°C was maintained by means of a water bath and agitation of 120 revolutions/minute was effected by means of a shaker.

Using the above procedure, 25 ml of inoculum resulting from the above process was aseptically transferred to a culture medium as above described and was subjected to an identical incubation process. In identical manner 125 ml of inoculum resulting from this induction transfer was thereupon employed to inoculate 1250 ml of the above described culture medium and again subjected to the same incubation stage.

In turn 1200 ml of the resulting built-up inoculum was transferred to a culture medium comprising 10.8 liters of the above first sterile solution, 0.540 liter of sterile 10 g/50 ml glucose solution and 0.108 liter of 0.002 g/10 ml FeCl_3 solution and incubated at 33°C with 200 rpm agitation and a 76 mm vacuum for 12 hours. The solutions were deaerated for about 10 minutes with sterile nitrogen gas and cooled to below 33°C prior to inoculation.

12 liters of the last resulting inoculum was then transferred to tenfold greater volume of the sterile culture medium described immediately above which had been deaerated and cooled to less than 33°C. A vacuum of 76 mm mercury was applied, a temperature of 33°C was maintained and agitation of 100 rpm was effected during incubation. The initial pH was about 7 to 8.

During incubation the pH rose but did not exceed 9.5. At the end of 12 hours 130 ppm $\text{NO}_3\text{-N}$ remained in the inoculum.

The inoculum resulting from the last buildup step was employed to denitrate the tobacco extract. Prior to use, 6,538 ml of sterile 20 g/50 ml glucose solution and 261.5 ml of sterile 0.002 g/1.0 ml FeCl_3 solution was added thereto.

261.5 liters of tobacco extract containing 5.4% soluble solids and having a pH of 5.6 was prepared for denitration by addition of 5.840 liters 4N KOH resulting in a pH of 7.3. To this extract mixture was added 3.0 liters of 0.933 g/ml K_2HPO_4 , 1.5 ml of 0.0349 g/ml $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.5 liters of 0.174 g/ml NH_4Cl . The overall extract mixture was autoclaved at 121°C for 30 minutes. The extract mixture was then deaerated for 10 minutes with 200 liters sterile nitrogen gas/minute and cooled to 33°C .

The above prepared tobacco extract was pumped at a rate of 328 ml/minute into a fermentor containing the prepared inoculum. Conditions in the fermentor were 33°C , 76mm Hg vacuum, 100 rpm, 0% DO (dissolved oxygen) and pH 7-8. At the end of 14 hours the feed had been completed and the nitrate in the fermentor was about 78 ppm. 2 hours later 0 ppm was achieved.

The denitrated extract was thereupon sterilized, concentrated to about 40% solids by evaporation and applied to a tobacco web to form reconstituted tobacco. The resulting sheet contained 10.8% OV, 44% hot water solubles, trace $\text{NO}_3\text{-N}$, 0.12% ammonia, 2.0% reducing sugars and 0.65% total alkaloids. Cigarettes formed wholly from the reconstituted tobacco delivered 0.08 mg NO, 17 mg CO, 0.03 mg HCN and 0.68 mg RCHO.

Example 2

Inoculum induction and buildup was effected in the manner employed in Example 1 except that the third incubation stage was effected using 220 ml inoculum in 1100 ml of culture medium.

At the end of the build-up the culture medium had been completely denitrified. Prior to denitrification of the tobacco extract, sterile glucose and FeCl_3 were added to the inoculum as in Example 1.

261.5 liters of tobacco extract containing 822.3 ppm $\text{NO}_3\text{-N}$ and 7.8% soluble solids having a pH of 5.44 was prepared for denitration by addition of 8.342 liters of 4M KOH to pH 9, followed by addition of 5.292 liters of 4M H_3PO_4 to pH 8. 52.23 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 1 liter of water and 261.5 grams of NH_4Cl in 1 liter of water were added to the extract solution. The extract was then autoclaved, cooled, and further 4M H_3PO_4 was added to pH 7.3. The extract was autoclaved at 121°C for 30 minutes. While cooling to 33°C, deaeration of the extract was effected with sterile nitrogen gas at a rate of 200 liters per minute and 200 rpm.

The prepared extract was pumped for 12 hours into the conditioned culture solution containing glucose and FeCl_3 at a rate of 405 ml/minute using a Cole and Palmer piston pump. Conditions in the fermentor were 33°C, 76mmHg vacuum, 100 rpm agitation, 0% DO and pH 7-8. After completion of the feed, conditions were maintained for two more hours to completely remove nitrate and glucose from the medium. Sampling during denitrification indicated 0% $\text{NO}_3\text{-N}$ at the end of 12 hours operation.

The denitrified extract was autoclaved at 121°C for 30

minutes. The resulting sterile extract was concentrated to 47.7% solids by evaporation and applied to a tobacco web to form reconstituted tobacco. The thus produced tobacco sheet was subjectively comparable to industrial grade tobacco. The sheet contained 12.70% OV, 48% hot water solubles, $<0.04\%$ NO_3N , 0.10% amino nitrogen, 0.12% ammonia, $<2.0\%$ reducing sugars and 0.67 total alkaloids. Cigarettes formed from the reconstituted tobacco delivered <0.01 mg NO, 11 mg CO, 0.02 mg HCN and 0.56 mg RCHO.

Example 3

The procedure employed for the inoculum build-up and denitrification conditioning was identical to that of Example 2 except that only a 700 ml inoculum rather than a 1200 ml inoculum, was employed to inoculate 6.839 liters of the first solution, 0.341 liter of 10 g/50 ml glucose solution and 0.0684 liters 0.002 g/10 ml FeCl_3 solution in the third transfer. At the end of this build-up stage 0 ppm $\text{NO}_3\text{-N}$ remained in the culture solution.

In addition the fifth inoculation involved 7.950 liters inoculum in 75.23 liters of the first sterile solution and 3.762 liters of 10 g/50 ml glucose solution and 752.3 ml of 0.002 g/10 ml FeCl_3 solution. To the culture resulting from this last stage were added 20.692 l of 20 g/50 ml deaerated sterile glucose solution and 4.14 ml of 40% weight/volume deaerated sterile FeCl_3 solution prior to denitrification of the tobacco extract.

275.9 liters of tobacco extract containing 17.9% soluble solids and 2240 ppm $\text{NO}_3\text{-N}$ and having a pH of 6.3 was treated with KNO_3 to bring the $\text{NO}_3\text{-N}$ to 3420 ppm. The extract was treated with 19.8 liters of 8 M KOH to raise the pH to 11.

Thereupon one liter of 276 g/l NH_4Cl and one liter of 55.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were added. The resulting extract solution was sparged with sterile nitrogen gas for 10 minutes at a rate of 200 liters/minute and 100 rpm. After 60 minutes at pH 11 sterilization of the extract had been effected. The pH was then reduced to 7.2 with 12 liters of 4M H_3PO_4 .

The resulting tobacco extract was aseptically and anaerobically fed by means of a Cole and Palmer peristaltic pump at a rate of 260 ml/min. into a fermentor containing the conditioned inoculum. The feed was completed in 18 hours. At this point 370 ppm $\text{NO}_3\text{-N}$ remained in the extract. During the feed, conditions in the fermentor were the same as in Example 1. Upon completion of the feed, these conditions were maintained for an additional two hours. 71 ppm $\text{NO}_3\text{-N}$ remained in the extract.

The denitrified extract containing 13.2% soluble solids was sterilized, concentrated by evaporation and applied to a tobacco web to form reconstituted tobacco. The resulting reconstituted sheet contained 47% hot water solubles, < 0.04% $\text{NO}_3\text{-N}$, 0.20% amino nitrogen, 0.30% alkaloids and < 0.10% soluble NH_3 . Cigarettes formed from the sheet delivered 2 mg CO and 0.45 mg RCHO. Neither the HCN nor NO delivery could be measured since both were too low and the cigarettes were difficult to keep lighted due to excessive moisture in the reconstituted tobacco.

CLAIMS

1. A method for reducing the nitrate or nitrite content of aqueous tobacco extract by dissimilatory denitrification characterized by forming a culture solution by feeding under anaerobic conditions a deaerated tobacco extract containing up to 21% soluble solids and up to 4000 ppm nitrate-nitrogen into a stirred inoculum, said inoculum having an initial volume equal to 15-30% that of the extract to be fed and having 10^6 - 10^8 cells/ml of conditioned denitrifying microorganisms, the rate of said feed being so controlled that the nitrate-nitrogen content in the culture solution does not exceed 1000 ppm; and maintaining said culture solution under dissimilatory denitrifying conditions during said feed and for at least two hours after the completion thereof.

2. The method of claim 1 characterized in that the feed rate is such that the nitrate-nitrogen content in the culture solution is maintained between 500 and 800 ppm, and preferably below 700 ppm.

3. The method of claim 1 characterized in that the microorganism is subjected to an induction process prior to commencing the extract feed.

4. The method of claim 1, 2 or 3 characterized in that the culture solution additionally includes

glucose as a carbon source, preferably in a quantity of 2.5 g glucose/1000 ppm nitrate where the tobacco extract contains up to 10% soluble solids.

5. The method of any of claims 1 to 4 characterized in that the culture solution additionally includes phosphate, ammonium salts and iron and magnesium ions.

6. The method of claim 4 or 5 characterized in that the additives are present in the inoculum at commencement of the feed.

7. The method of claim 4 or 5 characterized in that the additives are present in the extract at the commencement of the feed.

8. The method of any preceding claim characterized in that the culture solution is maintained under a partial vacuum, preferably in the range 50-500mm Hg vacuum.

9. The method of any preceding claim characterized in that the dissolved oxygen content in the culture solution is substantially zero, which level is preferably achieved by sparging the culture solution with an inert gas.

10. The method of any preceding claim characterized in that the feed of the extract is effected by means of a piston pump.
11. The method of any preceding claim characterized in that the microorganism is Paracoccus denitrificans.
12. The method of claim 11 characterized in that the pH of the extract is between 7-8, and preferably about 7.3.
13. The method of claim 11 or 12 characterized in that the pH of the culture solution is maintained between 7-9.5, and at a temperature of the culture between 20 and 40°C, preferably between 30 and 35°C, and especially at about 33°C.
14. Use of the method of any preceding claim for making reconstituted tobacco, characterized in that the denitrated extract is combined with a sheet formed from insoluble tobacco solids, the extract being preferably sterilized prior to formation of the reconstituted tobacco.